EuroResidue VIII

Conference on Residues of Veterinary Drugs in Food

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Egmond aan Zee
The Netherlands

Keynote, Oral and Poster
Proceedings

RESIDUES OF VETERINARY DRUGS IN FOOD

Edited by:
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Keynote, Oral and Poster Contributions

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PREFACE

Dear friends, dear participants, dear residue scientists,

Twenty-six years of EuroResidue Conferences! And, here is EuroResidue number 8 (VIII)!

Not a momentous number of years nor a memorable edition number. All editions were at their heyday at the moment they took place. With the evolutions in agricultural, industrial, economical, demographic, social, political matters related to what was once called "the residue problem" on the one hand, and the analytical technical advances at the other hand, each new edition of this conference series is an event-to-be. The well-visited alternating EuroResidue conferences and Ghent symposia reflect the worldwide interest in this vivid subject as it did in the past.

Speaking of 'worldwide' and 'the past': the number of participants coming from outside the Europe is ever-growing. The name Euro in EuroResidue is certainly not reflecting the audience. In particular, we warmly welcome all those colleagues coming from far. For you it is a much greater endeavour to take part in the event in Egmond aan Zee than the EU participants. Thank you.

With respect to 'the past', it is worthwhile to give you a short summary of the preceding EuroResidue conferences and what kept you busy then:

In 1990, ERI started with the 'dawning' of legislation on the subject, which we today know so very well. There was a clear and loud demand from the government and public to regulate the toxic risks from food. It was commented that much work had already been done, but that still lots had to be fixed. In the ERI edition, the emphasis was on toxicology and pharmacodynamics of various compounds, not only of veterinary pharmaceuticals.

The conference in 1993 (ERII) marked the continuous development of analytical techniques, which made a second conference "desirable"! It was concluded that more attention had to go to … LC-MS.

It was in 1996 that the organisers of ERIII actively sought a broader scope than the presentation of analytical methods to detect residues of predominantly "hormones and growth promoters". There was a clear and obvious need for more information on quality assurance and residue inspection systems.

Attention was going to "large scale screening of samples and advanced detection systems based on molecular spectrometry" in the edition of 2000 (ERIV). It was hypothesised that every five years, sensitivity of analytical methods is doubling as if it were a variant on Moore's law. Consequently, some of us asked themselves the following question for the first time: do we really need the available and ever-improving sensitivity to detect the last zeptomole of residues? What is the relationship between detecting the last attomole and public health? Without knowing your opinion when you read this, we can imagine the pro's and the cons of improving sensitivity in your response.

In 2004, it was concluded that everything was in place to guarantee safe food. A network of regulatory residue laboratories and CRLs was accomplished and ISO 17025 was mandatory since 2002. In addition, WADA and Codex Alimentarius were greatly adopting the EU system. The zero-tolerance substances remained a problem though, and there were worries about the use of MRPLs as "surrogate" MRLs. The influence of global trade on the residue problem became more apparent and important.

With ERVI (2008) it was noted that the number of symposia in the field was increasing despite the availability of modern ICT technology. "Apparently, there is a need to continue to meet each-other in flesh and blood", as it was concluded. Prominent items were risk-based monitoring and the problem of the handling of increasing number of data-points with even shorter acquisition times. Remarkably, it was concluded that public opinion settled and the problem of the residues faded from the attention of the "general politicians". Residue problems had become incidents instead of outbreaks and big scandals (this was pre-horse meat affair, but even this scandal was about … meat not really residues).

It was in the VIIth ER (2012) that the "–omics" technologies received increasingly more attention and HR-MS was announced as the direction to go if you want to perform modern instrumental residue analysis. Otherwise, the conference was largely on the improvement of existing methods and care for a sound validation. Although a longer considered problem, endogenous versus exogenous was discussed more intensively.

Now there is ERVIII, what can we conclude from the 2016 version of the meeting so far? The interest for methods detecting (multiple) antibiotics (simultaneously) has exploded. Almost all of the contributions on this topic refer to the development of bacterial resistance against antimicrobials and the steps to make to manage this problem as a motivation for the work.

When writing about bacteria: "*Bugs are better than drugs*" is a slogan in current microbiome research. This may be true as a vast growing number of micro-organisms, which have a beneficial effect on plants and animals, are found. They promote growth, immune system, reduce stress and have many other expected results. There is a microbiological revolution going on, but whether it can ever replace the chemistry in livestock management is very doubtful at the moment.

Shocking news is that some reports in this book suggest that farmers may use antibiotic-destroying enzymes to mask their use of antibiotics. Like the cat-and-mouse game we witness for a long time in the illegal use of anabolic compounds, we can expect this apparently also for other veterinary drug classes when they are shown in bad light.

Surprisingly, some professionals may think that a residue concentration in excess of the MRL is health threatening. Actually, that is barely the case if one realises that MRLs are estimated extremely conservatively. MRLs are determined for a 60 kg weighing person, who will reach an age of 70 years and who will consume daily(!): 1.5 L milk, 300 g meat, 100 g liver, 50 g kidney, 50 g animal fat, 100 g eggs and 50 g honey. That is, this diet daily and each food product shall contain the residue at a concentration of the MRL. Fortunately, some executive inspectors realise this very well. By the way, this is not a plea to do our work with less enthusiasm, it is only an attempt to realise what some non-compliant field sample results may mean when the product has already reached the market.

Evolutions do not stop with the changing socio-economical entourage of residue analysis or analytical technology, it also happens to the ER conference. This is the first version of the proceedings book that is solely available electronically. No multiple volumes of the physical book to carry home anymore. The point of view of the EuroResidue Conference Foundation has not changed though and it is to continue to publish the proceedings. We see namely that it accommodates residue scientists without an (academic) obligation to publish, to disseminate in a relatively low-demanding way their findings, method designs and other experiences. One other important function of the proceedings is that it is easily available for everyone worldwide unlike many scientific or technical journals.

Despite the beneficial effects of circulating proceedings, we see an upcoming friction. Time-pressure, cost-effective working demands and the prohibition of publishing the same data twice in the proceedings and in a peer-reviewed journal (in particular in the academic world) may jeopardize the series. We profoundly hope that you will continue to send your contributions to make up a unique collection of contemporary analytical developments in the residue field.

Finally, we want to acknowledge the efforts of the scientific and organising committees and we greatly thank all participants for coming to the conference and in particular those of you who have prepared orals, posters and the proceedings in this book. We are also thankful towards all sponsors. Their contributions broaden the scope of information presented at the event and at the same time made it possible to uphold the well-known all-under-one-roof setup of the event.

Aldert Bergwerff, Robert Schilt and Leen van Ginkel

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ABOUT THIS BOOK

Dear reader,

We are happy to present you the first version of the EuroResidue Conference proceedings that is available as a PDF file exclusively. We had several reasons for deciding to do so, and one of them is the current and strictly applied maximum weight of luggage at most flights (in some cases it is 7 kg). It was a reason for many participants in the last EuroResidue Conference to refuse the physical book. Indeed, the weight of the three volumes of the EuroResidue VII conference was 2.0 kg. People prefer to carry a versatile laptop, which is increasingly also their "labtop", as some contributions in this book showed.

We received 181 manuscripts for the proceedings, which is a "recovery" of 88% of the original total number of abstracts, which the Scientific Committee received for review. They are all included in this book. We emphasize that the authors are responsible for the content and the quality of the published contribution. Nevertheless, the manuscripts were edited so that they fit in the format and style of the rest of the book and in a way that the presentations form some degree of coherence. In some cases, editing was performed to improve the reading. The presentation style of the authors has remained untouched as much as possible.

As noted in the instructions, the authors have assigned the right to reproduce and distribute the manuscripts on a worldwide basis to the publisher, *i.e*. EuroResidue Conferences Foundation (*Stichting Euroresidue Conferenties*). It is assumed that, in individual cases, the authors have obtained permission to reproduce any figure, data or text from the respective copyright holders. The EuroResidue Conferences Foundation does not own the copyrights; the manuscripts remain the property of the authors. The Foundation considers it as one of its statutory tasks to distribute the proceedings to anyone who is interested, worldwide and free of charge.

Here is also a short instruction for using the proceedings. The core of the book is formed by the manuscripts. They are organised in three parts and represent the:

Keynotes = KNx
Orals = Ox
Posters = Px

Where x refers to a successive number. In case of the posters (Px), 'x' is the original abstract number provided to the submitting author. It is therefore that the numbering of posters sometimes misses consecutive numbers. That is not a flaw, it is correct. These missing numbers belonged to the abstracts which were selected for a keynote or oral presentation, or, otherwise, the manuscript was not received for this book. Numbering of the keynotes (KNx) and orals (Ox) follows the time-line in the programme, which is also included in this book starting on page 10. Each label at each contribution has an additional sequential number as well. It may be practical for some of you, it has no further function. It is given in italic between brackets.

The size of the 'paper' has changed compared to the earlier versions, namely A4 format. This gave more possibilities to accommodate large Tables and Figures, which grow with the introduction of multi-class, multi-residue analyses, which give many options/settings and results to present. Initially, we doubted on the size of the font to be used, but we decided that you can easily enlarge the pages on your screen if necessary, because we used a so-called 'true font'.

For your convenience, each part in the book and each contribution is tagged in the PDF. In addition, the file is searchable by using the two 'find' functions in your PDF-reader ('*Find*' and '*Advanced search*', resp.). This book, therefore, does not contain a keyword index, as you were used to in all previous versions of the proceedings. We included a manuscript and author index though to give easier access to the papers, as authors may also occur in the references and this may obscure the find results.

Another advantage of a PDF file, instead of a book, is that realisation of colours is easier than with a printed book, which has to be kept affordable. You may notice this from many figures and from the label attached to each contribution. The orange colour may help you to navigate easier in a thumbnail view.

Finally, we prepared also a 'light' version of the complete book of proceedings. The 'light' version only contains the programme and manuscripts of the keynote and oral contributions.

Lastly, we thank Sanne Biggelaar of the EuroResidue Secretariat for managing the receipt of the short abstracts and of the full manuscripts, and for chasing the authors when needed. Well done Sanne!

We hope you find this book worthwhile to read and to archive for later use.

The editor and the EuroResidue Conferences Foundation.

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PROGRAMME

Sunday 22nd May 2016

13.00-17.00 Pre-conference workshop:
"Residue analysis for dummies" RIKILT /SARAF- LABERCA

16.00-20.00 Registration open

17.00-20.00 Get-together party in Hotel Zuiderduin

Monday 23rd May 2016

8.45-9.15 **Opening of the EuroResidue VIII conference**
EuroResidue foundation

Opening address:
Dr. Ir. H. Paul, Inspector General of the Dutch Food Safety Authority

First session **Antibiotics, residues and resistance**
Chairman: T. Burnett
Co-chairman: A.A.M. Stolker

9.15-10.00 **Keynote lecture 1 (KN1)**
Prof. dr. Dan I. Andersson
Uppsala University, Sweden
Evolution of antibiotic resistance at very low antibiotic concentrations

10.00-10.20 Dr. Petra Gowik (O1)
BVL, Berlin, Germany
A monitoring study on the level and frequency of antibiotic residues in food producing animals in Germany

10.20-10.40 Dr. Brigitte Roudaut (O2)
ANSES, Fougères, France
Contribution of mass analytical methods to identification of antibiotic residues in meat – Application to antibiotic control in France

10.40-11.10 **Coffee break, poster viewing and exhibition**

11.10-11.55 **Keynote lecture 2 (KN2)**
Prof. dr. Pierre-Louis Toutain
Toulouse, France
Veterinary medicine needs new and innovative green antibiotics

11.55-12.15 Dr. Shabbir Simjee (O3)
Elanco Animal Health, Basingstoke, United Kingdom
Responsible use of antimicrobials in veterinary medicine: The EU vs. USA approach to AGPs

12.15-12.35 Dr. Andrew Cannavan (O4)
FAO, Rome, Italy and IAEA, Vienna, Austria
Global perspectives on antimicrobial resistance in the food-chain

12.35-13.45 **Lunch and poster presentations**
Lunch is served near the exhibition booths

12.45-13.30 **Vendor Seminar: AB Sciex**
Dr. Jens Dahlman
AB Sciex, Germany
What's in Your Food? Accurately Find and Identify Residues and Contaminants with Advances in LC-MS/MS

Second session **Residues and the environment**
Chairman: E. Daeseleire
Co-chairman: J. Polzer

13.45-14.30 **Keynote lecture 3 (KN3)**
Dr. Chris Sinclaire
Fera Science Ltd, York, United Kingdom
The Environmental Impact of Veterinary Medicines

14.30-14.50 Dr. Astrid Spielmeyer (O5)
Justus Liebig University Gießen, Germany
Spirits that we've cited our commands ignore - The fate of antibiotics in manure

14.50-15.10 Dr. Danny Chan (O6)
FERA Science Ltd, York, United Kingdom
Variability of residue concentrations of ciprofloxacin in honey from treated hives

15.10-15.40 **Coffee break, poster viewing and exhibition**

15.40-16.25 **Keynote lecture 4 (KN4)**
Dr. Jin-Wook Kwon
Ministry of Food and Drug Safety, Busan, Republic of Korea
Management of antimicrobials in the environment: What have we learned and what should we prepare for the future?

16.25-16.45 Dr. Steven Crooks (O7)
Agri-Food and Biosciences Institute, Belfast, United Kingdom
An investigation into the sources of contamination of cattle with the veterinary drug phenylbutazone

16.45-17.05- Dr. Tina van den Meersche (O8)
ILVO, Melle, Belgium
Quantification of five different classes of veterinary antibiotics in (treated) swine manure using a validated UHPLC-MS/MS method

17.15-18.00 **Agilent Technologies Netherlands drinks and seminar**
Martin Haex
Agilent Technologies, The Netherlands
Screening of Veterinary Drugs by LCMS
Solutions and workflows for Screening, Identification and Quantification by LCMS QQQ and LCMS QTOF

Wim van Duinkerken
Agilent Technologies, The Netherlands
The fast track to high quality results for food safety screening

18.15-19.15 **Dinner in Hotel Zuiderduin**

19.30-22.00 **Excursion, lobby Hotel Zuiderduin**

20.00-22.00 **Alternative: bowling in Hotel Zuiderduin (lanes reserved for EuroResidue participants)**

Tuesday 24th May 2016

Third session **New techniques, confirmatory analysis**
Chairman: L. Vanhaecke
Co-chairman: J. Boison

9.00-9.45 **Keynote lecture 5 (KN5)**
Prof. dr. Bruno Le Bizec
LABERCA, Nantes, France
An overview of latest advanced technological options for residue analysis

9.45-10.05 Dr. Anton Kauffmann (O9)
Official Food Control Authority, Zurich, Switzerland
Ion mobility coupled to high resolution mass spectrometry: The possibilities, the limitations

10.05-10.25 Ir. Christelle Robert (O10)
CER, Marloie, Belgium
Development of immunomagnetic precipitation methods for the detection of recombinant *bovine* somatotropine by UHPLC-MS/MS

10.25-10.45 Dr. Roberta Galarini (O11)
Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy
Confirmatory multi-class method for residues of antimicrobials in milk by LC-HRMS/MS

10.45-11.15 **Coffee break, poster viewing and exhibition**

Fourth session **Alternative matrices**
Chairman: S. Croubels
Co-chairman: K. Mitrowska

11.15-11.45 Robin Wegh MSc (O12)
RIKILT, Wageningen, The Netherlands
Hair and feathers: the matrix of choice for antedating the use of antibiotics, β-agonists and steroidesters?

11.45-12.05 Dr. Wim Reybroeck (O13)
ILVO, Melle, Belgium
Testing of Saliva as ante-mortem screening for antimicrobials in pigs

12.05-12.25 Dr. Eline Kowalski (O14)
Ghent University, Merelbeke, Belgium
Insects on your plate: monitoring chemical contaminants and residues

12.25-12.45 Dr. Javiera Cornejo (O15)
University of Chile, Santiago, Chili
Depletion study of oxytetracycline (OTC) and 4-epi-oxytetracycline (4-epi-OTC) residues in claws of broiler chickens by liquid chromatography tandem mass spectrometry

12.35-14.00 **Lunch and poster presentations**
Lunch is served near the exhibition booths

13.00-13.45 **Vendor seminar; Thermo Fisher Scientific**
Wouter Gebbink
RIKILT – Wageningen UR The Netherlands
Steroid hormone analyses in livestock urine by GC-Orbitrap

Laszlo Hollosi
Thermo Fisher Scientific, The Netherlands
Advantages and Challenges of High Resolution Accurate Mass Spectrometry in Residue Analysis

Fifth session **Validation and criteria approaches**
Chairman: E. Verdon
Co-chairman: U. Vincent

14.00-14.45 **Keynote lecture 6 (KN6)**
Dr. Bjorn Berendsen
RIKILT, Wageningen, The Netherlands
A unique collaborative study to assess confirmatory analysis performance criteria in veterinary drug residue analysis

14.45-15.05 Dr. Monique Bienenmann-Ploum (O16)
RIKILT, Wageningen, The Netherlands
Validation of multiplex bead-based assays for the simultaneous (on-site) detection of coccidiostats and antimicrobials

15.05-15.25 Dr. Joe Boison (O17)
Canadian Food Inspection Agency, Saskatoon, Canada
Approaches to validation of methods for regulatory use

15.25-15.45 Dr. Katrin Kittler (O18)
Federal Office of Consumer Protection and Food Safety, Berlin, Germany
Investigations on the influence of hydrolysis on the total amount of marker residue and consequences

15.45-16.05 Dr. Mark Sykes (O19)
Fera Science Limited, York, United Kingdom
Chloramphenicol proficiency tests on a global scale – unforeseen consequences

16.05-16.25 Dr. Jens Hinge Andersen (O20)
National Food Institute, DTU Food, Søborg, Denmark
Sample-based reporting of official national control of veterinary drug residues

16.25-16.45 **Tea break, poster viewing and exhibition**

16.45-18.15 **WORKSHOP Risk based approaches for monitoring**
Dr. Matthew Sharman
Fera Science Ltd, York, United Kingdom

Hans van Rhijn, MSc
Dutch Food Safety Authority, Utrecht, The Netherlands

19.30 **Conference dinner and party by the seaside**

Wednesday 25th May 2016

Sixth session **Broad screening**
Chairman: G. Dervilly-Pinel
Co-chairman: R. Granja

9.00-9.20 Dr. Kathrin Schmidt (O21)
BVL, Berlin, Germany
Application of LC-QTOF technology for screening for hormonally active substances in matrices of animal origin

9.20-9.40 Dr. Roberto Stella (O22)
Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy
Targeted proteomics for the indirect detection of dexamethasone treatment in *bovines*

9.40-10.00 Dr. Mario Botta (O23)
Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta, Turin, Italy
The histopathological approach for the monitoring of the illegal administration of growth-promotors in food-producing animals

10.00-10.45 **Coffee break, poster viewing and exhibition**

Seventh session **Broad screening continued**
Chairman: G. Hamscher
Co-chairman: J.-W. Kwon

10.45-11.05 Dr. Pilar Marco (O24)
CSIC/IQAC, Barcelona, Spain
Site-encoded DNA strategies for residue analysis

11.05-11.25 Jérémy Marchand (O25)
LABERCA, Nantes, France
Lipidomics: an alternative and complementary tool to highlight biomarkers of growth-promoting practices

11.25-11.45 Marco H. Blokland, MSc (O26)
RIKILT, Wageningen, The Netherlands
The Dutch approach for the detection of (synthetic) natural steroids in The Netherlands:
A retrospective overview

11.45-12.05 Dr. Gaud Dervilly-Pinel (O27)
LABERCA, Nantes, France
Are biomarkers universal and transferable?

12.05-12.25 Lieven van Meulebroek (O28)
Ghent University, Merelbeke, Belgium
Discovery of a urinary biomarker to discriminate between semi-endogenous and exogenous thiouracil in cattle

12.25-13.00 **Closing of the EuroResidue conference**

KEYNOTE LECTURES

CONTRIBUTIONS

KN2
(001)

VETERINARY MEDICINE NEEDS NEW AND INNOVATIVE GREEN ANTIBIOTICS

Pierre-Louis Toutain[1], Aude Ferran[1], Alain Bousquet-Mélou[1], Ludovic Pelligand[2] and Peter Lees[2]

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[2]The Royal Veterinary College, Hawkshead Campus, Hatfield, Herts., AL9 7TA, United Kingdom

Abstract

Veterinary medicine needs new and innovative antimicrobial drugs for food-producing animals, not for animal health reasons but for public health requirements. We have termed these new drug classes "green antibiotics". They should have minimal (ideally no) impact on animal commensal microbiomes and, therefore more broadly, no ecological impact on environmental resistomes. The aim is to suppress the animal contribution to the human resistome. In this overview, we first outline current ecological issues associated with veterinary antimicrobial drugs, then define the ideal pharmacokinetic and pharmaco-dynamic properties of a veterinary green antibiotic. We conclude that novel drugs can be developed through screening currently-used classes of veterinary antimicrobial drugs.

Introduction

Food-producing animal medicine is not currently facing the same critical situation as human medicine, in respect of the emergence of resistance, because there are no septic or chronic conditions, in poultry, pigs or cattle, which have to be treated obligatorily with antibiotics. However, we urge renewal of the veterinary antibiotic armentarium with new drugs for public health reasons in order to break the resistance link between human and veterinary medicine. We have termed "green antibiotics" these - new compounds, as they will have a minimal or no ecological impact on the animal commensal microbiome and hence on the environmental resistome (Toutain *et al*., 2015; Toutain and Bousquet-Mélou, 2013). The rationale for this proposal is that the worldwide consumption of antibiotics to treat or prevent the many health conditions encountered in food-producing animals will ineluctably increase over the next decades, Moreover, the so-called prudent use of antibiotics will not be able to stem the rise in resistance of animal origin and its subsequent impact on the human resistome. In this review, we first explain that the animal and human commensal microbiota constitute a "turnstile" for exchange between the two corresponding resistomes. We then describe the properties of an ideal green antibiotic and, finally, discuss the regulatory aspects that should be addressed to facilitate the promotion of green antibiotics.

Current issues with veterinary antimicrobial drugs

Veterinary medicine is faced with three types of antimicrobial resistance (AMR): for specific animal pathogens, for zoonotic pathogens and resistance of the commensal bacteria harboured by animals. AMR relating to specific animal pathogens may raise specific veterinary concerns in terms of reduced efficacy in treatment but it has no direct impact on human medicine. AMR to foodborne zoonotic pathogens are of greater concern. However, the emergence of foodborne pathogen resistance is not the most relevant hazard of veterinary origin for human medicine, because it is currently a medical issue for individual humans and is not a global ecological, economic and political hazard now or for the future. The hazards associated with AMR at the level of the animal's commensal microbiota, *i.e*. organisms of the gastro-intestinal tract, are potentially much more serious from an overall ecological perspective. This is because their biomasses greatly exceed those of the specific or zoonotic pathogens harboured by the same treated animals. It is reasonable to assume that the amplification of pre-existing or emerging genes of resistance displays proportionality to the size and genetic richness of the various microbiota, whether pathogenic or not, harboured by treated animals.

There are several pathways of exchange between the animal and human microbiomes. The two most relevant are: (1) directly from animal-to-man via the food-chain and (2) indirectly via the multiple intricate pathways of the environment. Most proposed scenarios for the transmission of AMR of veterinary to humans are consistent with the hypothesis of a pivotal role of the human commensal microbiota in the natural history of many human infections. There are two major routes for the emergence and spread of AMR to pathogenic bacteria in humans : the first is by direct selection of resistant mutants within the population of pathogenic bacteria at the site of infection followed by dissemination to a new patient by direct exposure, for example in a confined hospital environment (epidemic pathway); the second is indirect and involves selection of resistant bacteria in the commensal microbiota, attributable to the horizontal transfer of resistance genes from non-pathogenic to pathogenic bacterial species, and subsequent transfer within the pathogenic population and a possible delayed effect on the host. Thus, the human digestive tract can be viewed as the open door to AMR determinants from various external sources, including food animals. Any human subject (patient or not) is more or less permanently exposed to resistance determinants by the food chain and from both terrestrial and aquatic environments. These non-disease producing bacteria and resistance

determinants are ingested, exposing the gastro-intestinal tract commensal microbiota and forming within the microbiota a pool of resistance genes, which may subsequently undergo horizontal transfer to pathogenic species.

Shortcomings of currently used veterinary antibiotics and ideal pharmacokinetic and pharmacodynamic profiles for a green antibiotic to minimize public health issues

The most used veterinary antimicrobial drugs are eliminated in significant amounts into the gastro-intestinal tract, where they may impact negatively on commensal microbiota of animals. For orally administered drugs, the principal reason is their poor absorption (low bioavailability) leading to exposure of the distal intestinal microbiota to the unabsorbed drug fraction. Potentially, this can exert a local selective pressure, thereby increasing the density of the resistant bacteria and resistance genes. This load of enriched resistant bacteria and resistance genes is then released into the environment by faecal excretion. After systemic antimicrobial drug administration, most agents used in veterinary medicine are eliminated, to varying degrees but often extensively, in the digestive tract, either by biliary secretion or by intestinal clearance as a consequence of efflux pumps in the gastro-intestinal wall. As with oral dosing, there is unavoidable exposure of the intestinal microbiota. These shortcomings of currently used antimicrobial drugs justify the promotion of a new generation of green antibiotics.

Novel drugs should possess appropriate pharmacokinetic/pharmacodynamic (PK/PD) selectivity. Ideally, they should be distributed only to the biophase, the location of the targeted pathogen, and should have no pharmacodynamic impact on both the commensal microbiota of the treated animal or on the various matrix/ ecosystems of the environment. Our contention is that it is possible to achieve this goal by re-evaluating current classes of veterinary drugs and applying the existing wealth of knowledge of their medicinal chemistry and PK profiles. This will lead to more hydrophilic analogs of these drugs, so that there will be no requirement to discover and develop new drug classes with novel mechanisms of action. Such novel agents could be immediately challenged on the grounds of new additional AMR risks for human medicine. Eco-friendly drugs, such as ceftaroline, telavencin and dalbavancin, are in current use in human medicine (Rashid *et al*. 2012) indicating the feasibility of their development in veterinary medicine.

In summary, the ideal green antibiotic should have (1) high oral bioavailability, (2) a low volume of distribution to ensure PK selectivity (limiting exposure to organisms in the biophase) (3) a low clearance to have a rather long half-life (4) neither intestinal or biliary clearance as a mechanism of elimination, to avoid exposure of the animal gastro-intestinal microbiota (5) elimination by hepatic metabolism forming inactive metabolites (5) a high renal clearance with the eliminated active drug being rapidly degraded in the environment or immobilised by physical sorption.

Regulatory considerations

The regulatory climate should be favourable to provide incentives for industry to develop innovative antibiotics. Regulatory authorities should also consider the case of green antibiotics as alternatives to currently-used drugs, because they will be developed to fulfil the main requirement for the One Health policies *i.e*. to be devoid of side-effects for human health and the environment. To promote such green antimicrobial drugs and to give them a competitive advantage over existing drugs, regulatory authorities will need to consider the impact of any drugs (new or old) on the environmental resistome, in granting a marketing authorisation. The regulatory pathway should facilitate the development of green antibiotics. This will include provision of robust PK/PD data and confirmation of efficacy in a non-inferiority clinical trial. In addition, the licensing submission should provide data which clearly demonstrates the ecological advantage of the proposed green AMD over conventional comparators. According to some economic models, AMR can be reduced by extending the duration of the patent, as the patent gives the owner an incentive to protect the value of their drug by limiting usage. Pricing of green antibiotics should recognise the cost to industry for their research and development programmes. This will be difficult to ensure if the market is dominated by inexpensive drugs, because it is unlikely that the more expensive green antibiotics, whose only advantage is to mitigate public health issues, would be prescribed in preference to the less expensive drugs (both pioneer and generic) that work well in treating disease, that is from the animal health perspective but not from the animal health perspective.

Conclusions

Veterinary antimicrobial drugs should be innovative and expensive, and their marketing regulated to encourage their use in preference to older, less expensive and widely available drugs. The proposition made in this article is that we urgently need new antibiotics in veterinary medicine, because most of the drugs currently used in veterinary medicine ineluctably expose the animal gastrointestinal microbiome, through their lack of PK selectivity, and thus may enrich the human resistome *i.e*. increase accumulation of AMR genes harboured by both pathogenic and non-pathogenic human bacteria.

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MANAGEMENT OF ANTIMICROBIALS IN THE ENVIRONMENT: WHAT HAVE WE LEARNED AND WHAT SHOULD WE PREPARE FOR IN THE FUTURE?

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Abstract

Over the past decade, major advances have occurred in both understanding and practice with regard to environmental fate and occurrence of antimicrobial, food and ecological safety. It is important to understand how antibiotics are used in both humans and food animals and how these uses affect the evolution of antimicrobial resistance, ecosystem, food and feed safety. The increasing global concern for this issue primarily comes from the use at the farm and management by responsible authorities. This presentation will highlight a number of monitoring, fate studies, antimicrobial resistances from the terrestrial and aquatic farming systems and management practices of authorities over the last few decades. To find out the link between antimicrobial resistance and the occurrence of residues of antimicrobials, the life cycle study of the antimicrobials from farm to farm through the manure, soil, ground water, animal feed, animal product at the terrestrial farming system has intrinsic value. In this respect, it is necessary to extend this kind of study to aquaculture system. Registration of veterinary medicinal products should be also strengthened as a preventive measure. Because sustainable development could be achievable on the basis of the responsible care of manufacturer, correct use and refined management by authorities.

Introduction

Livestock, poultry and fish are often administered antimicrobials to treat and prevent diseases, as well as to promote animal growth and feed efficiency. Many of the antimicrobials used for treatment of food-producing animals are also used as human clinical pharmaceuticals. The majority of the antimicrobial use is estimated for animal growth rather than for medicinal reasons. Research indicates that sub-therapeutic use of antimicrobials can select for antibiotic resistance in bacteria. The purpose of this study is to encourage better strategies for prudent use of and limited unnecessary exposure to antimicrobials for food animals and in the environment by providing the current information. For this purpose, this study investigated four areas: i) antimicrobial use and consumption, ii) improvement of antimicrobial bioavailability to food-producing animals, iii) antimicrobial resistances, and iv) fate and occurrence of antimicrobials in the environment.

Antimicrobial use and consumption of food-producing animal

It is essential to understand the estimates of the quantity and types of antimicrobials administered to livestock, poultry and aquacultured animals for the policies of judicious use of antimicrobials in food-producing animals. Healthy animals help to guarantee a safe food supply. Enhanced sanitary conditions, vaccination strategies, and appropriate management are the cornerstones of a comprehensive animal health programme to control and prevent infectious diseases. Despite all preventive measures, animals still get sick and need to be treated. Drugs for livestock protect consumers from harmful food-borne pathogens or zoonotic agents. Despite the significant and potential consequences of antimicrobial resistance, there are no reliable quantitative data on global and national antimicrobial consumption by livestock and aquatic animals. Recently, Boeckel *et al*. (2015) estimate the global average annual consumption of antimicrobials per kilogram of animal produced was 45 $mg{\cdot}kg^{-1}$, 148 $mg{\cdot}kg^{-1}$ and 172 $mg{\cdot}kg^{-1}$ for cattle, chicken and pigs, respectively. They also estimated that between 2010 and 2030, the global consumption of antimicrobials will increase by 67%, from 63,151 ± 1,560 tons to 105,596 ± 3,605 tons.

If consumption data of antimicrobials are available, estimation and mapping the antimicrobial consumption could provide several insights of regulation and management. First, it provides an objective data-driven estimate of the potential magnitude of antimicrobial consumption at the global scale, whereas previous estimates were based on expert opinions (Wise 2002). Second, it identifies regions at higher risk of emergence of drug-resistant pathogens— places where surveillance and intervention efforts should be targeted. Third, this baseline estimate can be used to evaluate the progress (Gething *et al*., 2014) of future antimicrobial stewardship efforts.

Antimicrobial resistance in animals, humans and meat have not only been associated with Intensive farming practices but also with numerous livestock diseases such as highly pathogenic avian influenza H5N1 (Boeckel *et al*., 2012) and *porcine* reproductive and respiratory syndrome (Chung *et al*., 2005). Beyond animal health and antimicrobial resistance, other negative externalities associated with poorly regulated intensive farming include water and soil pollution (Gerber *et al*., 2005), loss of biodiversity (Tscharntke *et al*., 2005) and decline of meat nutritional quality (Sami *et al*., 2004). All of these have severe consequences that potentially outweigh long-term benefits of increased productivity. Data on antimicrobial consumption in aquaculture is insufficient, but consumption in fish-farming in Chile (Cabello 2006) and shrimp farming in Vietnam (Le *et al*.,

2005) demonstrated that aquaculture is associated with extremely high rates of antimicrobial consumption per PCU (population correction unit). Up to 1,400 mg PCU^{-1} has been reported for salmon farming in Chile (Cabello, 2006). As the aquaculture industry grows (World Bank 2013) and shifts toward more efficient production systems, it could constitute a major source of antimicrobial contamination of the aquatic environment over the coming decades.

Despite the widespread adoption of antibiotic use in food animals, reliable data about the quantity and patterns of use (*e.g.*, dose and frequency) are not available (Sarmah *et al.*, 2006). Quantifying antibiotic use in food-producing animals is challenging due to variations in study objectives. Investigators may for example measure only therapeutic uses, only non-therapeutic uses or a combination of those depending on their outcome of interest. In addition, there is lack of clarity on the definitions of therapeutic and non-therapeutic use (Mellon *et al*., 2001). The Union of Concerned Scientists reported that the total antimicrobial use was up to 35 million pounds in human and in agricultural and companion animals in the USA (Mellon *et al*., 2001). The non-therapeutic use of antimicrobials in the three livestock sectors was up to 70% of the total. All agricultural uses (livestock, non-therapeutic and therapeutic, plus pesticides) represented 84% of the total. All non-human uses (livestock, pesticides, and companion animals) were 87 percent of the total. The U.S. Government Accountability Office (USGAO) also reported that quantifying antimicrobial use in livestock is challenging and that estimates vary widely because there are no publicly available, reliable data on antimicrobial use in food-producing animals (USGAO 2011).

Pharmaceutical companies are not required to disclose veterinary drug sales information (Shore *et al*., 2009), and the types used at operations may be deemed proprietary information (Sapkota *et al*., 2007). Furthermore, use estimates based on dose rates can be complicated. Recently, US EPA reported that over 29 million pounds of antimicrobials were sold for livestock use in 2010 in the USA (US EPA 2013). This is about 3 to 4 times more than the amount used by humans.

About 60% to 80% of livestock routinely receive antimicrobials, the majority of which are estimated to be used for animal growth, rather than for medicinal purposes. The WHO has noted that sub-therapeutic antimicrobial use by livestock and poultry is an area of concern because of the selection for antimicrobial resistance. Antimicrobials generally do not biodegrade easily and may be more mobile in aquatic environments. Recognizing the importance of quantifying livestock and poultry antimicrobial use, USGAO has been advocating for better tracking and reporting mechanisms of antimicrobial use in livestock and poultry since 1999 (USGAO 2011). In accordance with a 2008 amendment to the Animal Drug User Fee Act, the USFDA released estimates of the annual amount of antimicrobial drugs sold and distributed for use in livestock and poultry in 2009 and 2010 (USFDA 2010 and 2011) and revealed a 62% increase over 1985 use estimates (U.S. Congress, OST 1995). Tetracyclines and ionophores were the largest class of antimicrobials reported, accounting for over 70% of all livestock and poultry antimicrobials sold during that year (USFDA 2011a).

Overall, estimations of annual antimicrobial use in food animals in the U.S. range from 11 to 29.2 million pounds. The WHO has developed criteria for the classification of antibiotics as 'critically important', 'highly important' and 'important' based on their importance in the treatment of human disease (WHO 2007).

There are many differences between aquatic and terrestrial management systems, such as the methods used for administration of drugs. Unique problems are related to the application of drugs in aquatic environments. Aquaculture is a growing agribusiness. It has grown more rapidly than either regular fisheries or terrestrial food animal production. Aquaculture is an important component of the world's food supply in both developed and developing countries. In recent years, fish have been widely recognized as a highly nutritious source of protein, essential fatty acids, micronutrients and minerals. It is well recognized that antimicrobial use in food animals is of global concern, and the problem has resurfaced recently in aquaculture. However, despite the controls and regulations introduced by governments to prevent environmental risks in many countries, antimicrobial resistance and toxicity are increasing. This has been well documented in developing countries where the aquaculture industry is growing rapidly. The culture of animals and plants in aquatic environments differs significantly from the culture of animals in terrestrial environments. The administration of drugs and chemicals indirectly, into the culture water, influences the speed and extent of exposure of non-target organisms such as other vertebrates, algae, invertebrates and bacteria, in contrast to the direct administration of drugs and chemicals in a land-based setting.

The threat to public health from the overuse of antimicrobials in food animal is real and growing. But Approximately 80% of antibiotics sold in the United States are used in meat and poultry production. The majority is used in healthy animals to promote growth, or prevent disease in crowed or unsanitary conditions. In this respect, it is necessary to find out the way 'How to reduce the use of antimicrobials?'. There were various efforts to reduce the use of antimicrobials in food-producing animals. Legislation to address the problem has been introduced several times since the 1980s, but the powerful agribusiness and pharmaceutical lobbies have continuously managed to obstruct their enforcements (Kammerle *et al*., 2009). A timeline of EU and US policies on non-therapeutic antimicrobials in food animal production is summarised in Table 1.

The first ban on farm use of antibiotic growth-promoters was enacted in 1986 in Sweden. The United Kingdom and other countries of the European Union introduced bans on the use of antibiotic growth promoters (AGPs) in food animal production. With the appropriate adjustments in practices to ensure continued animal health and safety, these countries continue to thrive animal food production despite of the ban. During the 1990s, vancomycin-resistant *Enterococcus (VRE)* was detected among patients, in meat and also in manure on farms where avoparcin was used as a growth-promoter in Europe. In

1997, the EU banned avoparcin for all uses in agriculture, then in 1999, EU officials discontinued further use of antibiotic growth-promoters from drug classes also used in human pharmaceutical, imposing a ban on tylosin, spiramycin, virginiamycin, and bacitracin. Other antimicrobials were phased out in 2006.

The Danish Integrated Antimicrobial Resistance and Monitoring and Research Programme (DANMAP) facilitated and encouraged these decisions. The activities of DANMAP were mainly: i) monitoring the occurrence of antimicrobial resistance, ii) monitoring the consumption of antimicrobial agents, and iii) investigation of associations between use of antimicrobial agents in animals and humans and between the occurrence of resistance among bacteria from animals, foods and humans. DANMAP data demonstrate that the ban on non-essential antibiotic use is working without major consequences for animal health. The Danish approach includes extensive monitoring systems to track drug resistance and antimicrobial use as well as services for research and analysis. Between 1992 and 2008, Danish farmers increased swine production by 47%, maintaining their standing as being among the largest exporters of pork in the world while exporting 90% of pork they produce. During this period, antimicrobial use in swine was reduced by 51%, from 100.4 to 48.9 mg kg^{-1} meat. Since the ban, production in poultry has increased slightly, while total antimicrobial use dropped with 90%: from about 5,000 kg in 1995 to less than 500 kg used (for therapy) in 2008 (Frank Aaerstrup, Technical University of Denmark, Lyngby) (Carol *et al*. 2011).

FAO (2007) reported that fish contribute more than 60% of the world supply of protein, especially in the developing countries. Fish accounts for about 17% of the global population's intake of animal protein. However, this share can exceed 50 percent in some countries. the contribution from fish as a source of protein is also significant: 44%in Senegal, 49% in Gambia, 51% in Ghana, 70% in Sierra Leone, 54% in Indonesia, 56 % in Bangladesh, 57% in Sri Lanka, 65% in Cambodia and 71% in the Maldives (World Bank 2013).

Global wild-catch fishery production was 93.7 million tonnes in 2011 and was the second highest ever (93.8 million tonnes in 1996) and 91.3 million tonnes in 2012. World aquaculture production continues to grow, albeit at a slowing rate. According to the latest available statistics collected globally by FAO, global aquaculture production attained another all-time high of 90.4 million tonnes (live weight equivalent) in 2012 (US$144.4 billion), including 66.6 million tonnes of food fish (US$137.7 billion) and 23.8 million tonnes of aquatic algae (mostly seaweeds, US$6.4 billion), with estimates for 2013 of 70.5 million and 26.1 million tonnes, respectively.

In addition, some countries also reported collectively the production of 22 400 tonnes of non-food products (US$222.4 million), such as pearls and seashells for ornamental and decorative uses. China alone produced 43.5 million tonnes of food fish and 13.5 million tonnes of aquatic algae that year. China has been responsible for most of the growth in fish availability, owing to the dramatic expansion in its fish production, particularly from aquaculture (FAO 2014).

However, this production is hampered by unpredictable mortalities that may be due to negative interactions between fish and pathogenic bacteria. To solve this problem, farmers frequently use antibiotic compounds to treat bacterial diseases (Cabello *et al*., 2006). Primary antimicrobials used in aquaculture include oxytetracycline, sulfamerazine, sulfadimethoxine-ormetoprim combination, and formalin. Estimates of total antimicrobial use in US aquaculture vary widely. MacMillan et al. (2003) estimated that 54,000 to 72,000 pounds of antimicrobials are used annually in aquaculture, while Benbrook (2002) estimated that this use is closer to 200,000 to over 400,000 pounds per year. Both estimates are significantly less than the estimates for livestock and poultry. However, in contrast to livestock and poultry, antimicrobials used in aquaculture enter surface waters directly, since they are added to the water through simple addition or via feed pellets. Research suggests that, an estimated 70% to 80% of drugs administered in aquaculture operations are released into the environment and that it is related to over-feeding and poor adsorption in the gut (Boxall *et al*., 2003). Current levels of antimicrobial use in aquaculture worldwide are not easy to determine because different countries have different distribution and registration systems. Nevertheless, Burridge *et al*. (2010) reported that the amount of antibiotics and other compounds used in aquaculture differed significantly between countries. Defoirdt *et al*., (2011) estimated that approximately 500-600 metric tons of antibiotics were used in shrimp farm production in Thailand in 1994. The large variation between countries is also illustrated by the use of 1 g per metric ton of production in Norway to 700 g per metric ton in Vietnam.

It strongly suggests that aquaculture, like terrestrial animal farming, is an important source for passage of large amounts of a variety of antimicrobials into the environment. Better information is needed to provide more accurate assessment of the classes and amounts of antimicrobials used in aquaculture in order to determine their potential impact on the general environment and on animal and public health. Usage data are unavailable for many groups of veterinary medicines and for several geographical regions, which makes it difficult to assess the risk of these substances to the environment.

As the preceding should be carefully taken into consideration for solving antimicrobial problems linked to environmental soundness lead to human and animal health, the following questions should be answered:
- What is the total quantity of pharmaceuticals used annually in each country?
- Which share of the total quantity of antimicrobials is used in animals and which share is used in humans?
- What portion of antimicrobial use is for non-therapeutic purposes?
- What portion of the antimicrobials used in agriculture is delivered through the feed and what portion through water?
- Which drugs and which portion is in food animals?

- Which uses are increasing and which are decreasing?

That is, continuous reliable data on the consumption of pharmaceuticals is essential and is the basis to set adequate and effective antimicrobial use policy.

Improvement of antimicrobial bioavailability to food-producing animals

Antimicrobials are often only partially metabolized in food-producing animals. They can be excreted as the parent compound (Kumar *et al*., 2005, Boxall 2008). For instance, up to 80% of tetracyclines are excreted by swine and poultry as the parent compound (Kumar *et al*., 2005, Khan 2008). Antimicrobial excretion of animal through manure have been mainly focused on environmental issues, but practical and actual problem of medication is mainly due to the low bioavailability and low absorption rate of formulated drugs with medication method across the species. To overcome bioavailability/solubility challenges for active pharmaceutical ingredients, rational formulation approach is necessary.

Veterinary drug faces the unique challenge of having to treat many types of domestic animal species, including mammals, birds and fishes. The main challenge for veterinarians is not to select a drug but to determine, for the selected agent, a rational dosing regimen because the dosage regimen for a drug in a given species may depend on its anatomy, biochemistry, physiology, and behaviour as well as on the nature and causes of the condition requiring treatment.

The World Watch List for Domestic Animal Diversity [WWL-DAD:3; (FAO 2000)] issued by FAO provides inventories of the species and breeds of the domestic animals used for food production. Of this number, 13 species from 7,616 breeds contribute to most of the world's food and agricultural production and are of veterinary interest. DAD is available to meet the increasing massive global demand for food and agriculture. The DAD component of biological diversity is essential to sustain efficient production from the world's broad range of food production environments required to satisfy many different needs of human communities Diversity of species and breeds are very important factors for medication of veterinary drug.

The multiplicity of species of veterinary interest with their large interspecies differences in pharmacokinetic (PK) and pharmacodynamic (PD). For effective treatment of disease in food-producing animals with a higher bioavailability of drugs, physiologically based pharmacokinetic modelling (PBPK) is a very useful tool, applicable to different species and breeds. Application of PBPK to food animals makes it possible to predict the rate of depletion of residues from edible tissues, which ensures food safety. Differences in the modalities of drug administration across species, in the case for food-producing animals, result from anatomical, physiological, and/or behavioural differences, depending on animal and management husbandry procedures.

Drug administration methods, such as intramammary infusion, intramuscular administration, subcutaneous administration, oral administration, injection site across species make differences between species for the bioavailability of drugs. In case of oral administration, as the most natural route of drug administration, there are many cases of interspecies differences in the modalities of oral administration, related to feeding behaviour. Administration of oral preparations, such as tablets or capsules, requires handler's skill sometimes. To get high bioavailability while saving medication efforts at the animal farm, a rational drug formulation across the species should be considered. Especially, a unique feature of veterinary drug are the collective treatments. Veterinary drugs may be treated to food-producing animals either individually or, at a herd or flock level. In general, the oral route has the benefit to be easily determined, due to the simultaneous and cheap treatment to large numbers of animals at the animal farm. The oral administration of drugs in drinking water or as medicated feed enable to avoid stress that may occur with individual treatments process, catching, restraining and injection, and to avoid both tissue damage and the presence of local residues. But, collective drug administration increases drug consumption more than selective/individual curative treatments, that is to say, overuse of drug occurs without prevalence of disease and inducing antimicrobial resistance and environment contamination.

Pour-on administration is the topical application of a pour-on formulation drug on the skin in a liquid formulation; it is a general mode of administration for endectocides (ivermectin, doramectin, eprinomectin, moxidectin) and to kill ectoparasites. Topical administration is easy and convenient, without risk of tissue damage and without persistent residues at the site of administration, in contrast to products containing the same drugs administered by subcutaneous or intramuscular injection. However, similar to drug treatment at aquaculture farm, the drugs typically remain in the environment and may flow out of animal farm facilities into sewage. In addition, considering the lick behaviour of cattle, pour-on is not a purely topical route of administration in cattle, under all normal husbandry conditions. As a natural behaviour, cattle lick themselves (allolicking) and lick other cattle (heterolicking). Due to the physiological behaviour of cattle, most of the ivermectin poured on skin was actually absorbed by the digestive tract (Laffont *et al*. 2001). This explains why the systemic availability of the pour-on drug formulation is both highly variable, unpredictable and harmful to environment.

Table 1. EU and US policies on non-therapeutic antimicrobials in food animal production over time.

Year	Event
1951 & 1954	US FDA approves penicillin, chlortetracycline and oxytetracycline as feed additives
1969	The UK government's 'Swann Report'. Recommendation: antimicrobials for animals be divided into two groups: feed additives used without a prescription and therapeutic agents used with a prescription; recommends restricting use of antimicrobial growth promoters, Hypothesised: Rising rates of multi-drug resistance bacteria are due to agricultural use.
1972–-1974	European bans on use of tetracycline, penicillin, and streptomycin for growth-promotion.
1973	US FDA proposes to withdraw its 1951 and 1954 approvals, unless industry can prove they are safe.
1986	Sweden bans use of antibiotics for growth-promotion in agriculture, as requested by Federation of Swedish Farmers.
1988	The Institute of Medicine of the National Academy in US issues a report that resistance from sub-therapeutic use if antibiotics is a 'potential human health hazard'. FDA tells the House and Senate committees that it has all the research it needs.
1988	Sweden stops use of all general prophylactic medications.
1993	Vancomycin-resistant *enterococci* (VRE) is reported in food animals in the UK.
1994	Denmark restricts direct sale of therapeutic antimicrobials from veterinarians and limits veterinary profits from antimicrobial sales. Denmark bans routine prophylactic use of antimicrobials.
1995	Denmark bans the use of avoparcin for all purposes in agriculture. DANMAP (Danish Integrated Antimicrobial Resistance Monitoring and Research Program) is initiated. Sweden and Finland join the European Union and lobby for EU-wide ban on agricultural growth promoters.
1996	Germany bans use of avoparcin.
1997	EU bans use of avoparcin. Netherlands bans use of olaquindox and carbadox. WHO Berlin meeting, "The medical impact of the use of antibiotics in food animals", concludes that use of medically important antimicrobials as growth-promoters should be stopped.
1998	The Copenhagen Recommendations: recognition of antimicrobial resistance as a global threat; call for development of new antimicrobials and establishment of a European Surveillance System. Denmark bans use of virginiamycin.
1999	Scientific Steering Committee of the European Commission recommends phasing out antimicrobial growth-promoters that are medically important and implementing disease-preventive methods. EU bans olaquindox and carbadox; suspends authorization of bacitracin, tylosin, spiramycin, and virginiamycin. EARSS (European Antimicrobial Resistance Surveillance System) established. Sweden bans use of remaining AGPs: flavophospholipol and avilamycin. UK's Advisory Committee on the Microbiological Safety of Food issues a report recommending improved veterinary training and surveillance of resistance.
1999	Citizen Petition filed by Center for Science in Public Health Interest (CSPI), Food Animal Concerns Trust (FACT), Public Citizen, Union of Concerned Scientists (UCS), and Environmental Defense Fund (EDF) requesting FDA to ban use of AGPs.
2001	ESAC (European Surveillance of Antimicrobial Consumption) launched to collect data on antibiotic use in ambulatory and hospital care.
2003	The Institute of Medicine says in a second report: "Mounting evidence suggests a relationship between antimicrobial use in animal husbandry and an increase in bacterial resistance in humans." FDA issues non-binding guidelines outlining that medically important antibiotics are unlikely to receive approval for use in food animals henceforth. Guidelines do not affect those antibiotics that have already been approved.
2004	The FDA tells feed manufacturers that it considers use of AGPs to be "high risk".
2005	FDA bans the use of fluoroquinolones for poultry following evidence that resistance rates for this antibiotic are rising. UCS, FACT, EDF, the American Academy of Pediatrics and the American Public Health Association file another Citizen Petition asking that the FDA ban medically important antibiotics already approved for use in growth promotion.
2006	EU bans use of all AGPs (Monesin sodium, Salinomycin sodium, Avilamycin, and Flavophophlipol).
2008	Amendments to the Animal Drug User Fee Act of 2003 require that manufacturers report on the quantity of antimicrobial drugs sold or distributed annually for use in food animals to the FDA.
2008	ESVAC (European Surveillance of Veterinary Antimicrobial Consumption Project): European Commission asks the European Medicines Agency to harmonize surveillance programs collecting data on antimicrobial sales and usage.
2009	Strategies to Address Antimicrobial Resistance Act (STAAR) introduced in Congress. The bill seeks to establish an Antimicrobial Resistance Office within the HHS Secretary's office as well as a public health advisory board to channel advice and expertise on the issue, and would reauthorize a number of antimicrobial resistance programs authorized in a previous law that have since expired. PAMTA re-introduced to Congress.

Table 1. (Continued)

2010	The FDA issues its draft voluntary guidance on limiting sub-therapeutic dosing. STAAR Act re-introduced in Congress. The FDA issues draft guidance "The judicious use of medically important antimicrobial drugs in food-producing animals
2011	FDA denies Citizens Petitions. Natural Resources Defense Council (NRDC) representing CSPL, FACT, Public Citizen, and UCS sues the FDA for failure to respond to the 1999 and 2005 Citizen Petitions. PAMTA introduced in Congress for the third time.
2011	EU commission proposes 5-year Action plan against the rising threats from antimicrobial resistance

Drinking water is the preferred mode of administration for drugs, especially for antimicrobials, because diseased poultry usually tend to stop eating but will usually continue to drink. To achieve an effective dose, drug concentration in the drinking water should be considered with the species specific daily water consumption. Within the same species, biological properties like body weight, age, gender, environment, lighting period, temperature and managerial factors, such as flock size, composition of the feed and Concentrated Animal Feeding Operation (CAFO) may influence individual animal water consumption.

Waste produced in aquaculture generally consists of faeces, excess feed, dead fish and other aquatic organisms, nutrients, antimicrobials, minerals, vitamins and pigments. Up to 15% of feed may be uneaten or spilled. Between 60% and 80% of dietary dry matter may be excreted in intensive aquaculture (Amirkolaie 2011). Antimicrobials used in aquaculture are administered to fish mostly in food and only rarely by injection or bath. Unconsumed medicated food is deposited by gravity in sediments under and around aquaculture sites. Of the ingested antimicrobials, approximately 80% pass into the environment in unabsorbed form in faeces or after absorption, in secreted forms in urine and other secretions (Björklund *et al*., 1990). These also accumulate in the sediments under and around the aquaculture pens from where they can be carried by water currents to sediments at distant sites (Buschmann *et al*., 2012).

Antimicrobials leached from sediments as well as from ingestion of uneaten medicated feed can also potentially affect free-ranging fish, shellfish and other animals in proximity to aquaculture sites (Björklund *et al*., 1990). Most antimicrobial use in livestock and fish requires a veterinary prescription, although individual treatment decisions are often made and administered by lay farm workers in accordance with guidelines provided by a veterinarian. But low bioavailability of drug leads to overuse and unnecessary use.

For a long time, various strategies have been widely investigated to enhance the bioavailability of poorly absorbed drugs in order to increase their clinical efficacy when administered orally. In case of aquaculture purpose, formulation development to enhance the physico-chemical properties to overcome the poor solubility, unstability/hydrolysis in water is considerable. Development of new antimicrobial needs high investments, long time, and efforts to register rather than improvement of formulation of existing antimicrobials. Furthermore, there is also a need to develop alternative therapies for bacterial pathogens in animal production, especially in aquaculture. Formulation development could contribute to the effective use with enhancing bioavailability and to reduce excretion of metabolized and parent antimicrobials for prevention of environmental exposure. For this, pharmacokinetic and pharmacodynamic based on the behaviour, physiology and biochemistry of species and breeds under farming environment should be also considered.

Antimicrobial resistances

Over the past several decades, recognition of the environmental load of resistant organisms has increased. With increased antimicrobial use in communities and among food-producing animals, antimicrobials from various sources are being discharged as active drugs into the environment through inadequate waste management. Resistance may occur in the absence of an antibiotic substrate, particularly in soils where organisms may bear resistance traits to drugs not detected in that environment. Moreover, the environment aids in the spread of resistance through gene transfer. Antibiotics affect not only the microbial flora of the treated individual or animal, but also the people (and animals) sharing the environment (Levi 2002). There have been long-time debates about recognition of antimicrobial resistance, mechanisms, definition of terminologies, pathway, and endpoint determination, *etc*. Therefore, this study describes and summarizes the current scientific information about antimicrobial resistance and recognition change of antimicrobial resistance from pharmaceutical-based-concept to environmental contamination.

1. Development of scientific recognition of antimicrobial resistance: from principle to surveillance

- Current understanding of the mechanisms associated with the development of antimicrobial drug resistance, international differences in definitions of resistance and the differences between bacterial tolerance and bacterial resistance

- Therapeutic strategies should be grounded in pathogen identification, an understanding of the susceptibility characteristics of that pathogen, and knowledge of the relationship between drug dose, pharmacodynamics and effect.

- Antimicrobial mechanisms of action: the magnitude of the inhibitory effect of some agents is primarily dependent upon the duration of drug exposure, for others it is largely a function of the rate and/or extent of drug exposure.

- Drug classification of concentration-dependent or time-dependent killing is primarily a function of the shape of its concentration-effect curve. Fluoroquinolones, which are typically classified as concentration-dependent agents, can act in a time-dependent manner if drug concentrations at the site of action decrease below bactericidal drug concentrations.

2. Definition of the term 'Resistance'

Natural resistance even prior to the use of antibiotics (Davies 1994), the emergence of resistance in previously susceptible bacterial populations has been attributed to the use of antimicrobial agents. Terms such as intrinsic and acquired resistance and single, multiple and cross-resistance have been introduced to describe the nature and clinical implications of these changes in bacterial subpopulations (Prescott and Baggot 1993). The Committee for Veterinary Medicinal Products (CVMP) in the European Medicine Evaluation Agency (EMEA, 1999) defined microbiological resistance as either:
(a) organisms that possess any kind of resistance mechanism or resistance gene; or (b) an infection where the bacteria do not respond to therapy. Davison *et al*. (2000) defined resistance;

1. Resistance must be regarded as a quantifiable (qualitative or quantitative) variable at the level of either the bacterial or host population and must be defined with respect to a reference population.

2. The detection methodology must possess known and quantified sensitivity, specificity, repeatability and reproducibility.

3. The target bacterial and host populations must be precisely defined.

4. The sampling framework must be fully specified, indicating how the samples are selected from the bacterial, host populations or the environment, including the various levels of organisation within these populations or ecosystems and the number of units from which samples are selected.

Table 2. Antimicrobial mechanisms of action versus resistance mechanisms.

Antimicrobial Mechanisms	Resistance Mechanisms
- Inhibition of cell wall synthesis. - Alteration of the 30s and 50s ribosomal subunits, resulting in a reversible inhibition of protein synthesis. - Inhibition of bacterial protein synthesis or the synthesis of aberrant proteins via the binding of drug to the 30s ribosomal subunit. - Alteration of nucleic acid metabolism. - Anti-metabolite activities.	A change in proteins made by the cell: - Alteration in ribosomal binding sites - Gene up-regulation (*e.g*. synthesis of inactivating enzymes such as β-lactamases) - Changes in target site binding (*e.g*. alteration in penicillin binding proteins) Changes in membrane permeability: - Porin closure - Efflux pumps Changes in protein synthesis caused by changes to DNA: - Plasmid-mediated - Mutational - Conjugation - Ingestion of DNA-materials

Table 3. Global examples of how 'resistance' has been defined from the differences in global surveillance systems.

Source	Resistance definition
National Antimicrobial Resistance Monitoring System –- Enteric Bacteria (NARMS)	Clinical Breakpoints (Clinical Laboratory Standards Institute)
European Union	Epidemiological cut-off values
The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP)	Epidemiological cut-off values
Enter-Net (the European Centre for Disease (ECDC) Prevention and Control)	Dependent on country but varies across Europe

Similar to the mentioned EMEA 1999 report, resistance is defined both from a clinical and an epidemiological perspective by the USFDA as it applies the term 'resistance' in their FDA/CVM Guidance #152. Table 2 describes the antimicrobial mechanisms of action versus resistance mechanisms. Table 3 represents how 'resistance' has been defined in the different global surveillance systems. Besides, the inconsistent definitions for resistance, there may be differences in the methodologies used to determine minimum inhibitory concentration (MIC) values as well.

3. Human versus animals

Since humans share a number of pathogens with food-producing animals (pathogenic or benign), it is frequently alleged that particular resistance patterns detected in human pathogens result from veterinary antibiotic use. The use of antibiotics as growth-promoters in agriculture was challenged and was subsequently banned in Europe (Phillips 2007). Certain antimicrobial agents may contribute to the prevalence of plasmid-mediated drug and indeed chromosomal resistance in bacteria that infect both humans and animals (Turnidge 2004). Since animals and humans provide overlapping reservoirs of antimicrobial resistance determinants, the issue cannot be ignored. The concern is, therefore, that indiscriminate use of antimicrobial agents in food animal production may result in the transfer of resistant bacteria and resistance genes to human pathogens, thereby compromising the treatment of infectious diseases in public.

Wassenaar and Silley (2008) concluded that multidrug resistance is a more severe problem in human pathogens than in veterinary pathogens. Although pathogens isolated from both human and veterinary hosts appeared to have higher incidences of resistance as compared to those restricted to a single animal host, pathogens expressing the most marked (broad) drug resistance profile were found to exclusively infect humans. Martinez and Silley (2010) suggested 'Resistance endpoint determination by pharmacokinetic considerations':

-Relationship between AUC/MIC value with dose change;
-Mutation Selection Window (MSW);
-Duration of dosing;
-PK/PD parameters;
-MIC;
-Clinical susceptibility breakpoints.

The Veterinary Antimicrobial Susceptibility Testing Subcommittee (VAST) of the Clinical and Laboratory Standards Institute (CLSI) has recently published their M37 guidance, which describes the data and data interpretation used to establish veterinary susceptibility criteria (Clinical Laboratories Standards Institute 2007).

For veterinary drugs, these susceptibility breakpoints are disease-indication and target-animal-species specific, thereby allowing for potential differences in the susceptibility breakpoints across target animal species and within a target animal species. By establishing the criteria correlating the required levels of drug exposure and the probability of success of therapy, clinically derived susceptibility breakpoints can minimise the risk of repeated exposure to suboptimal antimicrobial drug concentrations, which is a major factor contributing to the development of resistant bacterial strains.

4. Tolerance versus Resistance

Drug resistance and drug tolerance differ, in that resistance mechanisms prevent the antibiotic from hitting a target, whereas tolerance works by shutting down the targets (Lewis 2008). Unlike resistance, which frequently involves genotypic changes, tolerance is the result of phenotypic changes. Moreover, in contrast to resistance, which is a function of individual cells, tolerance reflects a community based synchronisation of gene expression that changes the bacteria from a growing to a slow or non-growing state (mostly by biofilm formation).

5. Antimicrobial resistance in the environment: sources, fate, relationship between occurrence of antimicrobial substance and resistance and identification of effects and risks

Antimicrobials from various sources are being discharged as active drugs into the environment through inadequate waste management and are delivered to crops in manure from animals fed antimicrobials for therapy and growth promotion.

The vast majority of the literature over the past half-century has focused on resistance in clinically relevant human pathogens and to lesser extent bacteria that cause disease in animals. One of the hallmarks of antimicrobials use has been that resistance emerges relatively rapidly in pathogens, within a year or as long as a few decades following the deployment of new drugs. However, there is a growing understanding that antimicrobial resistance is a natural (and perhaps default) property of virtually all bacteria (Wright, 2007, 2010; Allen *et al*., 2010).

It is estimated that ~5 10^{30} bacteria occur on the planet and only a relative handful cause human or animal disease (Whitman *et al*., 1998). Since environmental bacteria vastly outnumber human pathogens both in numbers and in genetic diversity, these organisms are potential reservoirs of resistance genes. Thus,

- The environment represents an enormous reservoir of antibiotic resistance genes.

-The generalized misuse and overuse of antimicrobial compounds over the last decades have resulted in an accelerated evolutionary process leading to bacterial resistance. Microorganisms are being exposed to relatively high doses of these compounds due to human activity not only in the clinic, households, and industrial settings but also in natural environments.

The induction of resistance determinants by certain environmental cues is known as adaptive resistance. This type of resistance is transient and, in general, the initial levels of susceptibility can be restored after removing the inducing signal.

As mechanisms of adaptive resistance, up-regulation of efflux pumps, modification of the cell envelope, stress responses, production of inducible enzymes, and muticellular behaviors are suggested.

- A better understanding of the possible triggers and mechanisms of stepwise and adaptive resistance is essential in order to plan new treatment strategies, the design of novel therapeutics, the use of combination therapy and rational planning of more adequate programs in terms of dose and timing. it is very important to avoid the exposure of the pathogens to sub-inhibitory concentrations of antimicrobials during treatment.

- Antibiotic production is a common attribute among soil bacteria, and examples can be found in groups of *pseudomonads* and *Erwinia* strains, within *actinobacteria* and *myxobacteria*, and *Bacillus* species. The *actinobacteria* are often regarded as notable antibiotic producers, as members of the class *Actinobacteria* synthesize some of the most economically important antibiotics still used clinically, including drugs such as erythromycin, gentamicin, tetracycline, the antitumor agent daunorubicin, the immunosuppressant rapamycin, and the antihelminthic agent avermectin (Paradkar *et al*., 2003).

-Thus, it is important to appreciate that resistance in soil bacteria is common, considering the antibiotic biosynthesis in natural environments.

Although antimicrobial concentrations in most soils are not at the therapeutic levels to cause inhibitory effects on bacterial population, they may still influence the selection of antimicrobial resistant bacteria in the environment (Gavalchin and Katz 1994; Nygaard *et al*., 1992; Kümmerer 2003; US EPA 2002). Many antimicrobials have a strong tendency to bind with soil particles and these soil-bound antimicrobials also possessing the antibacterial activity (Chander *et al*., 2005). Antimicrobials can therefore retain their bactericidal properties whether free or adsorbed and are thus biologically relevant in soil antagonism even when they cannot be detected.

-Both antibiotics and antibiotic-resistant bacteria are moved by water and wind as well as by transportation of goods and people around the world. One result of this has been the spread of specific strains around the world.

- Antibiotics are used for both human and agricultural activities for prevention and treatment of infections, as well as food additives and growth-promoters in food production in some part of the world and include animal husbandry, aquaculture, fruit crops, and bee-keeping. All of these activities contaminate the environment, which provides selective pressure on the resident environmental bacteria to become antibiotic resistant and in some cases increases transfer of specific tet genes (Facinelli *et al*., 1993).

- Antibiotics can be found in domestic animal manure, which may be transferred when this manure is applied to fields or stored in lagoons. Antibiotics are sprayed onto crops, which contaminate the surrounding soil, sediment, and groundwater. Antibiotics may be incorporated into the food given to farm animals and fish, which will, in turn, contaminate the surrounding area. Antibiotics from human therapeutic use, especially from hospital effluents, are a continuous source of pollution and are considered part of the 'emerging contaminants' in municipal waste with concentrations of tetracycline varying from nanograms to micrograms per liter (Verlicchi *et al*., 2010).

- There is continuous mixing of environmental and non-environmental bacteria that provides multiple opportunities for horizontal genetic exchange of antibiotic resistance genes.

Extensive research has been conducted on this subject and several conclusions have been drawn.

6. Resistome

The term resistome was coined by D'Costa *et al*. (2006) referring to 'all resistance determinants present in soil' as 'the soil resistome'. Wright (2007) redefined resistome as the collection of all the AR genes in microorganisms: 'the resistome' is a collection of all the antibiotic resistance genes and their precursors in pathogenic and non-pathogenic bacteria. It includes resistance elements found in both pathogenic bacteria and antibiotic-producing bacteria, and cryptic resistance genes (which are not necessarily expressed) that are present in bacterial chromosomes. The resistome also includes precursor genes that encode proteins with modest antibiotic resistance activity, or affinity to antibiotics, that might evolve into effective resistance genes.

Thaker *et al*. (2010) used the original concept: 'the resistome concept refers to the aggregate of all antibiotic resistance mechanisms'. The implications of wide pool of resistance genes to human and ecological health have been extensively discussed in recent years, and concern increases due to the ability of antimicrobial resistance genes to rapidly evolve and to be mobilized into other hosts. However, the characterization of the environmental reservoirs of antimicrobial resistance (AR) is far from being satisfactory and clearly our understanding of the microbial ecology of resistance is incomplete. The reasons many microorganisms cannot be cultivated under laboratory conditions are not entirely understood, but in general this results from the impossibility to mimic the natural conditions, using instead highly artificial and restrictive growth conditions (Barer and Harwood 1999). The employment of culture-based methodologies introduces biases not only due to the unculturability of most microorganisms but also to the unpredictable different levels of cultivability of different phylogenetic groups.

Culture-independent approaches potentially useful for AR assessment and to summarize novel findings that have already arisen from studies based on such methodologies. AR, which can potentially be clarified using culture-independent approaches and as culture-independent molecular techniques, PCR, quantitative PCR, hybridization, PCR-DGGE, Gene libraries, metagenomics, pyrosequencinf, and exogenous plasmid isolation will be very useful tools.

Fate and occurrence of antimicrobials in the environment

Contrary to pesticide registration, veterinary medicinal products' environmental impact data are not mandatory required in most of countries. Pesticides are still perceived as environmental contaminant from the 1950's, but pharmaceuticals including veterinary drugs are mostly regarded as medicine not as environmental contaminants. Environmental and food safety issues of pesticide have been investigated widely with the development of analytical instrument such as gas-chromatograph, gas-chromatograph linked to a mass-spectrometer and nuclear magnetic resonance *etc*. Physico-chemical properties of pesticides, lipophilic, hydrophobic, volatility and stability, are employed to analyse them in various matrices with these instruments. The physico-chemical properties of veterinary drugs including antimicrobials are water soluble, non-volatile, stability depending on pH. These characteristics may often act as barrier to analyse them in various matrices. With the first launch of commercial LC-MS/MS in 1989, it became possible to analyse antimicrobials in diverse matrices with identification of the metabolites/degradation products as well. LC–MS enables identification and quantification without derivatisation and typically results in lower detection limits (below 1 ng L^{-1} and 1 ng g^{-1} for liquid and solid samples, respectively) and better precision than GC–MS methodologies.

Once in the environment, it is difficult to predict how quickly antimicrobials will degrade, whether they come from animal use, human use or manufacturing, as they are very diverse chemically. Some degrade easily, while others bind to organic matter and can persist in their active states for long periods of time. This adds to the need for further study of this issue. From the 1990's, a number of researchers began to study wastes from intensive animal farms and their sewage often contained residue of pharmaceutical chemicals, in the name of PPCPs (Pharmaceuticals and Personal Care Products, Daughton and Ternes 1999).

The best way to reduce overall antimicrobial use is to establish targets or limits for antibiotic use in agriculture and aquaculture, to an agreed limit for each country, whilst allowing individual countries to work out the best way to meet their goals. Some companies have already made efforts to improve their environmental management through risk assessment frameworks. However, these do not currently consider the risks associated with rising resistance (Murray-Smith *et al*., 2011).

Veterinary medicinal products' (VMP) environmental risk assessment (ERA) is formally different from that for human pharmacologically active substances (PAS). VMP ERA, which had been developed independently in several countries has been harmonised between the EU, Japan and USA in the so-called International Cooperation for the Harmonisation of technical requirements for the registration of Veterinary medicinal product (VICH) process. Actually, similar to the current situation for the human pharmaceuticals environmental risk assessment (PERA). Canada, Australia and New Zealand have adopted (and further countries may accept) the VICH PERA guidelines, which are divided into two phases. Phase I is mainly a decision tree for filtering out those VMPs where no ERA is needed (VICH 2004). Phase II is divided into three major branches, aquaculture, intensively reared terrestrial animals and pasture animals, due to different entry pathways into the environment. General data requirements for Phase II (Tier A) are:

- Water solubility (OECD 105)
- Dissociation constants (OECD 112)
- UV–visible absorption spectrum (OECD 101)
- Melting point/range (OECD 102)
- Vapour pressure (OECD 104),
- *n*-Octanol/water partition coefficient (OECD 107 or OECD 117)
- Soil adsorption/desorption (OECD 106)
- Soil biodegradation (OECD 307)
- Degradation in water/sediment systems (OECD 308)
- (Optional) photolysis in water (OECD 316)
- (Optional) hydrolysis (OECD 111)

These requirements are almost the same as US EPA's 'Test guidelines for pesticides and toxic substances'. US EPA test guidelines are more detailed than VICH's, for instance the 'Fate, transport and transformation test guidelines' are divided into 'Laboratory transport test guidelines', 'Laboratory abiotic transformation test guidelines', 'Laboratory biological transformation test guidelines', 'Transformation in water and soil test guidelines', 'Field dissipation test guidelines', 'Ground water monitoring test guidelines' and 'Volatility from soil test guidelines'. Some of the 'Ecological effects test guidelines' deserve to consider as an experimental standard, for example, soil microbial community toxicity test, seedling emergence and seedling growth, plant uptake and translocation test.

But in contrast to pesticide registration data, it is rare to find out the VMP data achieved by test guideline from the governmental document and scientific literatures. Although, most of antimicrobials for food-producing animal have been already

developed and registered before suggestion of the VICH guidelines, it is necessary to identify the respective characteristics through the tests following the guidelines. Especially, whenever drug formulation changes, these experimental works should be accomplished.

In 1998, there were three notable articles. Arcand-Hoy *et al*. (1998) highlighted the importance of considering human estrogen agonist and veterinary androgen agonist pharmaceuticals as potential causative toxicants from point and nonpoint source effluents. In addition, review papers on pharmaceuticals in the environment by Halling-Sorensen *et al*. (1998) and Ternes (1998) were published. In 1999, another review paper, by Daughton and Ternes (1999], considered Pharmaceuticals and Personal Care Products (PPCP) in the environment and by doing so coined the PPCP acronym, which remains pervasive. Subsequently, a precipitous number of workshops, symposia, special meetings, and publications related to pharmaceuticals in the environment have occurred (Brooks *et al*., 2012). Some of the important developments and events related to pharmaceuticals in the environment included formation of the Society of Environmental Toxicology and Chemistry (SETAC) Pharmaceuticals Advisory Group in 2005, Pellston workshops by the SETAC on veterinary medicines in 2006 (Crane *et al*., 2008), a special issue of Environmental Toxicology and Chemistry entitled 'Pharmaceuticals and Personal Care Products in the Environment' in 2009, International Conferences on the Occurrence, Fate, Effects, and Analysis of Emerging Contaminants in the Environment in 2011, and 'Effects of Pharmaceuticals and Personal Care Products in the Environment: What are the Big Questions?' was held by Health Canada/SETAC in April 2011 were mainly held by environmental scientist and government authorities *etc*.

These events mainly focused on the current state of science and provide recommendations in following areas:
1) Risk assessment, management, and communication for veterinary medicines in the environment
2) Exposure assessment of veterinary medicines in the terrestrial environment
3) Effects assessment of veterinary medicines in the terrestrial environment
4) Exposure assessment of veterinary medicines in the aquatic environment
5) Effects assessment of veterinary medicines in the aquatic environment

Though the information in this timely area continues to rapidly expand, it appears critically important to now consider the lessons learned from the study of veterinary drugs in the environment and formulate directions for future efforts.

Veterinary drugs enter the environment by a number of different pathways. The environmental risk assessment of veterinary medicinal products is still concerned with emission at or after use of the product. However, emissions may occur at any stage in a product's life cycle, including during production and during the disposal of the unused drugs, containers, and waste material containing the product, such as manure, fish water, and other dirty water (Montforts 1999). The possible emission routes to the environment are followings:
- Manufacturing and formulation: air, water (waste water effluent), land (solid waste).
- Aquaculture: medicated feed, injection, topical applications, bath formulation, deposition of drugs from uneaten feed or faeces on, or in, under-cage sediment can be a major route.
- Agriculture (Animal Farming): waste material (including manure or slurry and 'dirty' waters), via excretion of faeces and urine-manure, spillage during external application, via wash-off from farmyard hard surfaces (*e.g*., concrete), or by direct discharge to the environment, topical applications, plunge dipping or sheep dipping; pour-on formulations; and the use of showers or jetters.
- Companion and domestic animals: runoff or leaching from on-ground faecal material *etc*.
- Disposal of unwanted drugs (waste/obsolete pharmaceuticals): if collection (return) system is not available on-farm disposal or landfill.

From these emission sources, exposure scenario could be summarized as below:
• Runoff during or following during external application;
• Releases of veterinary medicine in waste material (manure, dirty drinking water, and aquaculture water) during clean-up, storage, removal, and land application;
• Excretion via faeces and urine (grazing animals);
• Spillage at external application site or direct exposure outdoors;
• Disposal of containers (bottles and flea collars).

Experimental studies on the entry, fate and transport of veterinary drugs in soil and aquatic systems
Veterinary drugs will either be excreted directly to soil or applied to soil in manure, slurry, or compost. Veterinary drugs applied to soil can be transported to aquatic systems via surface runoff, subsurface flow, and drainflow. The extent of transport via any of these processes is determined by a range of factors, including the solubility, adsorption/desorption behaviour, and persistence of the contaminant; the physical structure, pH, organic carbon content, and cation exchange capacity of the soil matrix; and climatic conditions such as temperature and rainfall volume and intensity (Boxall *et al*., 2006).

Recently, a number of studies have explored the fate and transport of veterinary medicines. Lysimeter, field plot, and full-scale field studies have investigated the transport of veterinary drugs from the soil surface to field drains, ditches, streams,

rivers, and groundwater (*e.g*. Aga *et al*., 2003; Kay *et al*., 2004, 2005; Stoob *et al*., 2007, Kwon 2011). These studies did not suggest the standard or guideline for experimental design, only for providing the tendency or probability of dissipation or degradation of veterinary drugs (*cf*. Figures 1 and 2). In case of a dissipation study, like estimates of half-life, it is prone to depend on residual concentration, matrices' properties *etc*. Kwon (2011) suggested an experimental design 'Guidance on estimating soil persistence and degradation kinetics from environmental studies on veterinary pharmaceuticals for environmental risk assessment' in Korea. Proposed guidance consisted of three parts: i) Laboratory Soil Experiment, ii) Field Soil Dissipation Study, and iii) Estimation of DT_{50}/DT_{90}. Proposed guidance is a requirement for registration of veterinary pharmaceuticals fit for purpose in Korea. Kwon (2011) also suggested soil column design (*cf*. Figure 3) and leaching experiments to estimate ground water contamination with a modification of the method described by the US EPA (1985).

The degradation of veterinary drugs approved for use in aquaculture is very important in the evaluation of the impact of these drugs on the environment and to ensure safe food production.

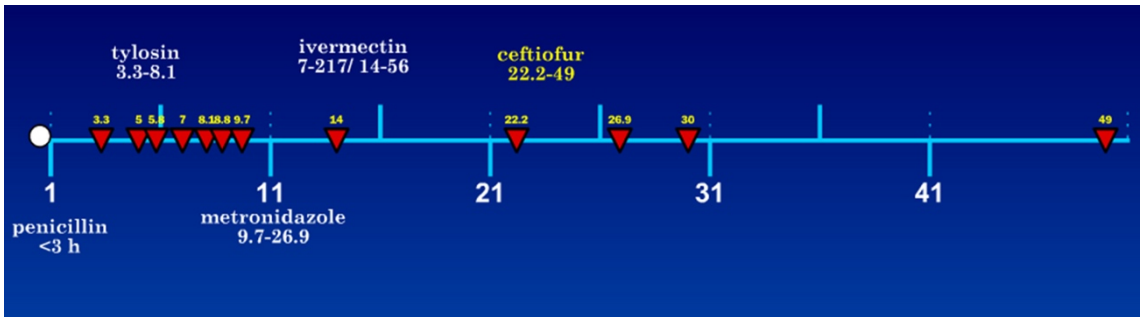

Figure 1. Persistence of some major classes of veterinary drugs in manure.

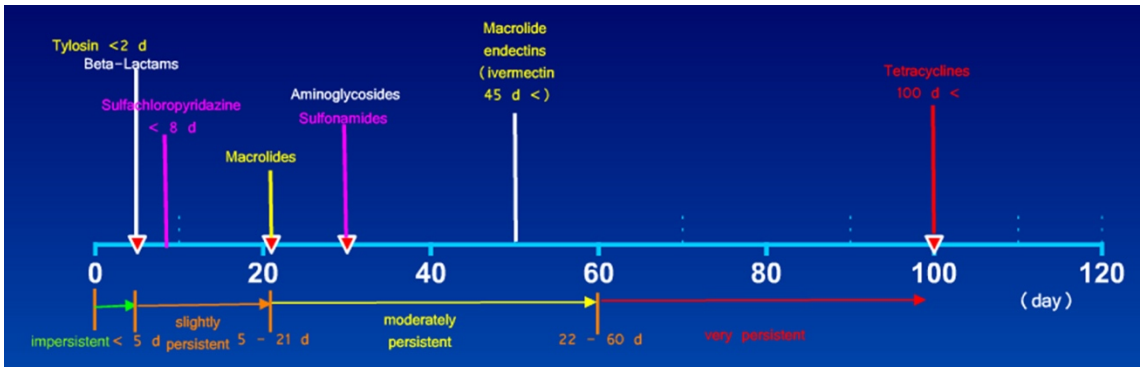

Figure 2. Persistence of some major classes of veterinary drugs in soil.

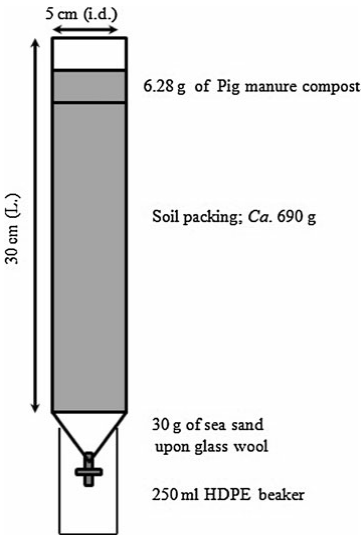

Figure 3. Schematic design of the soil column for experiments with manure compost.

Table 4. Monitoring concentration of veterinary drugs in swine and chicken Farms in Korea

Farm	Water (ng g^{-1})	Compost (ng g^{-1})	Soil (ng g^{-1})	Remarks
Chicken-A	SMTZ: 323.16 PeniC: 0.01	SMT: <PQL~5.14 SMTZ: 26.93~2,558.07 STZ: 2.80~4.38 PeniC: <PQL~0.37 OTC: 88.24 CTC: 207.21 AMOX: 7.19	N/A	>30,000 head Non-HACCP Composting Ground water
Chicken-B	SMTZ: 0.16 PeniC: 0.01 CTC: <PQL	SMT: <PQL~12.07 SMTZ: <PQL~11.05 STZ: <PQL~1.60 PeniC: 0.98~0.51 CTC: <PQL	N/A	>30,000 head Non-HACCP Composting Ground water
Chicken-C	STZ: 0.05 OTC: 0.51 CTC: <PQL	SMT: <PQL~4.39 SMTZ: 42.73~433.65 STZ: <PQL~4.52	N/A	>37,000 head Non-HACCP Composting Ground water
Chicken-D	SMTZ: 0.21 STZ: 0.02 PeniC: 0.01 CTC: <PQL	SMT: 4.52 STZ: <PQL~0.90 CTC: <PQL AMOX: 5.00	N/A	>55,000 head/farm HACCP Composting Ground water
Swine-A	SMTZ: 0.38 PeniC: 0.01 CTC: <PQL	SMT: 28.47~2,270.23 SMTZ: 2.75~10.60 STZ: <PQL~8.16 PeniC: <PQL~0.61 CTC: <PQL~361.2	N/A	>3,500 head Non-HACCP Composting Ground water
Swine-B	STZ: 0.04 CTC: <PQL	SMTZ: <PQL~17.44 STZ: 39.02~39.657 PeniC: <PQL~0.71 CTC: <PQL	STZ: 177.30	>300 head Non-HACCP Composting Ground water (Food Wastes)
Swine-C	SMT: 0.68 SMTZ: 0.46 STZ: 0.09	SMT: 101.34~1,457.96 SMTZ: <PQL~4.16 STZ: 50.49~566.19 PeniC: <PQL~0.36 OTC: 13.19~47.27 CTC: <PQL~51.21	N/A	>130 head Non-HACCP Composting Ground water (Food Wastes)
Swine-D	STZ: 0.03 FLO: 2.81	SMT: <PQL~2.12 SMTZ: <PQL CTC: <PQL	SMTZ: 21.15 STZ: 1.10	>300 head Non-HACCP Composting Ground water (Food Wastes)

PQL (ng g^{-1}): water, 0.01; compost & soil, 0.33

In case of aquaculture, there are generally four types of systems are used: i) ponds, ii) net pen cage, iii) flow-through systems and iv) recirculating systems. The potential exposure routes differ between the systems. Floating and bottom-culture systems are also used for culturing of mussels, clams, and oysters, but drugs are rarely used to treat these organisms. In each of these systems there are two major sources of medicine release: emissions from bath treatments or medicated feeds. Furthermore, the unique environment for aquaculture means that the administration of veterinary drugs to aquatic animals is more of a challenge than it is to terrestrial animals, depending on the species and the rearing system used (for example polyculture and aquaponic system). The environmental fate of drugs in aquatic system is dependent not only on the intrinsic properties of the drug substance but also on a range of other factors including the composition, the administrative route, and environmental factors including temperature, salinity, dissolved oxygen, and the number and species of microorganisms *etc*.

Therefore, the environmental fate and impact of veterinary drugs are also related and depend on the local aquaculture practices and circumstances.

As an internationally harmonized guideline, OECD 308 Test Guideline describes a laboratory test method to assess aerobic and anaerobic transformation of organic chemicals in aquatic sediment systems. The method permits the measurement of (1) the transformation rate of the test substance in a water-sediment system and in the sediment (2) the mineralization rate of the test substance and/or its transformation products, (3) the distribution of the test substance and its transformation products between the two phases during a period of incubation in the dark, at constant temperature, and (4) the identification and quantification of transformation products in water and sediment phases including mass balance.

In recent years there have been significant advances in our understanding of the sources and fate of veterinary drugs in aquatic systems. Alongside this, detailed guidance has been developed on regulatory approaches for assessing aquatic exposures, and a range of exposure modelling approaches and scenarios have been developed. However, there are still a number of significant gaps in our knowledge. Even though, lack of the international harmonized guideline and standard for validation of analytical method from the environmental samples, US EPA Method 1694 enable to analyse 73 pharmaceuticals and personal care products in 2007. Harris *et al.* (2012) reviewed the residue concentration and occurrence of antimicrobials in the environment (water, wastewater treatment plant, sludge and agricultural area). Occurrence of various antimicrobials and their metabolites, including amoxicillin, bleomycin, chloramphenicol, sulfamethoxazole, trimethoprim, clarithromycin, roxithromycin, ciprofloxacin, norfloxacin, ornidazole, tetracycline, metronidazole, were reported with most levels at ppb concentration throughout the world, mainly Europe and United States. Investigation of relationship between antimicrobial residues and dissemination of resistance in the environment is meaningful for human and ecological health.

Table 5. Frequency of occurrence of residue of veterinary drugs for fish from the fresh water aquaculture system in Korea.

Water (31)	oxytetracycline	doxycycline	ofloxacin	enrofloxacin
Culturing water	7	2	1	8
Effluent (6)	4		3	4
Ground water (7)	3	1		6
Reservior (2)	1			1
Total Frequency of occurrence (%)	48.4	9.7	12.9	61.3

Water (31)	perfloxacin	ciprofloxacin	norfloxacin	sulfadimethoxine
Culturing water	9	6		10
Effluent (6)	4	4	3	3
Ground water (7)	4	2		4
Reservior (2)	1	2		1
Total Frequency of occurrence (%)	58.1	45.2	9.7	58.1

Water (31)	sulfamethoxazole	sulfadiazine	oxolinic acid	erythromycin	florfenicol
Culturing water	9	7	10	4	5
Effluent (6)	5	3	4		1
Ground water (7)	5	3	2	3	1
Reservior (2)	2	1	1	2	1
Total Frequency of occurrence (%)	67.7	45.2	54.8	29.0	25.8

Tables 4 and 5 present the occurrence of veterinary drug from animal farms and fresh water aquaculture system in Korea (Kwon J-W. unpublished). These indicate low concentration levels and occurrence of mixtures at the same location. The extent of direct adverse effects, particularly to low dose long-term exposure and mixture toxicity is uncertain. It is necessary to fully understand the risk associated with antimicrobials in the environment to make appropriate decision regarding mitiga-

tion and human and ecological health. When regarding the residue occurrence of antimicrobials related to agricultural farming system, governmental authorities should consider to set a maximum residue limit of antimicrobials in agricultural products like crops, vegetable and feed in the future.

Conclusions

It is encouraging that national and international publications suggesting policies and practical codes to protect environmental from contamination and to mitigate the occurrence of antimicrobial resistance appear increasingly. They suggest the reduction of unnecessary use and of waste of antimicrobials for food-producing animals.

The huge benefit of veterinary antimicrobial use is the production of healthy food animals in large quantities. The drawback is, however, environmental contamination and the prevalence of resistant microbes. The absolute reality and adverse effects are now proven by scientific research results over the last decades and are beyond simple estimation.

This study reviewed advanced theories, research results and altered policies of some countries and international organizations on antimicrobial issues. It is especially focused on food-producing animal and the environment and suggests perspectives and practical approaches to meet some of the challenges.

Above all, to enable active and effective management and to plan reactions against the raised issues, more detailed and reliable usage data of veterinary drugs and judicious use is needed. Furthermore, action is needed on bioavailability enhancement by development of drug administration method, formulations to reduce excretion quantities, sources and pathways, comprehensive recognition of antimicrobial resistance with strategic approach and strengthen and implementation of environmental risk assessment of veterinary medicinal products as preventive measures.

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A UNIQUE COLLABORATIVE STUDY TO ASSESS CONFIRMATORY ANALYSIS PERFORMANCE CRITERIA IN VETERINARY DRUG RESIDUE ANALYSIS

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Abstract
Performance criteria for confirmatory analysis are established in CD 2002/657/EC. Since its publication, severe technical advances occurred and new approaches are applied. Therefore, a critical assessment of these criteria is justified.
In this study a large number of challenging sample extracts were prepared containing 100 registered and banned veterinary drugs at relevant levels. In the initial study, robust data (Selected Reaction Monitoring mode) was collected using four different instrumental set-ups and instruments from different vendors. Based on this highly relevant data set (>39,000 data points), the performance criteria for confirmatory analysis were assessed. The outcomes were verified based on a collaborative trial including laboratories from all over the world.
Furthermore, we comprehensively evaluated the use of different MS techniques (QqQ, TOF, Q-TOF, orbitrap, Q-orbitrap) and different acquisition modes with respect to selectivity. The initial study included full scan, targeted MS^2 product ion scanning (mimicking SRM at low and high mass resolution), and all ion fragmentation at high mass resolution. The unique collaborative study included LR-MS SRM mode, HR-MS full scan, AIF and targeted MS^2. The outcomes should serve as input for the revision of currently applied criteria and the establishment of a new, globally accepted, criterion document for confirmatory analysis.

Introduction

It is of utmost importance to ensure that decisions on the approval or rejection of food products are based on sound analytical science and evidence. The reliability of an analytical result obtained during residue testing depends on the type of methodology used and the performance criteria applied. These conditions come together in the statement that the method used was: “fit for purpose and adequately validated”. For confirmatory methods, the phrase “fit for purpose” includes that the reliability of the confirmation of the identity is beyond reasonable doubt. For this purpose European Commission Decision (CD) 2002/657/EC introduced the concept of ‘identification points’. In residue analysis, mass spectrometry (MS), either in combination with liquid chromatography (LC) or gas chromatography (GC), was assigned as the main technique for confirmation of the identity of banned and regulated substances (2002/657/EC).

Besides statements about techniques and methods being ‘fit for purpose and adequately validated’, confirmatory analysis involves the comparison of several physico-chemical properties, such as the chromatographic retention time (RT) and (selected parts of) the mass spectrometric fragmentation pattern of the detected compound with a reference compound. Selectivity, which is defined as “the power of discrimination between the analyte and closely related substances…” , is the main parameter of interest in the confirmation of the identity of a compound. Criteria for confirmatory methods in the analysis of food contaminants and residues, sports doping and forensic sciences, are laid down in several regulations. Recently, the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) included performance criteria in annex C of CAC/GL 71-200912, primarily based upon the EU guidelines (2002/657/EC).

Since the publication of CD 2002/657/EC, now more than a decade ago, technology available to the residue testing laboratory has advanced significantly with the development of fast scanning and sensitive mass spectrometric (MS) instruments that can be combined with orthogonal separation techniques like ultra-high performance liquid chromatography (UHPLC) (André 2001). These technological innovations have triggered the development of new analytical multi-residue and even multi-class screening and confirmatory methods that include simplified sample preparation procedures (Berendsen 2013a).

In this study, we aimed to assess existing criteria in the light of currently applied methodologies and to develop new evidence-based criteria applicable to modern and emerging analytical methods applied in the field of veterinary drug residue testing. Furthermore we compared selectivity in high resolution mass spectrometry (HR-MS) using different acquisition modes to the gold standard for confirmatory analysis: tandem mass spectrometry in selected ion monitoring.

Results and Discussion

It is of utmost importance to ensure that decisions on the approval or rejection of food products are based on sound science and evidence. The reliability of an analytical result obtained during residue testing depends on the type of methodology used and the performance criteria applied. Performance criteria for residue analysis of veterinary drugs in products of animal

origin were established in 2002 in Commission Decision 2002/657. Since the publication of this document, technology available to the residue testing laboratory has advanced significantly. The technological innovations have triggered the development of new analytical multi-residue and even multi-class methods. Because current legislation does not sufficiently cover all new techniques available and multi-compound approaches used, a study was designed to re-assess the performance criteria and to propose new/amended criteria. Following a survey towards the instrumental state-of-the-art and ongoing new developments, a selection of instrumentation and techniques to include in the study was made. The selection includes instruments and techniques that are readily available and are applicable to residue analysis of veterinary drugs, including:

- HPLC using fully porous and core-shell particles
- UHPLC
- Micro-fluidics
- Triple-quadrupole mass spectrometry
- Qq-Linear Ion-Trap mass spectrometry
- Time of Flight mass spectrometry
- Orbitrap mass spectrometry
- Hybrid instruments (e.g. Q-TOF, Q-Orbitrap)

A method used for residue analysis should be "fit for purpose and adequately validated". For confirmatory methods the phrase "fit for purpose" includes that the reliability of the confirmation of the identity is beyond reasonable doubt. For this purpose Commission Decision 2002/657/EC introduced the concept of "identification points". Besides the identification point system to assure adequate set-up of instrumentation, CD 2002/657/EC includes performance criteria regarding relative ion abundances and retention time (RT). The relative abundance of the two product ions, the ion ratio (IR), should be within certain ranges for confirmation of the identity of a substance. The acceptable tolerance of the IR varies with the relative abundance of the two product ions. For RT, currently a 5% tolerance is allowed and a 2.5% range for the relative retention time (RRT). In this project, a substantial amount of data was collected to assess the ion ratio and (relative) RT criteria and, furthermore, new techniques were evaluated for applicability in confirmatory analysis.

Regarding the confirmation criteria, it was concluded that the ion ratio deviation is not related to the ion ratio itself, but rather to the intensity of the lowest product ion. Therefore, a fixed ion ratio deviation tolerance reflects the real life situation better. A criterion for the maximum ion ratio deviation of 50% (relative) is proposed. Furthermore, it was observed that RT shifts, when using gradient elution, as is common practice nowadays, are mainly observed for early eluting compounds. Therefore, a maximum RT deviation of 0.2 min and a maximum RRT deviation of 2% are proposed.

It is concluded that selectivity depends on several factors, including the compound, the matrix, the sample clean-up procedure, and the mass resolution. As a result, no general criterion for minimum mass resolution to be used in high resolution mass spectrometry (HR-MS) can be established and the sample clean-up procedure and mass resolution should be selected fit-for-purpose. Alternatively, it is suggested to state that, in HR-MS, the mass error should be ≤±5 ppm in confirmatory analysis. To be able to achieve this, in some cases 10,000 mass resolution will prove adequate, in other specific cases a mass resolution >50,000 might be necessary. As a result, during the validation of a method it should be demonstrated that mass resolution is adequately set resulting in ≤ 5ppm mass error in the relevant matrix.

Data obtained for comparison of different acquisition modes was very heterogeneous. Therefore, conclusions are drawn with care. From comparing different MS acquisition modes, it is suggested that, only targeted MS^2 monitoring the pseudo-molecular ion in full scan and a single product ion after precursor ion selection (both in HR-MS using a maximum of 5 ppm mass deviation) leads to comparable false positive and false negative rate as SRM monitoring two product ions in low mass resolution mass spectrometry (LR-MS). Here, obtained ion ratios are only trustworthy if several subsequent product ion spectra are recorded instead of applying only a single product ion scan. In multi-residue analysis, this is only possible using fast duty cycles or slow chromatography. However, in routine analysis, confirmatory analysis is carried out as a follow up of a screening analysis and therefore, in the confirmation phase a method usually targets only a single or a few compounds diminishing this limitation.

Full scan monitoring of the pseudo-molecular ion at high mass resolution results in higher false positive and false negative rates. All ion fragmentation (AIF) at a resolution ≤30,000, monitoring a single fragment only, results in a relatively high false negative rate due to the fact that ion ratios differ severely. This is attributed to the complexity of the AIF spectra. AIF, carried out at higher resolution or in combination with ion mobility spectrometry (IMS), might be applicable for confirmatory analysis.

Overall, besides LR-MS operated in SRM mode monitoring two product ions, for now, only HR-MS monitoring the pseudo-molecular ion in full scan and one product ion after precursor ion selection is found adequate for confirmatory analysis. Data on AIF at higher resolution should be obtained to investigate this aspect further. Also research on the use of a broader precursor ion selection window is needed to obtain more widely multi-compound analysis methods applicable for confirmatory analysis. Currently these acquisitions modes are readily available (e.g. variable data-independent acquisition (vDIA) and

SWATH) and their potential for confirmatory analysis should be further investigated. Also additional data, from different laboratories, should be obtained for IMS. Technical innovations, e.g. faster duty cycle in Orbitrap instruments and the further development of fit-for-purpose software tools for efficient data analysis are needed to apply the full potential of HR-MS as a confirmatory technique in routine multi-compound analysis.

To avoid false positive findings, it is suggested that the use of product ions at *m/z* 91, 105 and 121 and neutral losses of -17 and -18 Da should be omitted.

An overview of current performance criteria and the proposed ones for veterinary drug residue analysis are presented in Table 1.

Conclusions

During the last decades, many developments in the instrumental techniques applied for residue analysis of veterinary drugs have been made. Current legislation does not sufficiently cover all new techniques available and the current multi-compound approaches used. As a result, the current criteria, established in 2002/657/EC [1], should be revised.

It is concluded that the ion ratio is an important parameter in confirmatory analysis. It is concluded that the ion ratio deviation is not related to the ion ratio itself, but rather to the intensity of the lowest product ion. Therefore, a fixed ion ratio deviation tolerance reflects the real life situation better. Clearly, the ion ratio deviation is related to the concentration of the compounds present and to prevent a high false negative rate for especially banned compounds, a maximum ion ratio deviation of 50% is suggested.

Furthermore, it was observed that RT shifts, when using gradient elution, as is common practice nowadays, are mainly observed for early eluting compounds. Therefore, an absolute RT criterion instead of a relative one is better suited. A maximum RT deviation of 0.2 min is suitable and no additional criterion for RRT is needed.

No minimum mass resolution for HR-MS can be established because the minimal performance needed depends on many factors including the compound, matrix and sample clean-up procedure. Instrumental setting should be selected in such a way that the mass error is ≤ 5 ppm. To prevent false negative results for low mass compounds, it is proposed to extend this criterion to a mass error tolerance ≤ 5 ppm or 1 mDa. Furthermore, to prevent false positive results in LR-MS, product ions should not include *m/z* 91, 105 or 121. For both LR-MS and HR-MS neutral losses of -17 and -18 Da should be omitted and a second ion transition should not include any isotopes of the first ion transition.

From the different MS-modes covered, in this study only the combination of an accurate mass pseudo-molecular ion (acquired in full scan) and monitoring a single product ion after precursor ion selection using a maximum of 5 ppm mass deviation, leads to comparable selectivity, false positive and false negative rate as SRM monitoring two product ions in LR-MS. Confirmation based on the accurate mass of one ion (pseudo-molecular ion) acquired in full scan results in higher false positive and false negative rate. Full scan combined with AIF at ≤30,000 resolution, monitoring the pseudo-molecular ion respectively a single fragment, results in a higher false negative rate. Until further studies demonstrate the feasibility of AIF at higher resolution, the combination with IMS or the application of new acquisition modes, two techniques are found suitable for confirmatory analysis:

- LR-MS monitoring two product ions after precursor ion selection (SRM mode)
- HR-MS monitoring the pseudo-molecular ion in full scan and additionally one product ion after precursor ion selection in PRM.

An overview of current performance criteria for veterinary drug and pesticide residue analysis and the proposed ones for veterinary drug residue analysis are presented in Table 1. Further details on this study are presented elsewhere (Berendsen 2013b, Berendsen 2015).

Table 1. Current performance criteria according to CD 2002/657/EC, SANCO/12571/2013 and the proposed criteria.

Parameter	CD 2002/657/EC	SANCO/12571/2013	Proposed criterion
Ion ratio tolerance	Dependent of ion ratio: I > 50% ±20% 50 ≥ I > 20% ±25% 20 ≥ I > 10% ±30% I ≤ 10% ±50%	≤30%	≤50%
Retention time tolerance	±5%	±0.2 min	Deviation ≤ 0.2 min
Relative retention time tolerance	±2.5%		No criterion needed
Mass resolution	-	Typically > 20,000 FWHM	Fit-for-purpose resulting in ≤ ±5 ppm mass deviations.
Confirmatory techniques	- Full scan - SRM	Single stage HR-MS: - Full scan - Limited *m/z* range - SRM MS/MS: - SRM	- SRM - Parallel Reaction Monitoring (PRM)
Confirmatory techniques	≥4 identification points, e.g.: - Monitoring 2 product ions using LR-MS after precursor ion selection; - Pseudo-molecular ion in full scan HR-MS combined with monitoring 1 product ion in HR-MS after precursor ion selection.	- ≥2 product ions after precursor ion selection in LR-MS. - ≥2 diagnostic ions, preferably including the (quasi) molecular ion; mass accuracy < 5 ppm; at least one fragment ion in HR-MS.	- ≥2 product ions after precursor ion selection in LR-MS. - ≥1 product ion in HR-MS after precursor ion selection in combination with the psuedo-molecular ion in full scan HR-MS.

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ORAL PRESENTATIONS

CONTRIBUTIONS

O1
(004)

A MONITORING STUDY ON THE LEVEL AND FREQUENCY OF ANTIBIOTIC RESIDUES IN GERMANY

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Abstract
The National Residue Control Plans (NRCPs) of the European Member States and the German Food Hygiene Control Programme monitor the occurrence of antibacterial residues above the MRL. However, in the light of the discussions on and the increase in antimicrobial resistances in both man and beast, also lower concentration levels are of interest. Consequently, a monitoring programme was initiated, testing 18,000 samples of liver and muscle of pig, calf and cattle within two years. Three German federal states participated in this study. The statistical analysis showed that the compiled data could be extrapolated to the whole of Germany. An extensive data analysis was carried out. The results gave evidence that the frequency of antimicrobial contaminations in these species above as well as below the MRL might be considerably higher than what can be derived from the NRCPs.
A by-product of the study was the simultaneous determination of the proficiency of the applied inhibitory test method. In Germany, the three-plate inhibitory test is used. The false-positive as well as the false-negative rates of the test were determined.

Introduction
Antibiotics are currently an intensively discussed topic in politics, society and the media. This does not only concern the administration of antibiotics to food-producing animals and questions of distributed and consumed quantities, but also antibiotic residues in edible tissues, the development of resistances in the treatment of animals and their transmission to humans. Therefore, it is an issue of animal welfare and animal health on the one hand, and of the health protection of consumers on the other hand. In this context, the BVL has extensive responsibilities and competences, including the authorisation of veterinary drugs, the activities of the NRL for residues of veterinary drugs including antibiotics, and more than ten years of experience in the antibiotic resistance monitoring of pathogenic agents in animals (GermVet report 2014). Thanks to its responsibility as NRL and the coordination of the national residue control plan (NRCP), the BVL has an overview of the proficiency in residue control in Germany. The results are published in annual reports. In addition, an atlas of antibiotic resistance and usage in Germany (GERMAP 2012) is published approximately every two years in collaboration with human medicine on the initiative of the Paul-Ehrlich-Society, the Department of Infectiology of the University of Freiburg and the BVL.

Moreover, for more than 20 years meat samples have been examined for antibiotic residues with the three-plate inhibitor test (DPT) in Germany according to a German regulation (AVV LmH). A standardised method for examining inhibitors in animal tissues such as kidney, liver and muscle, elaborated by the working group of veterinary experts for food hygiene (ALTS), has existed since 1971 (Levetzkow, 1971). With this control programme, 0.5% of slaughtered cattle and pigs and 2% of slaughtered calves are to be tested, resulting in approx. 300,000 investigations annually. Positive DPT results have to be confirmed by instrumental analytical techniques.

In 2007, the German regulation was amended and the validation of the DPT in analogy to Commission Decision 2002/657/EC was requested (AVV Lmh 2007). The following extensive validation studies showed considerable deficits with respect to the detectability of MRLs. Therefore, a project on the monitoring of negative results of DPT investigations by means of the LC-MS technique was set up.

The aim of the project was the determination of the grade of contamination of food with antibiotic residues and the estimation of the proficiency of the three-plate test, especially the false-positive and false-negative rates. Furthermore, during the monitoring phase several optimisation studies were carried out in parallel. Options for action should be developed on the basis of the final results.

Materials and Methods
In total 18,164 DPT-negative samples were investigated during the project period in three German federal states. In addition, the results of 1,359 DPT-positive samples and their confirmation results were reported, originating from the original food hygiene programme of Germany. The applied methods for the three-plate tests followed the instructions of AVV Lmh (AVV LmH 2007 and 2010). The confirmation methods were based on LC-MSTOF and LC-MSMS techniques. The substance spectra were varying slightly between the laboratories (Table 1). Sampling was carried out from November 2010 to November 2012 in three federal states. The instrumental analyses were prolonged until the middle of 2013. The internal final report was submitted to the Federal Ministry in February 2014.

Table 1. Substances investigated during the monitoring programme (not all substances were included in all laboratories).

Substance group	Antibiotic substances
Aminoglycosides	apramycin, dihydrostreptomycin, gentamicin, kanamycin, neomycin, spectinomycin, streptomycin
Amphenicols	florfenicol*, florfenicolamine, thiamphenicol*
Cephalosporins	cefoperazone*, cefalexin*, cefapirin*, desacetyl-cefapirin, cefquinome*, ceftiofur*, cefazolin, cefacetrile, cefalonium
Quinolones	cinoxacin, ciprofloxacin*, difloxacin*, danofloxacin*, enoxacin, enrofloxacin*, flumequine*, lomefloxacin, marbofloxacin*, nalixic acid*, norfloxacin, ofloxacin, oxolinic acid*, sarafloxalin*
Diaminopyrimidines	baquiloprim*, trimethoprim*
Lincosamides	clindamycin*, lincomycin*, pirlimycin*
Macrolides	clarithromicin*, erythromycin*, josamycin*, oleandomycin*, spiramycin*, neospiramycin*, tilmicosin*, tulathromycin*, tulathromycin_marker, tylosin a*, 3-O-acetyl-tylosin, tylosin B, tylvalosin
Mutilins	tiamulin*, 8-a-hydroxymutilin, valnemulin
Penicillins	amoxicillin*, amoxicilloic acid, ampicillin*, benzylpenicillin*, cloxacillin*, dicloxacillin*, nafcillin*, oxacillin*, phenoxymethylpenicillin*
Sulfonamides	sulfachlorpyridazine*, sulfaclozine*, sulfadiazine*, sulfadimethoxine*, sulfadimidine*, sulfadoxine*, sulfaguanidine, sulfamerazine*, sulfamethoxazole*, sulfamethoxypyridazine*, sulfamoxole, sulfanilamidd*, sulfapyridine, sulfaquinoxaline*, sulfathiazole*, sulfisoxazole
Tetracyclines	chlortetracycline* , doxycycline*, epichlortetracycline*, epioxytetracycline*, epitetracycline*, oxytetracycline*, tetracycline*
others	dapsone*

**Substances analysed in all laboratories*

Results and Discussion

Proficiency of the DPT

For the assessment of the proficiency of the DPT the false-negative and false-positive rates were used. For these parameters different definitions may apply. The share of false-negative results among all contaminated samples (positive in LC-MS analysis) is in accordance with what is used in the validation of chemical analytical methods and is applied in the further discussion. As false-positive rate the share of all uncontaminated samples (LC-MS negative results) among all DPT positive results was chosen. Depending on the individual federal state and the species (cattle, calf or pig), the false-negative rates were between 71% and 94% and the false-positive rates were between 54% and 95%.

The strongly varying false-positive/false-negative rates between the laboratories very clearly showed that the results were strongly influenced by the person measuring the inhibitory zones, *i.e*. the results were interpreted and assessed differently.

The monitoring project results verify what had already been found in a former validation study, namely that the test procedure is inadequate for the detection of MRL exceedances of amoxicillin, sulfadiazine and oxytetracycline as important veterinary medical pharmaceuticals (VMPs), but also of other substances. At the same time, the results showed that for benzylpenicillin, chlortetracycline, doxycycline and, in some matrices, for enrofloxacin, the DPT works appropriately.

An example for the capability of the test to detect benzylpenicillin is presented in Figure 1. For benzylpenicillin, the DPT shows the best performance; nevertheless, a high variability of the results per concentration can be observed.

Calculation of the share of contamination and extrapolation to the whole of Germany

In most cases the collected data offer a sufficient basis for a solid estimation of the contamination in the different species in the different federal states.

Due to the above-mentioned high variability of the data especially between laboratories, statistically significant differences between the contamination situations in the different federal states cannot be determined. Evidently, a comparison of the contamination situations could only be carried out based on the substances analysed in all laboratories. By means of a very conservative estimation procedure, it was possible to deduce the residue contamination from the three federal states to Germany.

Amoxicilloic acid as a marker for the application of amoxicillin could be found in 0.5% of slaughtered pigs, 1% of slaughtered cattle and 0.4% of slaughtered calves with concentrations higher than the MRL. In 0.732%, 1.423% and 1.989% of the slaughtered pigs, cattle and calves respectively, concentrations above half the MRL could be found (without amoxicilloic acid). When considering amoxicilloic acid, the contamination rate increased to 1.102%, 1.559% and 1.989% of slaughtered pigs, cattle and calves, respectively.

Because of the very conservative estimation, the actual contamination rate could be considerably higher. With the use of more sensitive screening procedures and with the inclusion of all known antibiotics the detection of contaminated samples could be enhanced.

The most important substances for which concentrations higher than the MRL could be detected were:

- Dihydrostreptomycin and gentamicin (especially in cattle and calves)
- Enrofloxacin and marbofloxacin
- Tulathromycin
- Amoxicillin and benzylpenicillin
- Sulfadiazine (especially in pigs and calves)
- Tetracycline, doxycycline

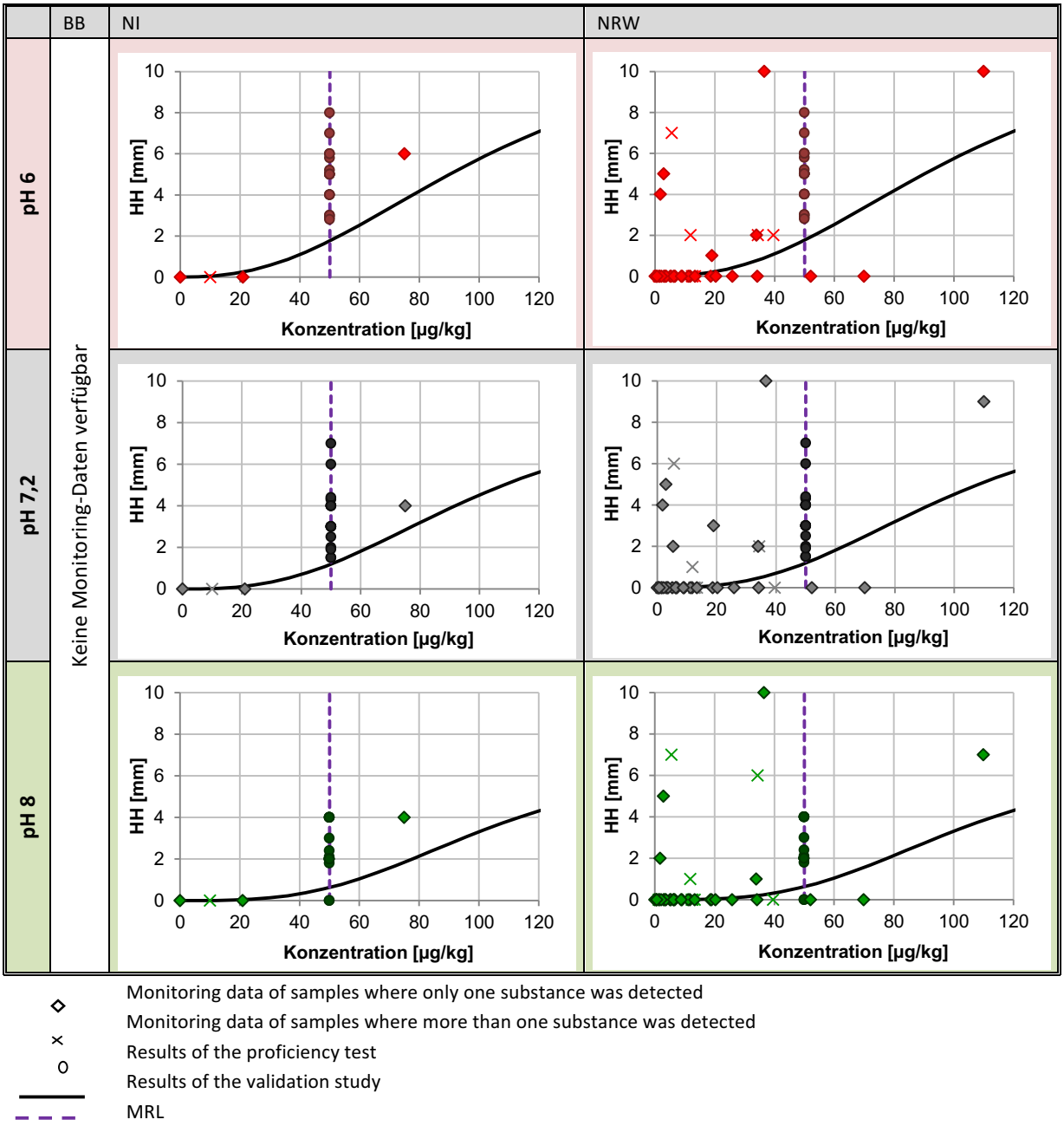

Figure 1: Benzylpenicillin – pig/muscle, results of the validation study, the monitoring project and the proficiency test

Possible optimisation options

During the project phase it was planned to amend the AVVLmh concerning the test procedure of the DPT in order to have the possibility to compare different situations. Therefore, the specification of the nutrient media was changed from a thickness of 2 mm to a volume of 11 ml (better control of this dimension). Furthermore, the nutrient media had to be prepared fresh

every working week. The decision whether a sample is a suspect sample should start already from 1 mm instead of 2 mm. Unfortunately, these optimisations did not lead to the expected improvements.

Conclusions

The results of the monitoring project give reason to principally recommend the use of multi-residue LC-MS methods with a defined analyte spectrum comprising all groups of antibacterial substances and the lowest possible critical concentrations.

The determined contamination situation showed a rate which was up to 10 times higher than the rate found by the usual NRCP programmes (0.21%; EFSA 2013) (0.08% pigs, 0.17% cattle; NRCP Germany 2012). Due to the high variability of false-positive and false-negative rates between the participating laboratories, no significant deviations of the contamination situation between the federal states could be detected. Therefore, a contamination rate of 0.5% to 1% above the MRL could be taken as a general basis. This estimation was carried out very conservatively so that the actual contamination rate could be considerably higher.

Possibilities for the optimisation of the test procedure could not be found.

Acknowledgements

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O2
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CONTRIBUTION OF MASS ANALYTICAL METHODS TO IDENTIFICATION OF ANTIBIOTIC RESIDUES IN MEAT – APPLICATION TO ANTIBIOTIC CONTROL IN FRANCE

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Abstract

Human exposure to drug residues especially antibiotics, is of increasing interest. To safeguard human health, the EU has established safe maximum residue limits (MRLs) for residues of veterinary drugs in food from animal origin. Monitoring of veterinary drug residues is governed by National Residue Control Plans (Council Directive 96/23/EC). Detection at slaughter mainly concerns the target control of authorized pharmacologically active substances and the control of banned substances. In France, besides microbial growth-inhibition tests used as screening approach for the detection of antibiotics in meat, a part of the antimicrobial residue control program is currently based on an antimicrobial residue screening by a multi-antibiotic LC-MS/MS (liquid-chromatography coupled to tandem mass-spectrometer) method validated according the European criteria. This strategy is now implemented for four years at the level of ten official laboratories using different LC-MS/MS systems. It allows getting rapid information about the presence and the identity of antibiotic substances (61 antibiotics) in meat. An outcome of the results for cattle, pig, sheep and goat will be presented as well as a comparison analysis with biological screening results. Less than 2 % of carcasses of all species showed non-compliant results (level above MRL). Failure to follow recommended withdrawal times is often implicated in residue problems.

Introduction

Monitoring large numbers of slaughter animals for antibiotic residues is often done using microbiological screening methods, because their high cost-effectiveness. However, these methods are not able to detect all antibiotics at their MRL level. Automation has been introduced to speed up many analytical procedures but instrumentation remains expensive. To improve the cost-effectiveness of LC-MS/MS method, it is necessary to increase the number of analytes that may be determined from a single analysis of a sample. Such an approach is extremely effective when multi-residue techniques, such as LC-MS/MS are used in screening. Modern instruments produce high signal-to-noise ratios and permit the simultaneous monitoring of an increasing number of specific transitions at a specific retention time window.

In France, since 2011, we have implemented a new control plan based on a screening step based on a LC/MSMS method in parallel to the classical strategy based on the bacteriological screening (4 plates test). Positive samples from the two screening methods are confirmed by LC/MSMS methods allowing quantification and identification of residues according criteria established in the Decision 2002/657/CE. The control plans implemented by DGAl (Department in charge of agriculture) are based on targeting criteria (Council Directive 96/23/EC) on some animals to detect bad farming practices and to control the MRL. In this paper, we compare the results obtained by the "microbiological" and the "LC-MS/MS" screening control plans implemented for three groups of animal species (*bovine*, pig, sheep and goat).

Materials and Methods

Analysis of antibiotics

An LC-MS/MS method was validated according the Decision 2002/657/CE by the National Reference Laboratory for Veterinary Drug Residues. The instrument was operated in the MRM mode and transitions were optimized and placed into the appropriate retention time windows. This permitted maximum sensitivity while maintaining the number of required data points across a chromatographic peak. The method was implemented in ten French official laboratories allowing the detection of at least 61 antibiotics among the main antibiotic families used in veterinary medicine: penicillins, cephalosporins, sulfonamides, macrolides, lincosamides, aminoglycosides, tetracyclines, and quinolones. The detailed conditions have been published elsewhere (Gaugain, 2013).

Two routes of extraction were used in order to recover all antibiotics. Extraction of penicillins, cephalosporins, macrolides and sulfonamides was performed by adding 8 mL acetonitrile to 2 g of sample. The sample was stirred for 10 min, then centrifuged at 14,000 *g* for 5 min. After evaporation of 6 mL supernatant under nitrogen flow, the residue was dissolved in 0.6 mL 0.2 M ammonium acetate, and then filtered using a 0.45 µm Millex HV filter of 13 mm diameter.

Extraction of tetracyclines, quinolones, aminoglycosides and lincomycin was performed by adding 8 mL 5% TCA solution to 2 g of sample. The sample was stirred for 10 min, then centrifuged at 14,000 *g* for 5 min. About 1 mL supernatant was filtered using a 0.45 µm Millex HV filter.

Twenty-five or 20 µL extracts were injected into the chromatographic system according to the gradient, and the separation was performed on a Symmetry C18 column (150 × 3.9 mm, 5 µm). Two different gradients mixing 0.1% PFPA with acetonitrile were used according to the appropriate series of antibiotics. A triple quadrupole mass detector set in a positive electrospray ionization (ESI) mode was used for the detection and identification of the targeted antibiotics. The network of laboratories used different types of apparatuses: Agilent 6460, Sciex API 3000, API 3200 Qtrap, API 4000 (2), API 4000 Qtrap (2), Quattro Micro, Thermo Ultra and Xevo TQ MS. The transfer of the method was validated by a collaborative study of the multi-residue method using spiked muscle materials (Roudaut, 2011).

In parallel, the classical strategy based on the bacteriological screening (4 plate test – 2011 version) was used by another network of official laboratories allowing the detection of some families of antibiotics after a long incubation time.

Positive samples from the two screening methods (LC-MS/MS and 4 plates test) were confirmed at the NRL level by specific LC-MS/MS methods allowing quantification and identification of residues according criteria established in the Decision 2002/657/CE.

Results and Discussion

The results obtained after screening by LC-MS/MS and microbiological analyses (non-compliant samples rate (%) and number of analysed samples in different species) are summarised in Table 1.

Antibiotics in bovine muscle

Globally, from 2011 to 2014, the LC-MS/MS screening leads to non-compliance rates around 2 % which are higher than those obtained with microbiological screening (0.61%) (Table 1). For the *bovine* control plan, physico-chemical screening leads to a non-compliant rate, which is 3.3 times higher than those obtained with microbiological screening. These differences are statistically significant (Khi 2 test). Furthermore, LC-MS/MS screening enriches the information through molecular information provided at the screening step. It increases the performance of the screening for several antibiotic families (macrolides, (fluoro)quinolones, sulphonamides etc). This big improvement of the sensitivity and specificity of the control plan performance is extremely important in the context of the implementation of an action plan to reduce the usage of antimicrobials.

Table 1. Non-compliant samples rate (%) and number of analysed samples (N, in italic) in muscle samples screened by LC-MS/MS or microbiological methods.

Screening	Non-compliant rates (%) CL-SM/SM method			Non-compliant rates (%) Microbiological method		
Year	*bovine*	*porcine*	*ovine*	*bovine*	*porcine*	*ovine*
2014	1.61	0.41	0.80	0.26	0.13	0
	497	*484*	*497*	*1135*	*794*	*42*
2013	1.71	/	0.72	0.90	0.16	0.5
	293		*279*	*1891*	*1921*	*200*
2012	3.33	0	/	0.35	0.10	0.4
	299	*299*		*2128*	*1943*	*750*
2011	1.36	0	/	0.93	0.10	/
	295	*283*		*2146*	*2020*	
Mean	**2.00**	/	**0.76**	**0.61**	**0.12**	**0.40**
	1384	*1066*	*776*	*7300*	*6678*	*992*

The most frequent found families in non-compliant samples are tetracyclines (41%), macrolides (21%), aminoglycosides (17%), (fluoro)quinolones (14%) and β-lactams (14%). In some non-compliant cases, there was also an association of antibiotics (tetracyclines, aminoglycosides, β-lactams). New molecules of macrolide (tulathromycin, gamithromycin) recently approved as veterinary drugs, have been detected the last years. It relates to the increased exposition of macrolides in *bovines* (+ 19% in five years). Figure 1 summarizes the distribution of antibiotics in non-compliant samples for cattle.

Non-compliances mainly concern dairy cows (44%), calves and young cattle (30%) and lactating cows (26%). In the case of cull cows, 42% of the targeting criterion was a medium visual state of the carcass; in the case of calves and young cattle in 50% of cases the targeting criterion involved an infiltration of the collar and finally for lactating cows in 43% of cases targeting criteria was information provided by the information package on food chain (FCI). The other targeting criteria used were: myositis, pleurisy, injuries, arthritis, pneumonia, peritonitis. In 2007, a survey (Cauzeau, 2010) conducted in France highlighted that 80% of antimicrobial treatments were for a single animal and nearly half were related to dairy cows, beef and calves were evenly represented with approximately 20%. Treatments were mainly related to udder diseases (37%) and to a lesser extent to musculoskeletal problems (14%).

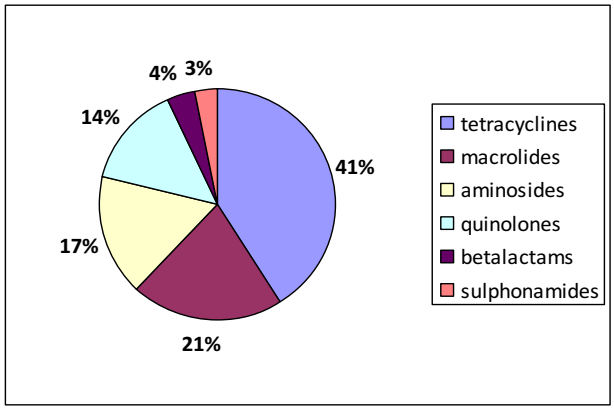

Figure 1. Distribution of antibiotics in non-compliant samples for bovine muscles (2011-2014).

The overall rate of 2.0% for non-compliance for cattle meat can be considered as low for a control based on targeting criteria. But efforts have to be made in this sector to reduce antibiotic administration in dairy cows and calves and to respect dose and withdrawal time.

Antibiotics in porcine *muscle*
Non-compliant results are scarce in the pig sector: 0.4% after LC-MS/MS screening in 2014 (Table 1) and 0.12% after microbiological screening (mean from 2011 to 2014). Finding of two non-compliant samples in 2014 (sulphadimethoxine and penicillin G) is likely in relation to the increase of the sample size (484 instead of 300) this year. If tetracyclines are the main family used in this sector, the non-compliance mainly concerns sulphonamides (sulphadimethoxine, sulphadiazine). Pigs are essentially treated by the oral route (87%). However, the practices are recently moved from treatments by feed to treatments by the drinking water (Chevance, 2014).

Antibiotics in sheep and goat muscle
The mean non-compliant rates after LC/MSMS and microbiological screening are respectively 0.76% and 0.40%. The non-compliant results only concerns sheep, mainly by dihydrostreptomycine (50%), oxytetracycline, sulphadimethoxine, and spiramycine). This is in relation to the uses of antibiotics in the *ovine* sector (Gay, 2012): penicillins, aminoglycosides, tetracyclines and macrolides. Extra-label use (disease indication or therapeutic scheme) was also frequently described in this sector. Chemical screening leads to a non-compliant rate 1.9 higher than this obtained after microbiological screening.

Comparison inter-species
It should also be noted that non-compliance rates in beef sector were significantly higher than those obtained in *ovine* and *porcine* sectors (statistically significant Khi 2 test) as well as after chemical or biological screening. If in the *bovine* and *porcine* sectors respectively 34% and 32% of the live weight are treated, only 2.8% are concerned in the *ovine* sector (Chevance, 2014). Globally, the non-compliance rates are 2 to 3.3 times higher after chemical screening. The antibiotics found are species dependent: tetracyclines in *bovine*, sulphonamides in *porcine* and aminoglycosides in *ovine* species.

Link with actions initiated to decrease antibiotic consumption
In France, the total sales of antimicrobial agents (in mg/ population correction unit) fell by 30% from 2010 to 2013 (EMA, Fifth European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) report). The decrease was 10.3% for cattle, 21.7% for pigs and 13.7% for poultry. The greatest decrease was noted in tetracyclines, sulphonamides and macrolides. Over the past five years, the overall exposure of animals to antibiotics decreased by 15.7% and in 2013 by 7.3% compared to 2012. For cattle, it decreased by 6.6% between 2012 and 2013 (Chevance and Moulin, 2014) although overall antibiotic exposure increased 0.2% over the past five years.

Inspections in farming have identified that the failure to respect the withdrawal time is the most common cause of non-compliance for the presence of antibiotic residues in food of animal origin (34%). We also found non-compliance of the veterinarian's prescription (16%), such as errors in dosing or on the treated animals. For 50% of non-compliance cases, the causes have not been identified. Inspections have also allowed to identify other non-conformities related to poor farming practices, such as lack of or incomplete breeding register, poor performance of the pharmacy of breeding. Warnings and/or reminders to the regulations were made by inspectors to the farmers concerned.

The overall decrease in the sales of antimicrobial agents can be linked to the French national action plan (Ecoantibio 2017), initiated in 2011 by the Department in charge of agriculture, to reduce the risks of antimicrobial resistance in veterinary med-

icine. The plan includes a target for reducing the use of all antimicrobial agents by 25% in five years. After two years, the decrease in use measured at animal level of exposure to antimicrobials (ALEA: animal level of exposure to antimicrobials) is 12.7%. ALEA is an indicator based on animal course dose which takes into account the differences in dosing between the various antimicrobials, forms and species. The animal sectors in which voluntary actions have been initiated showed a greater fall in the consumption of antimicrobial agents. For example, in the *porcine* sector with the use of an analysis tool of antibiotic uses in 2010 (Inaporc) and a qualitative and quantitative survey in 2014-2015 (Anses/IFIP). More recently, the veal sector (2013-2014) participate in actions (Idele) in the context of Ecoantibio 2017. Globally, France is among the European countries (with Slovenia, Netherlands and Italy) having the highest decrease (-30%) in the total sales of antibiotics between 2010 and 2013 (EMA, 2015). In 2013, the antibiotic consumption in France is lower than the European mean (Figure 2).

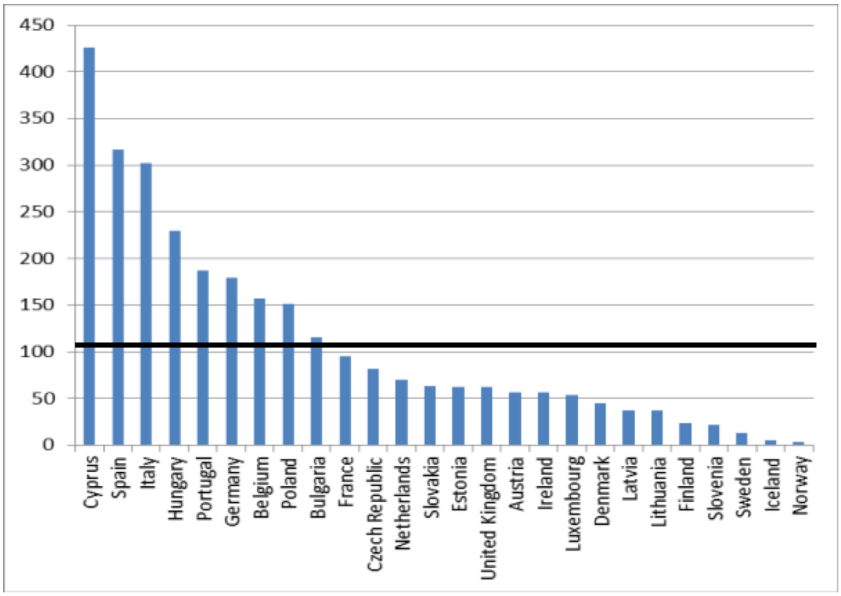

Figure 2. Sales of veterinary antimicrobial agents (mg PCU^{-1}) in EU countries in 2013 (EMA - Fifth ESVAC report 2015); — European mean sale : 109.7 mg PCU^{-1}

For critical antibiotics like 3rd and 4th generation cephalosporins and fluoroquinolones, the new French Law for the Future of Agriculture, Food and Forest, has set in October 2014 a 25% reduced use in three years (by referring to the year 2013). For *porcine* sector, an important decrease of exposure was achieved (-78% compared to 2010). However, achieving this goal requires the continuation of actions and the implementation of new actions, especially in sectors with high use of these families of molecules (*e.g*. *bovine* sector).

Conclusions

The results of the implementation of antibiotic screening by LC-MS/MS showed that this analytical strategy has a great interest. This interest comes from the ability of the technique to detect simultaneously all the main antibiotic families used in veterinary medicine (tetracyclines, macrolides, β-lactams, aminoglycosides, quinolones, sulphonamides) at or below the MRL level. It is also able to detect new use of authorized antibiotics in *bovines* with or without MRL in muscle (*e.g*. macrolides).

The ability to monitor a wider range of residues of various antibiotic families is fundamental to detect new practices. Thus, for 2015 and 2016, it was decided to increase the number of samples to be analysed directly with the method LC/MS-MS. This strategy was also applied to poultry and *equines* in 2014 and to fish in 2015 and is planned to be used in the future for rabbit. In 2016, programming samples for the presence of residues of antibiotics in cattle sector provides more than 3,000 meat samples analysed via multi-residue analytical strategy. In parallel, the NRL has developed and validated a new method to screen over 80 analytes, including eight aminoglycosides with a special extraction and new drugs. In 2016, this method will become operational in the network of official laboratories.

In the current context of promotion of prudent use of antibiotics to reduce the risk of antimicrobial resistance in veterinary medicine, the focus is set on breeding with the proper use of these veterinary drugs in the various animal sectors. A change of pattern of prescription of antibiotic drugs is expected and should be reflected in the control plan by a reduction of the portion of non-compliant samples as well as of the type of residue frequencies. Moreover, in the future official controls will be carried out regularly on a risk basis (Van Asselt, 2012) and with appropriate frequency.

Acknowledgements
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RESPONSIBLE USE OF ANTIMICROBIALS IN VETERINARY MEDICINE: THE EU VS. USA APPROACH TO AGPS

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Abstract

There has been much debate over the antimicrobial growth promoter (AGP) use of antibiotics in veterinary medicine and the EU, based on the precautionary principle, banned AGP use on 1st January 2006. The US FDA, in their guideline 213, divided antibiotics into three classes, human use, veterinary use and shared class. This guideline is requesting veterinary pharmaceutical companies to voluntarily limit AGP label claims for veterinary use only antibiotic classes.

Under the umbrella of responsible use, a number of actions could be implemented including but not limited to (1) Antibiotic resistance monitoring (2) Antibiotic Usage Monitoring (3) Antibiotic Availability by Prescription Only (4) National Formularies/Prescriber Guidelines (5) Consideration of the distribution channel and promotion of prescription only antibiotics.

With respect to antibiotic resistance monitoring, information on the change in minimum inhibitory concentrations over time to antimicrobials is needed at the local, national, and international levels to guide policy and intervention strategies. One area of confusion globally is in the interpretive criteria used in national monitoring programs.

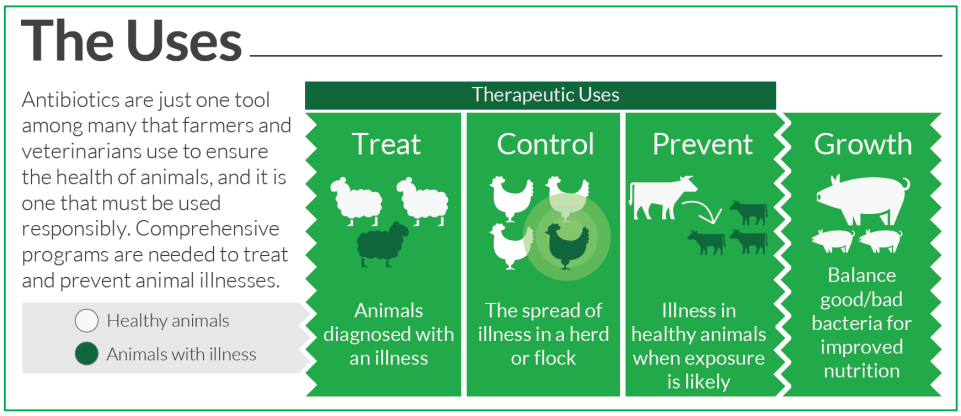
This paper will discuss the consequences of the EU AGP ban and the US FDA position with respect to AGPs and the urgent need for interpreting antibiotic resistance monitoring data using harmonised interpretive criteria.

Antimicrobial use in food animal medicine has become an important political and scientific issue, especially in relation to the contribution that animal sourced antimicrobial resistance might play in human medicine, leading to calls at a political level for national action plans to be developed. Globally, the World Health Organization (WHO) and World Organisation for Animal Health (OIE) have created critically important antibiotic list for human medicine and veterinary medicine, respectively. The responsible use of antibiotics in food animals and humans is essential to preserve the viability of antibiotics for use in medicine. For animals, maintaining their health is important for the production of safe food for human consumption, to meet the demands of an increasing world population that is seeking more animal sourced affordable protein in their diets.

Sound political policy can be developed based upon the best science and practices considering global experiences and lessons learned. Extensive experiences and learnings globally, including those in the European Union (EU) and the United States (US), can contribute to developing science-based policy and regulations for individual countries. Risk analysis principles and application, which includes risk assessment, risk management and risk communication, can serve to ensure countries incorporate best practices into their ever evolving food animal regulatory processes. Learnings and best practices globally have demonstrated that a strategic approach to addressing antimicrobial resistance is critical. Recognition of existing status and desired goals in a country and then building a process through laws, regulations and practices, that bridges from current state to desired state, is most important to ensure that policy and science can be and are implemented for a successful outcome. Political action without reasoned science and realistic practices will lead to unintended consequences and a waste of limited resources.

Antibiotics are normally regulated as part of a food animal drug approval process. The regulatory approval process includes assessment for safety (human, animal and environment), quality and efficacy (the label claim with approved uses). The regulatory human safety assessment for antibiotics historically considered toxicological and microbiological safety. This assessment provided for the establishment of an acceptable daily intake (ADI) and maximum residue limits (MRLs) to ensure safe use of the product. More recently, the safety assessment is incorporating a risk analysis specific to antimicrobial resistance. This process includes assessing the risk considering exposure, managing the risk through label use directions and communicating the risk to ensure responsible use. The primary focus is to reduce food borne pathogens, and the resistance associated with bacteria, that may contribute to untreatable human illness. Importantly, residues and resistance both need to be addressed through laws and regulations, however, each requires unique policies and practices to meet desired outcomes. Providing for the regulated uses of antibiotic products reduces the uses of unregulated antibiotic products. Regulation with proper enforcement contributes to a safe food supply while preserving the effectiveness of antibiotics for use in human and food animal medicine.

Antibiotic regulations and the associated risk analysis need to consider the indication for use and the categories for use of the compound. Antibiotics are used in food animal medicine either for a therapeutic indication which includes disease treatment, control and prevention (see definitions in box 1 below) or for growth promotion.



Box 1. Pictorial definitions of how antibiotics can be used in veterinary medicine

Global experiences and lessons can provide insights for best practices in developing sound policy and science-based regulation. The European Union and United States both have sought over the past two decades to address antimicrobial resistance by evolving their laws, regulations and responsible use practices. Each advanced different approaches over time and thus with different experiences and lessons learned. Globally, antibiotics continue to be used in all countries in food animal production; no country has eliminated all uses of antibiotics.

The European Union has evolved their antibiotic regulations to provide for uses as injectables, medicated water and medicated feed. Antimicrobial resistance risk analysis is a critical part of the regulatory approval process. Antibiotics can be approved for therapeutic uses of disease treatment, control and prevention. The veterinarian plays a major role as the prescriber of antibiotics.

In the EU, there has been much debate over the antibiotic growth promotion (AGP) use of antibiotics in veterinary medicine, the EU banned AGP use as of 1st January 2006. Importantly, the EU ban was not a molecule ban but rather an indication of use ban meaning that if a molecule had a therapeutic indication and an AGP indication the AGP indication was banned however the molecule remained on the market for therapeutic indications. The EU approach to banning AGP use has had a negative impact termed the 'unintended consequences' which has resulted in an increased incidence of necrotic enteritis in poultry and dysentery in swine which has resulted in increased quantities of antibiotic being used as therapeutic indications. Figure 1 below shows the increase in therapeutic use of antibiotics in Denmark after the AGP ban was implemented in 2000. The increase was mainly due to the extensive use of tetracyclines and penicillins such as ampicillin.

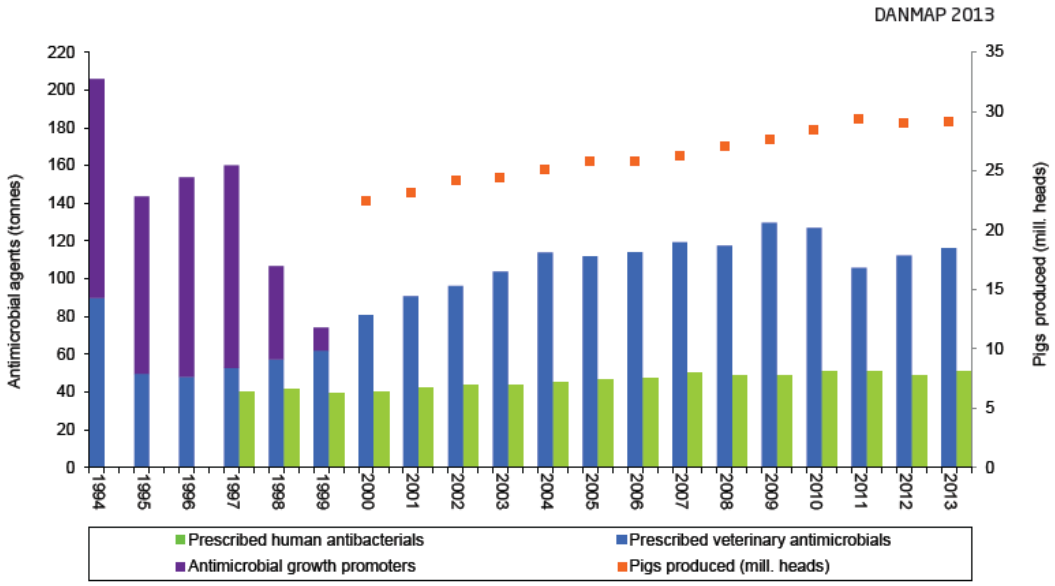


Figure1. Prescribed antimicrobial agents for humans and animals in Denmark (DanMap2013, 2014)

This increase in use of tetracyclines and penicillins resulted in increased levels of resistance to these antibiotic classes in foodborne pathogens especially *Salmonella* species (Figure 2). Data from DanMap 2013 clearly shows that post the AGP ban

the level of resistance to tetracyclines and ampicillin increased and this was reflected in the usage of these molecules for therapeutic purposes.

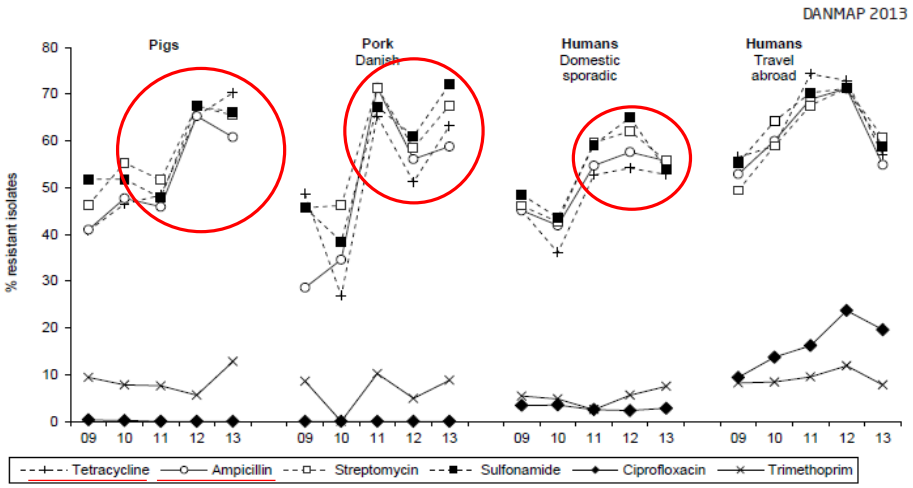
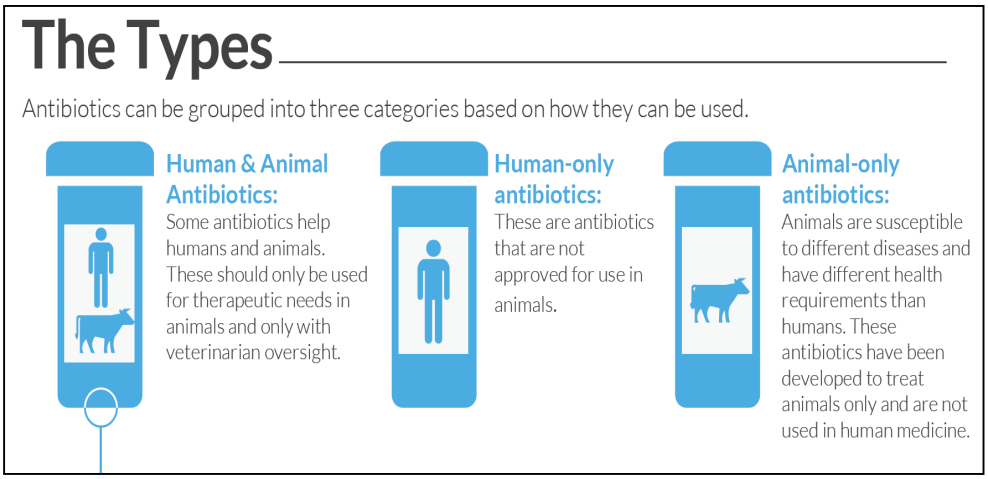


Figure 2. Resistance (%) in Salmonella Typhimurium *in pigs, pork and human cases in Denmark (DanMap2013, 2014)*

This decision in the EU was based on the precautionary principle rather than on scientific data.

The United States has evolved their antibiotic regulations with antimicrobial resistance risk analysis as a critical part of the regulatory approval process. Antibiotics can be approved for therapeutic uses of disease treatment, control and prevention, and for growth promotion. The veterinarian plays a major role as the prescriber of antibiotics, including for use in feed under a "veterinarian feed directive".

The US FDA has, in their guideline 213 for industry, divided antibiotics into three classes. Human use only, *i.e.* classes of antibiotics only used in human medicine, animal use only, *i.e.* classes of antibiotics only used in animal medicine and shared class use, *i.e.* classes of antibiotics used in both human and animal medicine. This guideline is requesting veterinary pharmaceutical companies to voluntarily remove AGP label claims of all shared class antibiotics while allowing the AGP claim to remain for those antibiotic classes that are animal use only (See Box 2).



Box 2. The three classifications of antibiotics, as defined by US-FDA.

A country needs to determine what laws, regulations and responsible uses policies are best for them considering learnings from global experiences. The approach should start fully understanding current policy and practices, the desired goals and then a practical approach that bridges current status to desired status over time.

As we look at addressing the issues of antibiotic resistance globally, under the umbrella of responsible use a number of actions could be implemented including but not limited to (1) Antibiotic resistance monitoring (2) Antibiotic Usage Monitoring (3) Antibiotic Availability by Prescription Only (4) National Formularies and Prescriber Guidelines (5) Consideration of the distribution channel and the promotion of prescription only antibiotics. This article focuses on the first point, Antibiotic Resistance Monitoring.

Antibiotic resistance monitoring forms the corner stone of risk assessment and a metric for measuring risk management policies. Embedded in a microbiological risk assessment to evaluate the potential of antibiotic resistant bacteria contributing to infections in humans that cannot be treated with the same antibiotic class that were used in food animals are the parameters of release, exposure and consequence. Once a risk assessment identifies a potential risk such as increased prevalence of antibiotic resistance and appropriate risk management is implemented, antimicrobial resistance needs to continue to determine if resistance has decreased post risk management intervention indicating appropriate action vs. resistance levels either increasing or remaining stable, which would indicate inappropriate risk management policies.

Zoonotic bacteria such as *Salmonella* and *Campylobacter* can develop resistance to antibiotics in the animal reservoir. These resistant bacteria may be transferred to humans via food and may subsequently cause infections that may not respond adequately to antibiotic treatment. Commensal bacterial populations from the intestinal tract of animals may reflect antibiotic selection pressure as a result of use of antibiotics over time. Animal-origin commensals may also act as a reservoir of resistance genes that can be transferred to pathogens or to other commensals in the human gut. For this reason, *Escherichia coli* and enterococci of animal origin are included in many resistance monitoring programmes as indicator bacteria representative of the Gram-negative and Gram-positive flora, respectively, in order to provide information about the general prevalence of resistance in healthy production animals. These zoonotic and commensal indicator bacteria are referred to as food-borne bacteria in the context of the discussion below.

Resistance monitoring programmes of food-borne bacteria typically collect bacterial strains from food-producing animals, generally at slaughter but also on farm and from retail meats, and then determine their susceptibility against a panel of antibiotics relevant for human medicine. Monitoring of the antimicrobial susceptibility of food-borne bacteria is performed on both a national and international level. Examples of monitoring systems on a regular basis at national level are CIPARS (Canada), DANMAP (Denmark), JVARM (Japan), MARAN (Netherlands), NARMS (USA), NORM-VET (Norway), and SVARM (Sweden). There are a few harmonized international programmes. In the late nineteen nineties, the European Antimicrobial Susceptibility Surveillance in Animals (EASSA) programme was initiated as a shared initiative of the veterinary pharmaceutical industry whilst over a decade ago the European Food Safety Authority (EFSA) established an overarching antimicrobial resistance monitoring programme in food-borne pathogens from food-producing animals and food thereof. The latter integrated EU monitoring has now replaced part of the national programmes in Europe. Hence, currently two international European monitoring programmes of antimicrobial resistance of food-borne bacteria in animals are in place. Additionally, various one-off monitoring studies continue to be published periodically.

Globally, regulatory agencies are relying on risk assessments models to base decisions with respect to responsible use of antibiotics in veterinary medicine. Whilst risk assessment models rely heavily on antibiotic susceptibility data, it is evident that data is not being generated or interpreted using harmonised protocols. In a number of recent conferences, as well as publications, a number of authors presented papers which, as a component, included minimum inhibitory concentration (MIC) evaluations. The studies were robust but lacked a fundamental understanding of interpretive criteria. Many of the authors interpreted the data using epidemiological cut-off values (ECVs) yet reported the interpretation as if clinical breakpoints (CBPs) were used i.e. as 'percentage susceptible' and 'percentage resistant' (%S and %R, respectively). Despite a number of peer-reviewed publications (Bywater *et al*., 2006; Schwarz *et al*., 2010; Silley *et al*. 2011) and the recent Clinical and Laboratory Standards Institute's X-08R report (CLSI, 2011) it is regretful that there is still a lack of distinct and clear understanding of the differences between ECVs and CBPs.

Clinical breakpoints are numerical values used solely to predict the probability of treatment success using standard dosing regimens. Their role is neither to predict the presence or absence of an acquired resistance mechanism, nor predict the outcome of using dosing regimens that are not similar on pharmacokinetic/pharmacodynamic (PK-PD) grounds to the standard dosing regimen. Clinical breakpoints are valued to generate surveillance data that are used to guide clinicians in their selection of empirical therapy, and to update treatment guidelines and protocols. They are less valuable for the detection of emerging resistance mechanisms, because in many circumstances, MIC clinical breakpoints are higher (sometimes much higher) than MIC ECVs (see Figure 3). The implication is that an etiological agent with acquired resistance mechanisms and MICs at or below the susceptible clinical breakpoint will still respond favourably to the standard dosing regimen. Although this concept has been controversial, the validity of treating pathogens with acquired resistance mechanisms is now more widely accepted because of supporting data from animal models.

ECVs are determined on the basis of the distribution of MICs for an antimicrobial and a bacterial species. The population that clearly departs from the normal population ("wild type") is categorized as "non–wild type". The differences between ECV and clinical breakpoints are outlined in Figure 3, the principles of which apply to all antimicrobial compounds. Although the use of ECVs is important for the early detection of decreased susceptibility, it is usually inappropriate to use this non–wild-type value to determine percentage clinical resistance. Figure 3 demonstrates that there are instances in which a bacterial isolate will have an MIC value above the ECV (≤0.25 μg mL^{-1}) but below the clinical susceptible breakpoint (≤4 μg mL^{-1}). Such an isolate will remain clinically susceptible and should therefore not be categorized as resistant.

Antimicrobial Susceptibility Testing (AST) studies seek to categorize bacterial isolates as susceptible, intermediate or resistant to each antimicrobial tested, on the basis of the MICs or the zone diameters obtained. Such classification requires approved interpretive criteria. Currently, two different types of interpretive criteria are available: CBP and ECV values. The precise emphasis of a particular study will dictate which criteria must be applied. If data are intended to guide a therapeutic approach (*i.e*. the aim of the study is to determine which antimicrobial agents are most likely to lead to therapeutic success), the CBPs must be applied. ECVs should be used to describe MIC distributions of bacteria without clinical context. CBPs and ECVs may be very similar or even identical for some bacteria/drug combinations; however, authors need to understand that ECVs are determined by a different approach than CBPs and do not necessarily take into account the results of clinical efficacy studies, dosing and route of administration of the antimicrobial agents, nor the drug's pharmacokinetic and pharmacodynamic parameters in the respective animal species. The term 'breakpoint' should be used exclusively for CBPs and 'susceptible', 'intermediate' and 'resistant' categories should also be reserved for classifications made in relation to the therapeutic application of antimicrobial agents.

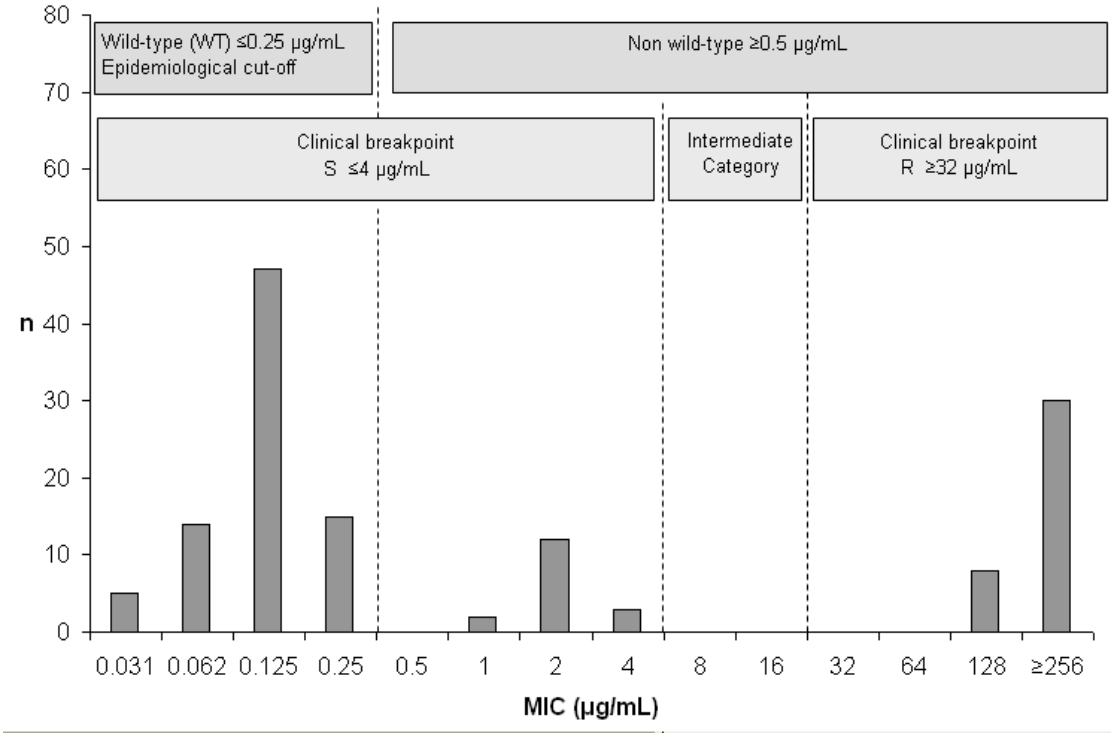


Figure 3. Distribution of MICs and Categorization by Clinical Breakpoints Contrasted to ECVs.

When reporting data using epidemiological cut-off values, the term 'resistant' is inappropriate; instead, bacteria should be reported as 'wild-type' if the MIC or zone diameter falls within the wild-type range, or 'non-wild-type' if the MIC is higher or the zone diameter smaller than the wild-type range.

Universal adoption and understanding of separate CBP and ECV terminology would enable veterinarians to choose appropriate treatment based on information relevant to the individual animal, yet would recognise that epidemiologists need to be aware of small changes in bacterial susceptibility which may indicate emerging resistance, and allow for appropriate control measures to be considered.

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GLOBAL PERSPECTIVES ON ANTIMICROBIAL RESISTANCE IN THE FOOD-CHAIN

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Abstract
Antimicrobial resistance (AMR) has become a priority issue in human medicine and animal health with many countries recognizing it as an important emerging threat to global public health and food security. There has been a concerted global effort in the last few years to bring this issue into the limelight and secure the political commitment to take action. While the development of resistance to antimicrobials is a natural phenomenon, the overuse and inappropriate use of antimicrobials are considered to be important factors in rapidly exacerbating the problem. Antimicrobials are an important part of our food and agriculture production systems and are used for both therapeutic and non-therapeutic purposes. With the increasing demand for food, particularly of animal origin, to meet the demands of a growing global population, it is estimated that antimicrobial usage in the food and agriculture sector will rise substantially in some parts of the world. Meeting the dual challenge of protecting the efficacy of antimicrobials while still producing adequate safe food is a major task facing the food and agriculture sector. This paper explores some of the issues around antimicrobial resistance in the food and agriculture sectors with a particular focus on food safety aspects along the food-chain.

Introduction
The availability and use of antimicrobial drugs in terrestrial and aquatic animal and crop production is essential for their health and productivity and contributes to animal welfare and food safety. This in turn contributes to the protection of livelihoods, sustainability of production and ultimately food security (FAO, 2015a). The global concerns about resistance to antimicrobial drugs threaten to reverse these benefits as well as the decades of benefits to human health. Antimicrobial resistance (AMR) has thus become a priority issue in human medicine and animal health (WHO, 2014a; FAO, 2015b).

In May 2014, following the publication of the first global report on surveillance on antimicrobial resistance (WHO, 2014b), the World Health Assembly adopted a Resolution, which called for the development of a Global Action Plan (GAP) on AMR and strengthened collaboration among what are often referred to as the three sisters, the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE) and the World Health Organization (WHO), to combat AMR within the context of the "One Health" approach. That GAP was developed by WHO with input from FAO and OIE and adopted in 2015 (WHO, 2015a). The GAP sets out five strategic objectives: 1) to improve awareness and understanding of AMR; 2) to strengthen knowledge through surveillance and research; 3) to reduce the incidence of infection; 4) to optimize the use of antimicrobial agents; and 5) to develop the economic case for sustainable investment that takes account of the needs of all countries, and increase investment in new medicines, diagnostic tools, vaccines and other interventions. It clearly recognizes a role for the food and agriculture sectors in the global efforts to combat AMR.

Commitment to AMR in the food and agriculture sector
Highlighting the importance of this issue for the food and agriculture sector, FAO members adopted a Resolution on AMR in 2015 (FAO, 2015b) setting out targets for the membership and the FAO in addressing this issue. The commitment of OIE was also concretised through an adoption of a resolution on AMR in 2015 (OIE, 2015). The challenge now is to channel and convert the high level political commitment to this issue into real action. To address this, FAO has defined its strategy through its plan of action to support the food and agriculture sector in addressing AMR. FAO's plan revolves around four pillars: 1) awareness of the issue, its impact on the sector and commitment to action; 2) evidence (through surveillance and monitoring) to support the development of locally relevant strategies and data against which to measure progress; 3) governance both locally and internationally; and 4) the promotion of good practices in food and agricultural systems to reduce the use of antimicrobials and the spread of antimicrobial resistance (FAO, 2015c).

The complexity and interconnectivity of the environment in which AMR develops and spreads means that the success of any efforts is dependent on achieving an effective multidisciplinary and multi-sectorial approach. This is the basis of the FAO approach, which is bringing together internal production specialists, veterinarians, lawyers, food safety and laboratory specialists (among others), which is further enhanced through its tripartite working arrangements with WHO and OIE.

AMR and food

FAO recognizes that food plays an important role in AMR. The food-chain provides innumerable niches where microbial populations abound and in which AMR can emerge and disseminate, and food plays a significant role in the very complex pathways of AMR transmission to humans. Pressures for the selection of AMR bacteria can occur in the food-chain both in natural environments and following the use of antimicrobials (including antibiotics, antivirals, antifungicides and antiparasitics) in food production, including aquaculture, livestock production and crop culture. It is also possible that the use of sanitizers and biocides through the food-chain can contribute to AMR (Tezel and Pavlostathis, 2011). As with any other sector, awareness of the issue and the appropriate governance mechanism or policy and regulatory framework within which to operate are key to meeting the AMR challenge. The two other major and inter-dependent aspects of management of the transmission of AMR via food are: i) prudent use of antimicrobials in food production and management of the whole food-chain to prevent or minimize the emergence and transmission of AMR; and *ii*) the need for monitoring and surveillance systems and the related laboratory infrastructure, to have an understanding of the current situation with regard to antimicrobial usage, the extent of resistance in the food-chain and the potential to monitor progress.

At the international level, the Codex Alimentarius[a] has for over a decade recognized the importance of AMR in food safety and quality. In 2005, Codex adopted its "Code of Practice to minimize and contain antimicrobial resistance" (Codex Alimentarius, 2015), with the stated objective of minimizing the potential adverse impact on public health resulting from the use of antimicrobial agents in food producing animals. It highlighted the importance of all those involved in "*the authorization, manufacture, sale, supply, prescription and use of antimicrobials in food –producing animals*" to "*act legally, responsibly and with the utmost care in order to limit the spread of resistant microorganisms among animals so as to protect the health of consumers*." This was not an isolated standard. It is intended to be used in conjunction with Codex guidelines on national regulatory programmes associated with the use of veterinary drugs in food producing animals (CAC, 2009). Subsequently in 2011, Codex adopted "Guidelines for risk analysis of foodborne antimicrobial resistance" (Codex Alimentarius, 2015). This highlighted the importance that the international food regulatory community placed on having a structured and harmonized framework for AMR risk analysis. The guidelines also give a perspective on the complexity of the issue and the related challenges in addressing AMR within the food-chain. Codex is currently undertaking work to review the extent to which countries are adopting and applying the existing Codex guidance and to identify major capacity development gaps and any other challenges they face in adopting and applying these standards. This work will also evaluate the need for the update of the existing Codex texts, taking into consideration the developments in the area over the past ten years and consider the need to review any new scientific evidence related to the AMR in the food-chain including risk management options for the containment of AMR in support of any revision of Codex texts. In this context it is likely that discussions on AMR in the food-chain will continue within this international food safety forum in the coming years.

AMR in food ecosystems

Bacteria can be intrinsically resistant to antibiotics due to their inherent makeup, or following exposure to antimicrobials they can acquire or develop resistance through gene mutation or by horizontal transfer of resistance encoding genes from other bacteria. Such exposure can be due to inappropriate use or misuse of antimicrobials. Food and food-chain environments are widely exposed to bacteria that may be intrinsically resistant and the use of antimicrobials, possibly also sanitizers and biocides, and as yet unknown factors can strengthen the selection pressures for AMR mergence. Alexander Fleming was already aware of the potential risk of resistance noting that "*there is the danger, that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant*". He may not have realised the extent to which his observation would become a reality or the extent to which resistant organisms would become part of our food ecosystem. Recent studies have indicated that resistant organisms are found in the environment around livestock production systems (Zhang *et al*., 2013), that our waterways and oceans are now serving as potential reservoirs for AMR (Hatosy and Martiny, 2015) and that the AMR resistant strains in our food-chain may come as often from the human sources as they do food and agriculture sources (Woolhouse *et al*., 2015). Food ecosystems provide ideal niches and conditions where bacterial genes can be mobilized and a food microbiome can be exposed to additional genetic material through interactions with those of animals and humans and the environment (*e.g*. soils, oceans). Transfer of AMR genes among food bacteria, including pathogens, has been demonstrated *in vitro* and *in vivo e.g*. between lactic acid bacteria, *Enterococcus* and *Listeria monocytogenes*, between *Salmonella* and *Escherichia coli*, among *E. coli* and between isolates of a species from animal and human origins (Economou and Gousia, 2015).

[a] The Codex Alimentarius or "Food Code" was established by the Food and Agriculture Organization of the United Nations and the World Health Organization in 1963 to develop harmonised international food standards, which protect consumer health and promote fair practices in food trade. For more information, see http://www.fao.org/fao-who-codexalimentarius/codex-home/en/

Food – a favourable niche for AMR transmission

Bacteria usually do not reside in food ecosystems as planktonic cells, rather they form assemblages or biofilms, free-floating or attached to surfaces where the same or multi-species communities are enclosed in a self-produced polymeric matrix (Madsen *et al*., 2012). Biofilms are found in animals and humans, on horticulture crops, and on every non-sterile surface along the food-chain. In biofilms, bacteria are present in high density in close contact; the matrix concentrates nutrients and compounds that facilitate cell interaction and protects the bacteria from biotic and abiotic exposures; and, periodically bacteria laden fragments slough off. Biofilms provide ideal protective structures that can enhance transfer of genetic elements, bacterial fitness and persistence and, in reverse, these elements may even promote biofilm formation.

Food reservoirs of bacteria and AMR elements

Except for commercially sterile foods, foods are contaminated with numerous bacterial species and our knowledge is limited to those culturable in laboratories and these represent only a small portion of those present. Raw foods have the highest bacterial concentrations followed by minimally and fully processed foods, while bacteria can also be purposefully cultured in foods or used as pro-, pre- and syn-biotics. Additional food contamination occurs when food is handled and from environmental exposure and this varies with the level of food-chain hygiene conditions. In FAO/WHO studies of food-chains in regions with poor hygiene management about half of raw chickens at markets were contaminated with >1,000 *E. coli* mL^{-1} rinsate, many were AMR and some multi-drug resistant. Even with good hygiene conditions, ready-to-eat foods can be considered of satisfactory microbiological quality when contaminated with up to 10^4 colony forming units (cfu) aerobic bacteria g^{-1} and can include pathogens *e.g*. a few *Escherichia coli* g^{-1} or up to 100 coagulase positive *Staphylococci* g^{-1} and others. Foods including those of acceptable microbiological quality can be considered as a virtual pool or sink of bacteria, bacteriophages, bacterial DNA and mobile genetic elements, some of which may include AMR genes (Economou and Gousia, 2015). A better understanding of the broader microbiome associated with a food and food-chain environments and its content of AMR genetic material will help better elucidate the role of the food-chain in AMR. With new techniques such as whole genome sequencing and next generation sequencing receiving increasing use and multiple efforts ongoing to sequence various food production biomes we will potentially have a much better understanding of these aspects in the near future.

Known foodborne AMR pathogens

Foodborne pathogens e.g. *Salmonella*, pathogenic *Escherichia coli*, *Campylobacter*, *Staphylococcus* spp., *Enterococcus* spp. and extended-spectrum β-lactamase (ESBL)-producing Gram negative bacteria, have been the obvious focus of AMR attention. This is due to increased observance of AMR resistance among these strains and links between the presence of AMR strains of these bacteria in food, the use of antimicrobials in animal production, and human illness have been made (Landers *et al*., 2012; ECDE/EFSA/EMA, 2015). This has placed emphasis on zoonotic foodborne bacteria and AMR in animal products although the role of other commodities indirectly contaminated or unrelated is less well understood. Microorganisms do not respect geographic borders and the global food trade pathways present ample opportunity for their global spread. For example, *Salmonella* DT104, which is typically resistant to five types of antibiotics (ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline), was first isolated in the United Kingdom in the 1980s and was later discovered to be endemic in cattle, which acted as a reservoir for contamination of meat. It spread worldwide with alarming speed during the 1990s and is now common, especially in Europe and North America. Of particular ongoing concern is the fact that it has shown an ability to acquire resistance to other types of antibiotic, including the clinically important fluoroquinolones and cephalosporins. A recent genomics study investigating the emergence and spread of *Salmonella* DT104 suggest that this may have first appeared back in approximately 1948 and that multiple transmission events led to its global spread (Leekitcharoenphon *et al*. 2016), highlighting the complexity of AMR in the food-chain. *E. coli* O104:H4, which caused a major outbreak of foodborne disease in Germany in 2011 (sprouted seeds), and resulted in 53 deaths and over 3,000 illnesses, was found to be resistant to a number of antibiotics (ampicillin, trimethoprim, cephalosporins and tetracycline) (Robert Koch Institute, 2011). Furthermore, it possessed a plasmid-borne gene for ESBL production, which can mean resistance to a wide range of therapeutic antibiotics.

Even these recognized pathogens may present unexpected challenges. Surveillance and monitoring of pathogens and AMR in human illness and non-human sources is required to identify and respond to threats in a timely manner. A better understanding of the role of food in AMR transmission is important together with an understanding of the potential associations between AMR genes and other important genes encoding virulence and the survival and persistence of pathogens in foods and their environments. For example, currently there is an epidemic of invasive (compared with usual non-invasive) non-typhoidal salmonellosis occurring among high risk individuals in regions of Africa and parts of Europe (Ao *et al*., 2010). The systemic clinical manifestations indicate antimicrobial treatment and AMR among the strains is becoming a major concern. Person-to-person transmission appears common; however, if the traditional role of food, water and animals in transmission occurs the epidemic could take a dramatically different path.

The role of food control systems in addressing AMR

Irrespective of its complexity, food presents a potential route of exposure for everyone to AMR organisms. With recent research indicating that antimicrobial usage in food animal production is estimated to increase by about two thirds by 2030, there is clearly a need for the food sector to play an active role in addressing AMR. While much of this rise is anticipated simply due to the need to produce more animal protein, at least a third of the increase is linked to changing production systems such as a move towards intensification (Van Bockel *et al*., 2015). This, combined with the current knowledge that food provides a favourable environment for resistance development and transmission, clearly highlights the need for action. It is also clear that the action taken will have to be multi-dimensional, involving different sectors and "One Health" in nature. However, for now let us look as the role of food control in addressing this issue and in the four key areas identified earlier in the paper 1) awareness, 2) governance, 3) management of the transmission of AMR via good practices, and 4) the need for monitoring and surveillance systems and the related laboratory infrastructure.

Awareness

A minimum understanding of the issue and why it is relevant to all stakeholders along the food-chain is an important precursor for any action that will contribute to addressing AMR. However, key-players in the food-chain often have limited knowledge of the relevance to this issue to the food sector in terms of both its impact on the sector and the role of the sector in addressing the problem. FAO has directly observed this lack of awareness at national level amongst many stakeholders and has defined it as one of its four key focus areas on AMR. An important aspect to awareness raising efforts is ensuring that it resonates with food stakeholders and their role and where possible using local and/or sector specific evidence a basis for the messaging.

Governance

Codex has already laid the foundations for food control authorities to tackle this issue with its various standards related to AMR, veterinary drugs and their residues, food hygiene, animal feed and others. These standards serve two purposes – to minimize occurrence of conditions which could lead to AMR and to minimize the transmission of AMR organisms and/or determinants through the food-chain. The challenge for countries is to take these standards and incorporate them into national legislation. An overview of how some national authorities are progressing in this regard will be included in a paper on AMR currently under preparation for the next session of the Codex Alimentarius Commission in July 2016. Understanding how to incorporate AMR into national legislation is also a challenge, which FAO has identified, due to the cross-cutting nature of the issue. However, as the capacity and resources of many countries to take action to address AMR is dependent on political commitment, appropriate policy and a relevant regulatory or legislative framework within which to operate this aspect cannot be ignored.

Management of the transmission of AMR via food

Management of the transmission of AMR via food is multi-pronged; it incorporates management of the whole food-chain to prevent or minimize the emergence and transmission of AMR and prudent use of antimicrobials in food production. At the practical level the overall aim of controlling AMR transmission via food is similar to general requirements for the control of other foodborne hazards e.g. food safety hazard control and hygiene all along the chain from inputs (*e.g*. animal feed) to food production through to the finished product. In addressing AMR greater emphasis on managing hygiene through the food-chain to control microbial populations that harbour the AMR gene pool as well as consideration of the food environment factors that may contribute to resistance development are required. In this context, hygiene is more than a pre-requisite for pathogen control but also an essential element for the control of AMR.

Sanitizers and biocides are used to support hygiene and sanitation programs along the food-chain and minimize conditions that can support the development of AMR. Differences in the mode of action of these compounds compared to antimicrobials such as antibiotics meant that they were not considered important in the development of AMR themselves. However, more recent research suggests that build-up of some of these compounds in the environment may be a risk for AMR development (Tezel and Pavlostathis, 2012). This area requires further understanding to guide appropriate and safe use.

A vital aspect of the control of AMR is the use of antimicrobial drugs in veterinary medicine. There are several aspects to this which range from better infection control and improved production systems to minimize the need for antimicrobials and prudent use of antimicrobials which remain critical for the food and agriculture sector. These aspects need to be supported with information on best practices which are based on local information to the extent possible and can guide key behavioural and practice change at farm level and beyond. Examples of FAO guidance which is supporting such an approach include guidance on "Improving biosecurity through prudent and responsible use of veterinary medicines in aquaculture food production" (FAO, 2012). Similarly, ongoing work within FAO, such as the development of guidance on risk-based meat inspection provide an opportunity to highlight the issue of AMR and its relevance in this context. Codex already provides a framework for minimizing use and as an example the codex code of Practice on animal feeding specifically states that "Antibiotics should not be

used in feed for growth promoting purposes in the absence of a public health safety assessment" which already provides a strong international reference point for countries to target in their national efforts.

With respect to veterinary drug use and their residues, two types of AMR are important (WHO, 2014c): 1) the incidence of antimicrobial-resistant bacterial veterinary pathogens, zoonotic bacterial pathogens and commensal microorganisms in food producing animals through the veterinary use of antimicrobial agents; and 2) the selection and emergence of resistant microorganisms in the human gastrointestinal tract through exposure to residues present in commodities from food-producing animals treated with antimicrobial agents. Both types of resistance have implications for human health, but only that arising from human exposure to residues of antimicrobial agents in edible foods is relevant to the work of the Joint FAO/WHO Expert Committee on Food Additives (JECFA[b]). The Committee has established procedures for evaluating this, as well as the possible impact of such residues on disruption of the colonization barrier function of the gastrointestinal microbiota. In this context it is important to note the work carried out by (JECFA).

Monitoring and surveillance systems and diagnostics

Understanding the extent of antimicrobial use and AMR is the food sector serves an important role in terms of securing commitment to address the issue and in determining the most appropriate interventions. This can ideally be achieved through integrated monitoring and surveillance systems programs would coordinate testing of samples from food animals, foods and humans for resistant organisms (WHO, 2013). There are currently only a few countries with AMR surveillance programs which produce somewhat comparable data. Another big gap exists in terms of antimicrobial usage. In line with the GAP for AMR, WHO and OIE are following up on mechanisms to collect data on consumption in human and animal health. However, this needs to be augmented with better data on all aspects of usage in the food production sector. Data from monitoring programmes also provide a means of monitoring the efficacy of interventions. In this context national monitoring plans for residues of veterinary drugs (including antimicrobials), supported by appropriately accredited analytical laboratories and methods, provide feedback on the effectiveness of controls and the implementation of regulations. Monitoring of veterinary drug residues and monitoring of targeted antibiotic bacteria, particularly food-borne pathogens in animals raised for food production, are both important for the provision of information and evidence of AMR. The possibility to combine both types of monitoring on the same samples, for example those collected under a veterinary drug residue monitoring plan, such as that outlined by Codex (Codex Alimentarius, 2009), would potentially provide useful information on possible direct linkages between antimicrobial drug use (or abuse) for veterinary purposes and the development of AMR and serve as a basis for more integrated surveillance systems. Another key aspect is the methodology available to support such programs. With the development and increasing uptake of new technologies, in the field of genomics for example, there is the potential for surveillance programs to be much more informative. However improved methodology and diagnostics is not only relevant to monitoring and surveillance programmes but also to more prudent use, with for example more rapid identification of infections facilitating a more targeted approach to use of antimicrobials.

Conclusions

Food is one of the potential routes of exposure to antimicrobial resistant organisms that is relevant to each and every individual. Given the complexity of current global food trade, geography becomes irrelevant as resistant organisms from one part of the world can easily be transported to another on the global food highway. A food-chain approach which recognizes the hazards, their sources and the related risks, and emphasizes the need for prevention, through implementation of good practices at the primary production level and through the food processing environment remains critical when it comes to the prevention and control of antimicrobial resistance. For example, good hygienic practices are integral to minimizing the introduction and spread of microorganisms in food; however, given the current level of foodborne illness due to microbial hazards (WHO, 2015b), it is clear that we have far from optimized our food hygiene efforts along the food-chain.

Improvements in food safety and hygiene are particularly required in regions of poor sanitation and hygiene, where there are no national strategies, or, where there is inadequate implementation of national or local programs for ensuring food safety and hygiene. Mapping of bacterial contamination including pathogens and indicators and characterization of isolates along food-chains can indicate key points where contamination occurs, the dynamics of the population concentrations and where interventions can be applied. These are useful studies to realistically demonstrate to food-chain actors and authorities the

[b] JECFA is a joint FAO/Who expert committee which elaborates principles for undertaking safety and risk assessments for residues of veterinary drugs (including antimicrobials); establishes Acceptable Daily Intake (ADIs) and other guidance values for acute exposure; recommends maximum residue limits (MRLs) for target tissues; and determines appropriate criteria for and evaluates methods of analysis for detecting and/or quantifying residues in food. The MRLs recommendations made by JECFA are based on the use of the drugs in accordance with Good Veterinary Practice (GVP). The recommended MRLs are then considered and eventually adopted by Codex as international standards.

roles they play and actions that need to be undertaken. Practical and economical ways to do this and to link in with surveillance and monitoring programs are important in achieving this. In all situations, monitoring and investigation is required to be sure that processing procedures and technologies, excessive use of sanitizes and biocides is not inadvertently contributing to AMR or cross-resistance and mobilization of other virulence, resistance or persistence capabilities.

As documented by the Codex Standards, for over a decade the food safety community have acknowledged that antimicrobial drugs are powerful tools for the management of infectious diseases in animals and humans and need to be protected. In recent years, there have been some advances in implementing risk-based approaches to the prevention and control of AMR. However, significant challenges remain in translating internationally accepted guidelines into appropriate policies and actions at national and regional level. Much still needs to be done in many parts of the world to strengthen policies, capacities and systems for the detection, monitoring, and management of AMR along the food-chain.

Effective approaches to combat AMR need to be put in action by coordinated activities at different levels. This requires a consolidated multi-sectorial effort, of which those involved in food safety along the food-chain can make an important contribution. These include improving regulatory frameworks based on internationally agreed principles and standards of Codex; reducing the need for antimicrobials in animal husbandry, by improving animal health disease prevention and good practices along the chain; strengthening food and human surveillance systems for AMR and the quantities of all antimicrobials being used and building on existing residue monitoring systems to provides inputs on where to focus these efforts; raising awareness (among food-chain stakeholders) about AMR; developing guidance on effective regulation, and specific guidelines for food-chain stakeholders from feed manufacturers to farmers and food processers, with particular emphasis on the prudent and responsible use of antimicrobials; and supporting locally-led research to generate data/information to underpin national policy making in relation to foodborne pathogen contamination and AMR. In simple terms the objective of these measures is to *remove* the need for antimicrobials where possible, *reduce* the amount of antimicrobials and *restrain* and control the transmission of resistance through the food-chain.

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SPIRITS THAT WE'VE CITED OUR COMMANDS IGNORE – THE FATE OF ANTIBIOTICS IN MANURE

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Abstract
The usage of antibiotics in veterinary medicine is still widespread. In Germany, more than 1,200 metric tons were applied in 2014. However, due to poor metabolism and adsorption in the animal gut, up to 90% of the parent compounds can be excreted and antibiotics may enter the environment via fertilization of agricultural soils with liquid manure. Manure is also used as substrate in biogas plants, but currently only little is known about degradation pathways and potential degradation products of veterinary antibiotics during the anaerobic fermentation process. Therefore, we investigated the fate of antibiotics in batch fermenters with both autoclaved and non-autoclaved inoculum to evaluate the impact of biotic and abiotic processes on the elimination. Whenever possible, transformation products were isolated and the structures were elucidated. Moreover, semi-continuous fermenter studies revealed a distinct influence of the experimental design on the elimination.

Introduction
Liquid manure is commonly used as soil fertilizer due to its high nutrient content (Insam *et al.*, 2015 and references therein). However, if animals are medicated, residues of the pharmaceuticals can be frequently detected in the manure due to poor absorption in the animal gut and/or unmetabolized excretion (Sarmah *et al.*, 2006 and references therein). Thus, if the manure is used for fertilization, the active ingredients can enter the environment. Currently, there is no safety limit for pharmaceuticals in manure regulating its applicability as fertilizer. Pigs and cattle excrete approx. 4 kg and 53 kg manure (faeces and urine) per day, respectively (Lange *et al.*, 2002 and references therein). In Germany, 12.4 million cattle and 28.7 million pigs are kept in agricultural holdings (Deutscher Bauernverband 2015). This corresponds to an amount of 279 million metric tons manure per year. If a limit value for pharmaceutical active compounds was introduced, this could lead to a huge challenge concerning manure disposal as the excrements might partly not be disposed on fields anymore.

Manure is also used as substrate in biogas plants (Weiland 2010). Several studies discussed the potential of the anaerobic fermentation process to serve as sink for compounds such as veterinary antibiotics. For different compounds such as sulfonamides and tetracyclines, a reduction of the initial content has been reported, in some cases even a complete elimination was observed (Arikan *et al.*, 2006; Mohring *et al.*, 2009; Spielmeyer *et al.*, 2015). However, many studies lack the elucidation of the transformation products and/or the elimination pathways. Especially the question whether biotic or abiotic processes are involved has been considered only in few studies (Zhang *et al.*, 2013). Thus, the potential of the anaerobic fermentation concerning the elimination of pharmaceutical active compounds cannot be conclusively evaluated so far. If compounds such as antibiotics were efficiently eliminated within the biogas production process or if their content was at least partly reduced, the input of these substances in the environment could be considerably minimized.

The elimination of sulfonamides in both batch and semi-continuous fermenters as well as in biogas plants was investigated. Whenever possible, corresponding transformation products were isolated and their chemical structures as well as their antimicrobial activity were determined. By using autoclaved and non-autoclaved inoculum, the proportion of biotic and abiotic elimination processes was evaluated.

Materials and Methods

Experimental setup
Biogas plant samples were taken from a biogas plant in Bavaria, Germany, during the course of one year. For input samples, liquid manure was taken from the storage basin and mixed with the respective amount of manure to simulate the actual biogas plant input (renewable primary products were not taken into account). For output samples, a one-hour mix sample was taken directly from the fermenter.

Batch fermentation studies were conducted in 20 L polypropylene vessels using fermented swine and cattle manure (ratio 1:3) as inoculum. One antibiotic compound was added to each fermenter to reach a final content of 5, 50 or 200 mg analyte per kg substrate. In addition, autoclaved inoculum was fortified and transferred into glass bottles. The fermenters and bottles were incubated for 35 days at mesophilic conditions (40°C). For all studies, sample aliquots were filled in PE bottles and stored at -20°C before analysis.

For semi-continuous fermenter studies 36 L fermenters consisting of plastic and stainless steel were utilized. Fermenter inoculum, liquid manure and maize silage were obtained from the biogas plant in Bavaria which was monitored over one year. The fermenters were fed daily with a substrate exchange rate of 1.1%. The liquid manure already contained antibiotics, so for control purposes no fortification was performed. Samples were withdrawn from the bottom of the fermenter two times per week before the addition of new substrate. Sample aliquots were filled in PP centrifuge tubes and stored at -20°C before analysis.

Sample preparation and analysis

For extraction, 1 g sample material was suspended in citrate buffer. The antibiotics were extracted with a mixture of ethanol, methanol and dichloromethane (samples of biogas plants and semi continuous fermenter studies) or isopropanol and a 1:1 volumetric mixture of ethanol and acetonitrile (batch fermenter study). After solvent evaporation the residue was reconstituted in methanol/water and analysed via UHPLC-MS/MS (Spielmeyer *et al.*, 2014). Results are given as mg antibiotic per kg fresh weight. For calculation of the recovery rates, the analyte content of day 0 was considered as 100%.

Results

Of all sulfonamides investigated, sulfadimethoxine, sulfamethoxypyridazine and sulfamethoxazole showed an almost complete elimination within 35 days during batch fermentation. The elimination was suppressed when autoclaved inoculum was utilized (Table 1).

Table 1. Recovery rates of selected sulfonamides in autoclaved and non-autoclaved inoculum during batch fermentation.

	sulfadimethoxine		sulfamethoxypyridazine		sulfamethoxazole		sulfamethazine	
day	non-ac	ac	non-ac	ac	non-ac	ac	non-ac	ac
0	100	100	100	100	100	100	100	100
1	55	100	84	92	26	88	97	100
3	19	91	62	91	1	95	91	94
7	2	71	42	92	0	80	93	90

recovery rates given in %, non-ac – non-autoclaved, ac – autoclaved; results shown for a fortification of 5 mg kg^{-1}

In case of sulfadimethoxine and sulfamethoxypyridazine, an O-demethylation occurred (Figure 1). This sort of reaction can be catalysed by acetogens like *Acetobacterium woodii*, an organism also common in biogas plants (Berman and Frazer 1992; Weiland 2010). O-Demethylated transformation products of sulfadimethoxine were previously detected in excrements of chicken, snails or turtles (Takahashi 1986, Vree *et al.*, 1989a, Vree *et al.*, 1989b)). For sulfamethoxazole, products of a reductive cleavage of the isoxazole ring and a rearrangement of the heterocycle were observed (Figure 1). Latter one was also detected in the sample extracts of the autoclaved inoculum implying an abiotic transformation pathway for this product.

The frequently used veterinary antibiotic sulfamethazine showed no elimination during batch fermentation (Table 1). However, different results were obtained using semi-continuous fermenters. As the manure utilized in this experiment already contained sulfamethazine, the course of the analyte could be monitored without artificial fortification of the substrate. The experiment lasted for 64 days, but already on day 29 the sulfamethazine content reached a plateau at 5.2 mg kg^{-1} (Figure 2). This result is even more remarkable when it is compared with the results obtained for biogas plant samples (Table 2). Those samples were withdrawn from the plant whose materials were used for the semi-continuous fermenter studies. Apparently, comparable elimination processes occurred both in the lab-scale fermenter and the biogas plant. These processes are currently unrevealed, but evidently they do depend on the experimental design (batch versus semi-continuous fermentation).

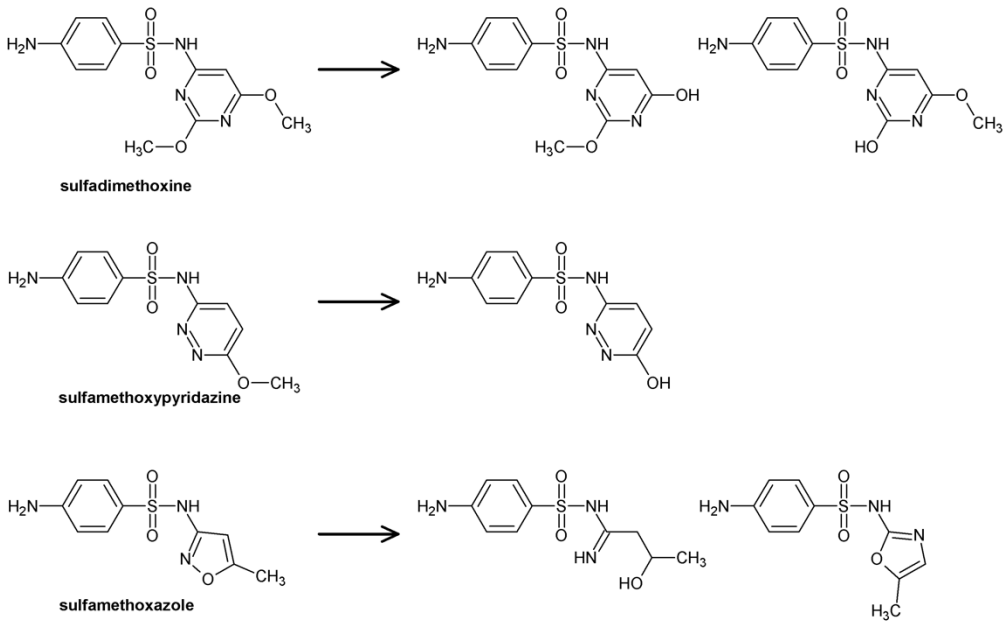

Figure 1. Transformation products of sulfonamides built up during batch fermentation.

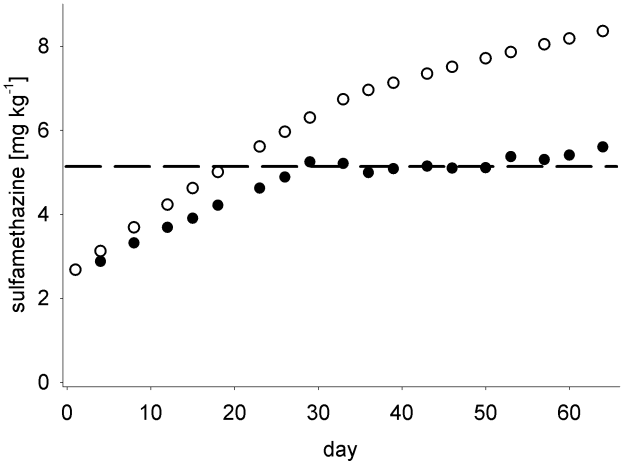

Figure 2. Sulfamethazine content during semi-continuous fermentation; open and black symbols represent the calculated and the measured content, respectively; the dashed line shows the sulfamethazine content of the biogas plant output which was used for incubation (see Table 2); results are given in mg per kg wet weight.

Table 2. Antibiotic contents of biogas plant input and output samples obtained during the course of one year.

compound	sulfadiazine		sulfamethazine		chlortetracycline		tetracycline	
sampling (month/year)	in	out	in	out	in	out	in	out
02/13	0.5		11.2		3.0		0.3	
04/13	0.1	0.2	12.3	5.2	1.7	<MLOQ	<MLOQ	<MLOQ
07/13	<MLOQ	<MLOQ	20.7	9.2	2.3	0.7	<MLOQ	<MLOQ
10/13	<MLOQ	<MLOQ	3.9	3.8	2.6	0.5	<MLOQ	<MLOQ
01/14		<MLOQ		2.8		0.7		<MLOQ

results given as mg per kg wet weight; <MLOQ – content below method limit of quantification

In general, higher antibiotic contents were detected in the biogas plant input samples than in the output samples (Table 2). Chlortetracycline showed the highest elimination rates both during lab-scale fermentation (data not shown) and in biogas plants (Table 2). The main transformation product of chlortetracycline is iso-chlortetracycline which is formed under neutral

or alkaline conditions (Halling-Sørensen *et al.*, 2002). In case of the other compounds, the elimination processes, potential transformation products and the relevant factors leading to the observed elimination have to be identified in future studies.

Conclusions

Veterinary antibiotics can be detected in biogas plant samples with input samples possessing higher antibiotic contents than output samples. Thus, the fermentation process might represent a potential sink for veterinary antibiotics. Using batch fermenters, the elimination of different sulfonamides was investigated in detail. Structure dependent elimination pathways were identified which involved biotic and abiotic processes. However, only minor structural modifications were found in all cases implying that especially sulfonamides are even more persistent than expected. Future studies should determine relevant process parameters leading to better degradation rates.

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VARIABILITY OF RESIDUE CONCENTRATIONS OF CIPROFLOXACIN IN HONEY FROM TREATED HIVES

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Abstract

Honey bees (*Apis mellifera*) were treated with a model veterinary drug compound (ciprofloxacin) in a three-year study (2012 to 2014) to investigate the variability of residue concentration in honey. Sucrose solution containing ciprofloxacin was administered to 45 hives (1 g of ciprofloxacin per hive) at the beginning of the honey flow in late May/mid June 2012, 2013 and 2014. Buckfast honey bees (*A. mellifera* – hybrid) were used in years 2012 and 2013. Carniolan bees (*A. mellifera carnica*) were used instead of the Buckfast bees as a replacement due to unforeseen circumstances in the final year of the study (2014). Honey was collected over nine scheduled time points from May/June till late October each year. Up to five hives were removed and their honey analysed per time point. Honey samples were analysed by liquid-chromatography tandem-mass spectrometry (LC-MS/MS) to determine ciprofloxacin concentration.

Statistical assessment of the data showed that the inter-hive variation of ciprofloxacin residues in years 2012/2013 is very different compared to that of 2014 with relative standard deviation (RSD) of 138% and 61%, respectively. The average ciprofloxacin concentration for 2014 at the last time point was >10 times the concentration compared to samples from 2012/2013 at the same time point. The difference between the 2012/13 data compared to the 2014 data is likely due to the different type of honey bees used in this study (2012/13 Buckfast vs 2014 Carniolan).

Uncertainty estimates for honey with high ciprofloxacin concentration (upper 95th percentile) across all hives for 55-day withdrawal samples gave a residual standard error (rse) of 22%, 20% and 11% for years 2012, 2013 and 2014, respectively. If the number of hives were to be reduced for future studies, rse was estimated to be 52% (2012), 54% (2013) and 26% (2014) for one hive per time point (nine total hives).

Introduction

Pollination by bees is essential for the sustainability of many sectors of the world's agricultural production. In the UK alone, the value of bees' services was estimated at £200 million a year, with the retail value of what they pollinate valued at nearly one billion pounds (DEFRA, 2009). Bee colonies are susceptible to a number of infestations and diseases, including Varroa and Foulbrood (Genersch, 2010), but worldwide there are relatively few drugs legally permitted for the treatment of bees. For instance, in the United States, oxytetracycline, tylosin and lincomycin are authorised to be used to treat American Foulbrood (US Food and Drug Administration, 2015), however the European Union (EU) has yet to officially authorise antimicrobial based veterinary drugs for the treatment of bees (Europe Medicines Agency, 2008). There is current debate internationally and within the EU, on how maximum residue limits (MRL) and withdrawal periods may be set for honey production.

Whilst comprehensive guidelines exist for calculating the withdrawal time for veterinary medicines in most food-producing species, the analytical variables are not that well defined for bees/honey. For example, the EU Notice to Applicants and Guideline for the establishment of MRLs for Residues of Veterinary Medicinal Products in Foodstuffs of Animal Origin (European Commission, 2005) states: 'Where relevant for the proposals for MRLs the expert should present and discuss a summary table of approximate withdrawal periods for each species of food-producing animal as well as their edible products, such as milk, eggs and honey, which could be realistically observed under conditions of good practice in the use of veterinary medicinal products'. The withdrawal periods for animal slaughter as well as for the production of milk, eggs, and honey for human consumption are determined from the results of suitable residue depletion studies using the formulation intended for marketing. For honey, this depletion study should comprise '5 samples from each of 5 hives, the time points to consider should be defined according to the period of treatment and the production of honey; the withdrawal period should provide a high degree of assurance both to the producers and the consumers that the concentrations of residues in foods derived from treated animals are not above the permitted concentrations.

The purpose of this study is to assess the variability of veterinary drug concentration in treated hives and criteria required for establishing a robust protocol for the determination of MRLs in honey. In a previous study, we investigated the use of ciprofloxacin as a model compound for determining the variability of residue concentration in honey using up to ten treated hives (Fussell *et al.*, 2012). Large intra- and inter-hive variability in ciprofloxacin residues were observed in the honey samples. It was evident from the study that larger sample numbers were required from each hive to reduce intra-hive variability and generate statistically meaningful data.

Statistical assessment of the results estimated that a minimum of 40 hives are required to meet a target uncertainty of 0.25. Furthermore, bees can often steal honey from other hives/colonies to supplement their honey stocks which introduces further variables into the study. This is compounded by variable effects due to seasonality of the treatment, even in the same

geographical area. In addition to this study, there have been a number of recent discussions within the VICH regions (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) on the study design recommendations for residue depletion studies in honey. Of particular importance is the timing of bee treatment in relation to the start of the honey flow and sample collection after the start of the honey flow. A "zero withdrawal period" is normally defined as two weeks after treatment when honey flow begins.

In the present study, we have attempted to assess some of the issues from our previous study by (a) using a larger number of hives and (b) bulking up honey from each hive (per superbox) to avoid large intra-hive variation of results.

Adverse weather conditions and season-to-season variation had a significant impact on the honey production throughout the project. This led to several amendments to the planned collection time points, and more significantly a change in the type of honey bees used in the 3rd treatment year (Buckfast bees reared at the National Bee Unit in year 2012 and 2013, and Carniolan bees procured from Poland in year 2014). Finally, the implication of using a reduced number of hives for future MRL determination studies is discussed below.

Materials and Methods

Standards and Reagents
Ciprofloxacin (Vetranal^{TM} grade) and ciprofloxacin-d8 (Vetranal^{TM} grade) were purchased from Sigma (Dorset, UK). HPLC grade methanol, acetonitrile, water and reagent grade formic acid were purchased from Fisher Scientific (Loughborough, UK). Oasis MCX solid phase extraction cartridge (60 mg/ 3 mL) was purchased Waters (Elstree, UK).

Treated Apiary setup
Forty-five colonies of healthy Buckfast bees (*A. mellifera – hybrid*), reared at the National Bee Unit (NBU) in the UK, were placed on a single apiary site and treated with ciprofloxacin towards the end of May in 2012 and June 2013. Bad weather leading up to May 2014 resulted in poor mating of the Buckfast bees at the NBU. This led to the procurement of new queens of the Carniolan variety (sourced from Poland) so that the project could commence without further delay.

Sucrose solution (50% w/v) containing ciprofloxacin (1 g) was applied to each hive by pouring the solution into an empty brood frame. The treated frame was placed two frames from the edge of the brood nest with the treated side of the frame facing outwards.

Control apiary setup
Five colonies were prepared and balanced in the same way as the treated colonies, but were located at a different apiary (10 km south of the treated apiary) and remained untreated.

Sampling protocol – treated colonies
Each colony initially contained one brood chamber/box at the bottom and one superbox on top. Depending on the productivity of the bees, additional superboxes were added to the top of the original superbox (superbox 1) as and when required (*i.e.* top "supering"). Honey from each superbox was sampled and analysed at nine pre-determined time points. In our previous study all treated hives remained on site and only small portions of honey were removed from each hive. This method of sampling gave large intra-hive variation of ciprofloxacin concentration, demonstrating that the residue is not homogeneously distributed throughout the hive. In order to reduce the sampling variability in this study, five hives were collected and taken off site at each time point. Honey from each individual super box was bulked up and homogenised. Two sub-samples were taken from each bulk honey sample and were analysed in duplicate.

Sampling protocol – control colonies
Where possible a minimum of five samples of super honey were collected from random discrete locations in each control hive. Each sample was analysed in duplicate.

Determination of ciprofloxacin in honey by LC-MS/MS
Honey sub-samples were diluted 50 to 500 fold with 0.1% (v/v) formic acid (*aq*) and analysed by LC-MS/MS. Honey samples found to contain <500 µg kg^{-1} ciprofloxacin were concentrated down by solid-phase extraction (SPE) clean-up using Oasis MCX cartridges.

LC-MS/MS analysis was carried out on an Alliance 2695 HPLC/autosampler coupled to a Quattro Ultima triple quadrupole mass spectrometer. HPLC separation was achieved on a Kinetex XB-C18 column (100 x 2.1 mm ID, 2.6 µm, Phenomenex) using 0.1% formic acid (*aq*) and 0.1% formic acid methanol:acetonitrile (50:50 v/v) mobile phases.

Assessment of ciprofloxacin homogeneity in treated superbox

Homogeneity of ciprofloxacin in bulk honey collected at Time Point T1 was assessed to determine the minimal number of samples required for analyses. Honey from individual frames was mixed during centrifugal extraction. Ten sub-samples of at least 25 g were collected with the sample being remixed manually before withdrawal of each sub-sample. Each sub-sample was analysed in duplicate by LC-MS/MS as described above. The result was assessed using ANOVA (Analyses of Variance).

Statistical analyses

The aim of the statistical analyses is to describe how sampling data were used to provide information on sampling protocols for field trials in support of the establishment of MRLs for residues in honey. It is assumed that the most useful quantities for this purpose are:

a) The mean residue concentration in honey from "treated superboxes" in the upper 95th percentile of hives (*i.e*. honey with high residue concentration). This represents a reasonable upper limit for smaller-scale production (*i.e*. Production A, assuming the super honey from each superbox is not mixed with that from other treated hives);

b) The mean concentration of residue from "treated superboxes" in the upper 95th percentile across all treated hives. This represents a reasonable worst case for larger-scale production (*i.e*. Production B, assuming that the super honey is mixed from all treated hives);

c) The estimated rate of change of concentration of residue over time;

d) The size of the uncertainty associated with these estimated concentrations, variation and rate of change;

e) The estimated effect of reducing the number of colonies on the precision of the final results.

A linear mixed model was fitted to log transformed results. The effect of using a reduced number of hives was estimated via parametric bootstrap methodology.

Results and Discussions

Homogeneity assessment to determine honey sampling protocol

Bulking and mixing of super honey from each superbox meant that the effect of large intra-hive variation in residue concentration was much reduced compared to our previous study where samples were taken directly from individual frames within the superbox. (Fussell *et al*., 2012). Homogeneity assessment of bulk honey using ANOVA gave a repeatability standard deviation of 7.9% and between sample standard deviation of 5.0%. The uncertainty associated with the mean results produced by duplicate analyses of two samples is very likely similar to that produced by duplicate analyses of ten samples. Based on this assessment, two sub-samples were analysed in duplicate from each bulk honey sample for the main study.

Ciprofloxacin residues in honey samples

In the majority of cases, the RSD% of ciprofloxacin residues in the bulk honey from each superbox is <10%. However, variation of residues between hives (based on superbox 1 measurements) at each time point remains very high. RSD% (inter-hive) ranged from 40 to 197% for 2012, 39 to 106% for 2013 and 25 to 75% for 2014. Average ciprofloxacin concentrations per time point are presented in Table 1. Figure 1 gives a qualitative summary of data collected for 2012, 2013 and 2014.

All control honey samples analysed for ciprofloxacin from years 2012 to 2014 were "negative" with residues <5 µg kg^{-1}.

Table 1. Summary of average ciprofloxacin concentration per time point. n = number of hives analysed per time point. RSD% refers to variation between hives at each time point

Time point	2012		2013		2014	
	Days	Ave conc. µg kg^{-1} (n, RSD%)	Days	Ave conc. µg kg^{-1} (n, RSD%)	Days	Ave conc. µg kg^{-1} (n, RSD%)
T1	25	25006 (n=4, 73%)	26	1382 (n=4, 29%)	13	33331 (n=4, 69%)
T2	28	11681 (n=4, 42%)	28	1081 (n=4, 106%)	21	16795 (n=4, 57%)
T3	35	8154 (n=3, 40%)	35	1671 (n=4, 79%)	29	13761 (n=4, 50%)
T4	43	14218 (n=4, 47%)	43	1061 (n=5, 42%)	35	18359 (n=4, 41%)
T5	56	2388 (n=5, 94%)	56	1539 (n=5, 70%)	52	8661 (n=5, 70%)
T6	77	1382 (n=5, 174%)	76	1426 (n=5, 53%)	73	10385 (n=4, 22%)
T7	98	1081 (n=5, 85%)	99	1081 (n=5, 56%)	94	10667 (n=5, 38%)
T8	127	807 (n=5, 103%)	132	1867 (n=5, 57%)	115	13705 (n=5, 29%)
T9	160	436 (n=5, 108%)	161	1595 (n=5, 90%)	144	9354 (n=3, 25%)

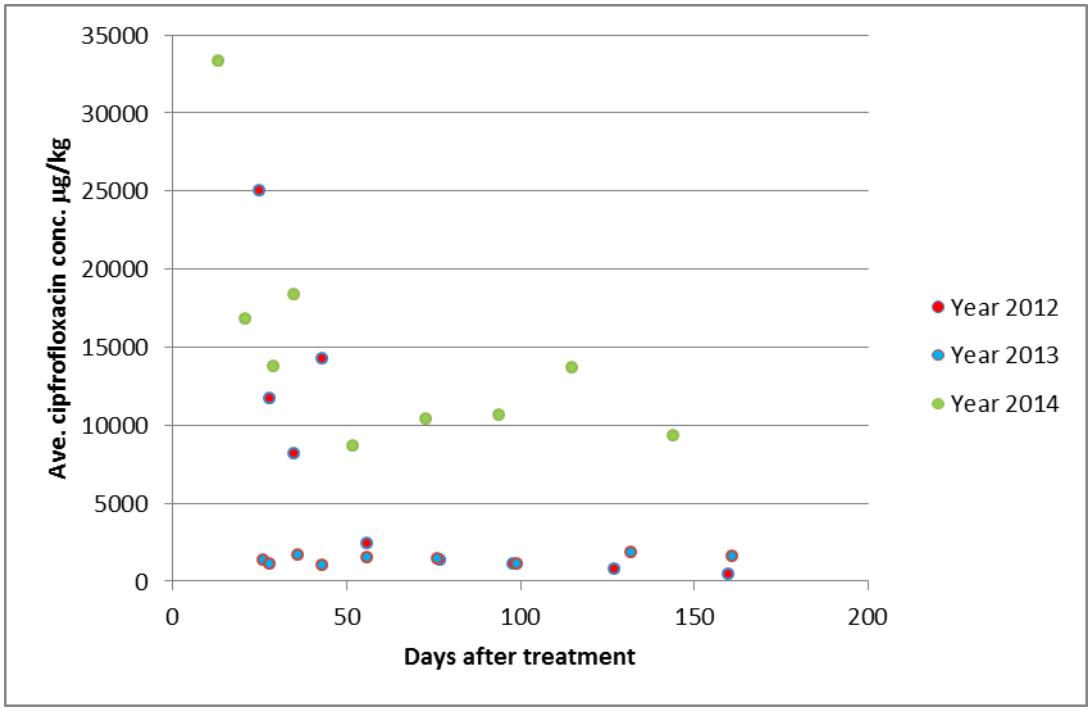

Figure 1. Average ciprofloxacin concentration for years 2012, 2013 and 2014.

Wet vs Dry Summer (2012 vs 2013)

Total rainfall in June 2012 at the treatment site was recorded to be over 120 mm (measured by the WS-GP1 portable meteorological station). This was nearly twice the total average rainfall normally expected in June in England (UK Met Office, 2012). These wet conditions continued for most of the summer and severely affected honey production and the experiment. Sampling was originally scheduled to be collected 3 days after ciprofloxacin treatment, but sample collection did not begin until 25 days after treatment due to the lack of honey production. Furthermore, it was not possible to collect 5 hives per sampling point for the first half of the sampling period, because some hives did not contain any super honey. In order to make a direct comparison of 2012 and 2013 results, the first samples were not collected until 25+ days after treatment in 2013 despite normal honey production. Figure 2 shows a summary of total monthly rainfall at the treated apiary site.

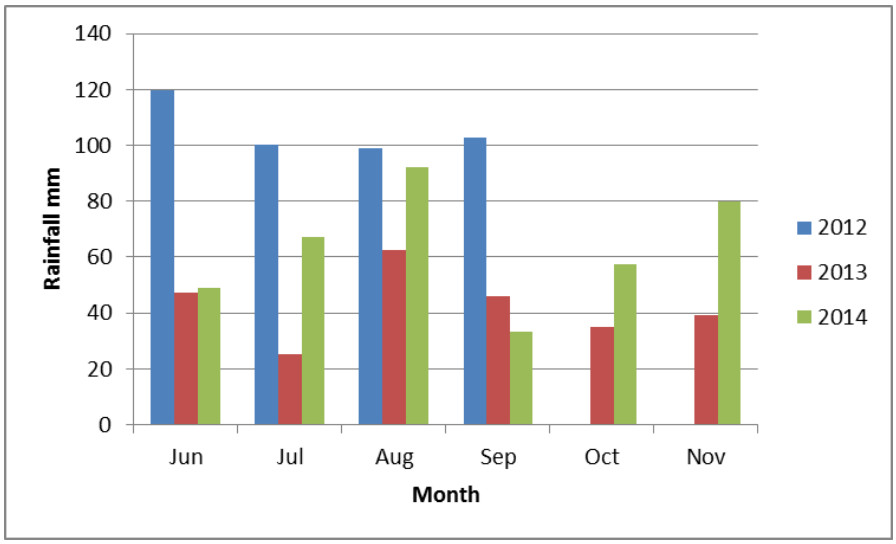

Figure 2. Total monthly rainfall at treat apiary site in 2012, 2013 and 2014. (Data not available for October and November 2012).

There is a large difference in average ciprofloxacin concentration at T1 for 2012 (25,000 µg kg^{-1}) and 2013 (1,400 µg kg^{-1}). The wet summer of 2012 seems to be the cause of this large observed difference in ciprofloxacin concentration at T1 (25/26 days). During the heavy rain period of 2012, the bees would not be foraging as much when compared to the drier 2013 summer, leading to less honey production. At the first sampling point T1 for years 2012 and 2013 (day 25/26) the average honey

production per hive was 1.2 kg for year 2012 ("wet" season) and 2.5kg for year 2013 ("dry" season). Honey dilution of the ciprofloxacin residue might go some way to explaining the higher ciprofloxacin concentration found during the "wet" 2012 since honey production is about 2 times less compared to the "drier" 2013 season. However, concentration of ciprofloxacin is almost 10 times higher in 2012 compared to 2013 at Day 25/26. Aliyah *et al*. (2013) have reported that honey bees *A. Mellifera L.* can consume up to 80% less supplemented sugar syrup solution as a result of heavy rain. The author suggested that during the heavy rain period, the bees were less inclined to consume the supplemented sugar syrup feed. Another suggestion is that during prolonged periods of heavy rainfall, the older worker bees are dying (20-40 day life span) and the younger bees were less adapted to consume the sugar syrup. Whatever the reason behind the reduced consumption of food during the heavy rain period, it is likely that the bees in our study also reduced their food consumption (*i.e*. treated ciprofloxacin syrup) during the heaving rain period in 2012. Since the bees are self-treating themselves by consuming the ciprofloxacin treated sugar syrup, the heavy rain period of 2012 may have in effect delayed the start of the treatment date, even though the treated sugar syrup was present all the time inside the hive. The summer of 2013 was much drier in comparison (Figure 2), and because of the decision to use the same sampling time points as for 2012, by the time the first sample was collected (Day 26) much of the ciprofloxacin would have been consumed by the bees and distributed throughout the hive or outside environment, as well as diluted by honey production.

Data from our previous investigation also showed that mean ciprofloxacin concentrations depleted significantly 20-30 days after treatment in 2009 (Figure 3). The rainfall and mean temperature for the summer of 2009 was similar to that of 2013, with an average total rainfall of 60-50 mm and a mean temperature of 14˚C (June, 2009). When sampling commenced on Day 26 in 2013, it is likely that the concentration of ciprofloxacin reflected levels that would be expected considerably sooner after drug administration under conditions more favourable for bee activity. Despite the differences of average ciprofloxacin concentration at the beginning of 2012 and 2013, the average concentration is similar from Day 56 onwards in both years (Figure 1). The results suggest that the wet weather can delay the depletion of residues in the honey.

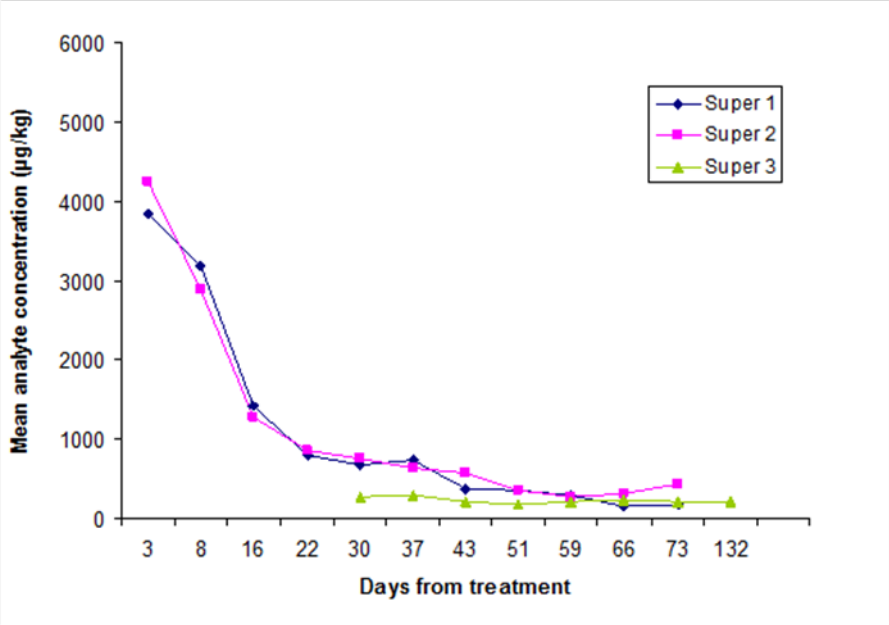

Figure 3. Depletion of ciprofloxacin from previous study (Fussell et al., 2012).

Buckfast vs Carniolan bees

Data from 2014 show average ciprofloxacin concentration starts at over 30,000 µg kg^{-1} (Day 13, T1) and drops to around 10,000 µg kg^{-1} (Day 52, T5) and the mean concentration does not vary too much for the rest of the sampling period. The mean concentration at time points T6 to T9 for 2014 is a lot higher compared to 2012-2013 (1,000-2,000 µg kg^{-1}). The different strain of bees (Buckfast in years 2012, 2013 and Carniolan in 2014) used is likely to be a significant factor behind the differences in the observed results.

Buckfast bees are known to have a much higher turnover rate of honey compared to Carniolan bees. This means food source in the brood will be consumed and replaced with fresh supplies at a much higher rate for Buckfast colonies compared to Carniolan colonies. Honko *et al*. (2002) have reported that Carniolan bees consumed significantly less food compared to Buckfast bees during their study.

The ciprofloxacin sugar solution was made in a 60% w/v sugar syrup. The bees would either consume the sugar solution straight away or reduce the water content to 20% and store it as "capped honey" within 24-48hrs. If the treated sugar solution is stored as "capped honey" it will not be possible to differentiate it visually from normal capped honey (unless the sugar solution is dyed beforehand). The "capped honey" from treated syrup can act as a reservoir of ciprofloxacin, *i.e*. it is possible the bees can redistribute residues at a later point in time, contaminating freshly produced honey. Since the Carniolan bees are much more efficient compared to the Buckfast bees, stores of "capped honey" made from the treated sugar syrup will last a lot longer in a colony of Carniolan bees compared to the Buckfast bees. This effectively means the Carniolan bees are being exposed to ciprofloxacin over a longer period of time compared to the Buckfast bees, resulting in higher concentration of residues in the super honey over a longer period of time.

Statistical analysis of data

Statistical assessment of the data shows that the results produced in 2014 displayed less inter-hive variation than those produced in 2012 and 2013. Additionally, the concentration of ciprofloxacin in honey taken from colonies in 2014 (the 95th percentile of colonies) was estimated to be 17 times higher than in 2012. The differences observed from the statistical assessment between 2014 and 2012/2013 is likely due to different type of honey bees used (2012/2013 Buckfast v 2014 Carniolan) as discussed above.

One of the most important quantities for planning future studies to estimate MRLs is the size of the inter-hive variation in concentration of ciprofloxacin. This was estimated to be equivalent to a relative standard deviation of 138% in 2012/2013 and 61% in 2014. Hence, for colonies behaving in accordance with the model fitted to 2012/2013 results we can expect the concentration of ciprofloxacin in an individual colony to deviate by a factor of approximately 7.5 (7.5 times or 1/7.5) from the expected mean concentration for a treatment; we can expect that for two randomly selected colonies undergoing a treatment the concentration in the highest-concentration colony may be approximately 15 times the concentration in the lowest-concentration colony. For colonies behaving in accordance with the model fitted to 2014 results we can expect the concentration of Ciprofloxacin in an individual colony to deviate by a factor of approximately 3 (3 times or 1/3) from the expected mean concentration for a treatment; we can expect that for two randomly selected colonies undergoing a treatment the concentration in the highest-concentration colony may be approximately 6 times the concentration in the lowest-concentration colony.

Uncertainty associated with estimates of upper limits for concentration and the effect of the number of colonies

Table 2 presents estimates for honey with high ciprofloxacin residues (95th percentile) with upper 95% C.I. at day 55 for Production A and B scenarios. 'Uncertainty' gives the multiplicative factor of the expected value within which 95% of estimates are expected to lie.

For example, in the Production A scenario, using data from all hives (around 40+) at day 55 in 2012, we can expect to produce estimates of upper limits for concentrations that vary by an uncertainty factor of approximately 1.6 around their expected value (*i.e*. the value that an infinitely large study would produce). Table 2 shows this uncertainty factor increases to 2.6 if the number of hives used in this study is reduced from 40+ to 9 (*i.e*. one hive per time point). Figures 4 and 5 show graphically the uncertainty estimates of the 95th percentile ciprofloxacin concentration for all withdrawal periods in Production A scenario.

The expected concentration in boxes not present at the time of treatment

The concentration of ciprofloxacin is 4.9 (95% C.I. 3.1 to 7.8) times lower in honey taken from super boxes that were not present during treatment compared to honey taken from equivalent superboxes that were present during treatment. This factor can be combined with the proportion of honey from superboxes present and not present at the time of treatment to provide an estimate of the expected concentration in non-worst-case scenarios.

Factors affecting ciprofloxacin depletion in honey.

The withdrawal period of any particular drug compound will be dependent on a number of factors. For traditional animal dosing experiments, the major factors will be based on the percentage uptake of the drug to target organs, excretion rate from the animal, and metabolism/degradation to non-active compounds. For honey, we envisage the withdrawal period will be based initially on percentage re-distribution of the drug from the brood chamber to the superbox (and environment outside the hive), ingestion/excretion by the bees (metabolism/degradation), degradation of the drug during storage in the honey and dilution of the drug by honey production. We know from the previous study that ciprofloxacin is a very stable compound and for all intents and purposes does not degrade in honey. The depletion parameters will therefore be based primarily on percentage re-distribution of ciprofloxacin from the brood, to the super honey (and environment outside the hive), ingestion by the bees themselves, and dilution by super honey production. Furthermore, external factors like poor weather conditions can reduce bee activity and significantly delay the depletion of residues in honey as discussed above.

The statistical assessment of the data shows ciprofloxacin depletes on average from 3% to near 0% per day. This means that if a honey MRL was ever to be considered for ciprofloxacin, concentrations would need to deplete, or be diluted to below a certain concentration in relation to its Acceptable Daily Intake. This concentration could be estimated using the upper 95th percentile of ciprofloxacin concentration (honey with high residue concentration) calculated in this study.

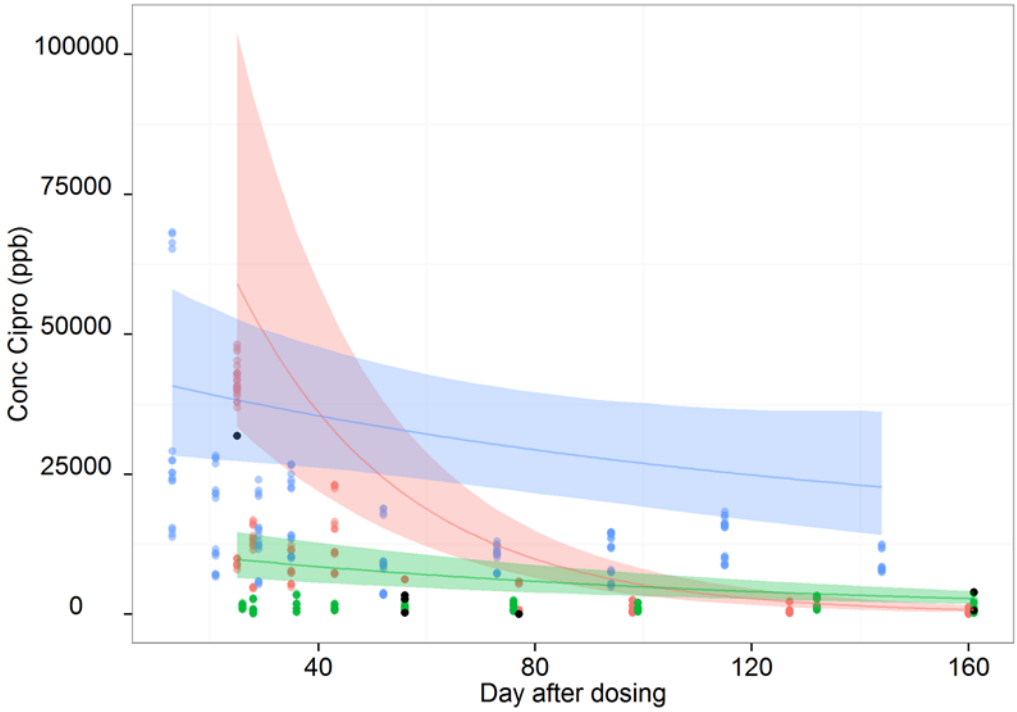

Figure 4. 95% tolerance interval for estimates of the relation between the concentrations of ciprofloxacin in the 95th percentile of hives estimated using parametric bootstrap samples consisting of all hives (+4 hives per time point) for Production A scenario.

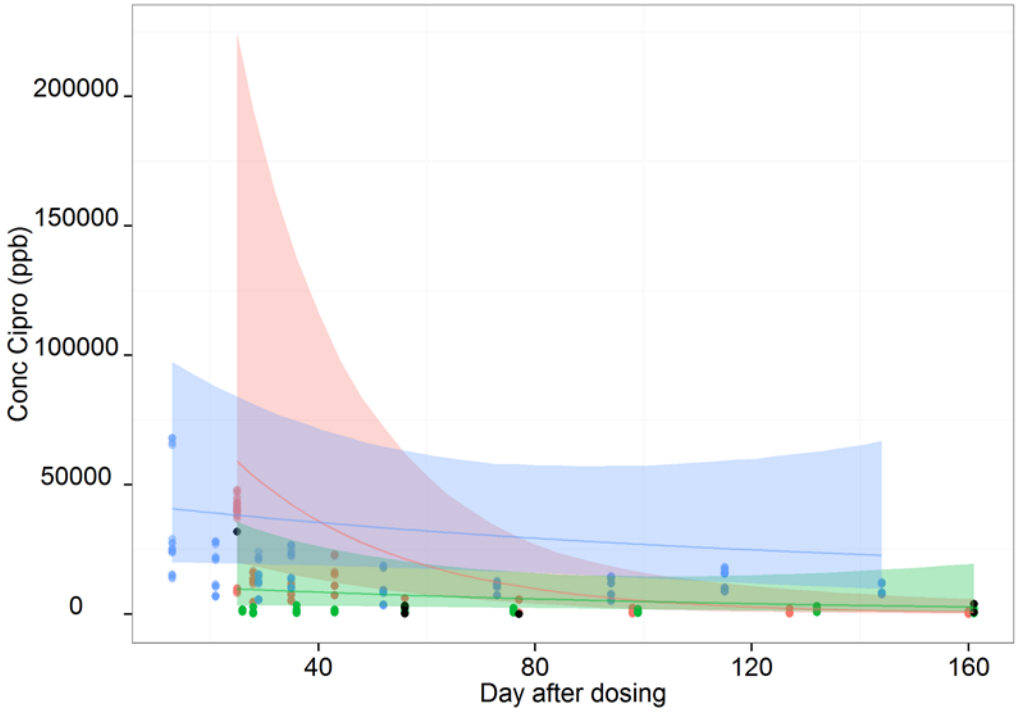

Figure 5. 95% tolerance interval for estimates of the relation between the concentrations of ciprofloxacin in the 95th percentile of hives estimated using parametric bootstrap samples consisting of one hive per time point for Production A scenario.

Table 2. Variation of the value of the upper 95% CI for mean Cipro concentration in honey from 'treated boxes' and for ciprofloxacin concentration in honey from hives at the 95th percentile at day 55 estimated using parametric bootstrap samples consisting of 1,2,3 and 4-5 colonies per time point (rse = relative standard error).

No Hives	Year	Upper 95% C.I. for mean conc. Across colonies (Production B)				Upper 95% C.I. of colonies (Production A)			
		2.5 (ppb)	7.5 (ppb)	Rse (%)	Uncertainty	2.5 (ppb)	7.5 (ppb)	Rse (%)	Uncertainty
1	2012	5195	36380	52%	2.6	9028	61648	52%	2.6
2	2012	5698	20533	34%	1.9	11553	44117	35%	2
3	2012	5968	16264	26%	1.6	12964	37814	28%	1.7
All	2012	6083	14262	22%	1.5	13987	34753	23%	1.6
1	2013	1749	12449	54%	2.7	3040	21163	53%	2.7
2	2013	1942	6891	34%	1.9	3985	14761	35%	1.9
3	2013	2005	5507	26%	1.6	4369	12904	28%	1.7
All	2013	2049	4436	20%	1.5	4876	11257	22%	1.5
1	2014	12905	35417	26%	1.7	18250	62388	32%	1.9
2	2014	13818	26651	17%	1.4	21724	50917	22%	1.5
3	2014	14134	23645	13%	1.3	23466	46087	17%	1.4
All	2014	14284	21949	11%	1.2	24708	43693	15%	1.3

Conclusions

This study has provided information on how residue levels can vary between hives during different seasons and when different types of honey bees are used. The effect of different drug application regimens (*e.g*. treatment concentration strength, dusting or trickling methods, etc.) and the timing of dosing (e.g. how long before honey flow, during honey flow, etc.) will need to be studied further since this is expected to have an impact on the depletion rate of residues in honey.

To reduce cost for future studies for establishing MRLs in honey, we estimated relative standard error of residue concentration if the number of hives were reduced. For day 55 samples, relative standard error ranged from 11-52% depending on number of hives and production scenarios (large or small scale). Further guidance from the EU on an acceptable relative standard error value will be a useful reference point for future studies. These discussions are currently ongoing within the VICH community.

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AN INVESTIGATION INTO SOURCES OF CONTAMINATION OF CATTLE WITH THE VETERINARY DRUG PHENYLBUTAZONE

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Abstract

Phenylbutazone is licensed for use in horses but is unauthorised for use in any animals, including horses, which are destined for human consumption. Given this, there is potential for misuse of phenylbutazone in food producing animals and indeed residue analysis within the European Union has, in recent years, displayed non-compliance in both horses and cattle. This study describes a number of small investigations undertaken to determine if non-compliance in *bovines* could be as a result of contamination from authorised use of the drug. Trials investigated if an untreated *bovine* could become non-compliant by feeding from a contaminated vessel, from close contact with a treated animal or from grazing contaminated pasture. Concentrations of phenylbutazone were measured in plasma by UHPLC-MS/MS. Results indicate that contamination via feeding vessels, pasture or through contact with treated animals is clearly an issue and may be a source of at least some of the non-compliance recorded in EU monitoring plans. It was found that all sources of contamination investigated had the potential to generate non-compliant blood samples in bovines.

Introduction

Phenylbutazone (PBZ) is a non-steroidal anti-inflammatory drug (NSAID) used to treat horses for musculoskeletal disorders such as lameness associated with osteoarthritic conditions, acute and chronic laminitis, bursitis and carpitis, and in the reduction of post-surgical soft tissue reaction (National Office of Animal Health, 2015). In 1949, PBZ was introduced to human medicine to treat rheumatoid arthritis but was subsequently discovered to induce blood disorders including aplastic anaemia, leucopoenia, granulocytosis and thrombocytopenia (Lees *et al*., 2013) and as a result, its license was revoked for use in humans in the UK in 1984 (Tobin *et al*., 1986). In 1997, the Committee for Veterinary Medicinal Products (CVMP) found the main risks of PBZ residues to the consumer were idiosyncratic blood dyscrasias and the genotoxic/ carcinogenic potential for which no thresholds could be identified and, as such, no maximum residue limits could be established (European Medicines Agency, 1997). Consequently, in the EU, PBZ cannot be used in animals destined for the food-chain; a stance also adopted by other countries such as the USA, Canada and Japan.

In 2013, after the "horse meat scandal," the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) issued a joint statement on the presence of residues of phenylbutazone in horse meat, where the risks to humans were reaffirmed (European Food Safety Authority and European Medicines Agency, 2013). Clearly these risks are not species-specific and the fact that 0.1% of *bovines* tested in the EU in 2013 were non-compliant (European Food Safety Authority and European Medicines Agency, 2014) is of concern. There is potential for unauthorised use of PBZ in cattle. However, there is also anecdotal evidence of animals, which have not been treated with the drug, giving non-compliant results through cross-contamination.

This work investigated, through a number of small studies, the impact of some potential sources of contamination on untreated animals. These included contamination through inappropriate feeding practices, animal to animal contact and contamination via pasture.

Materials and Methods

Collection of blood samples

Holstein Friesian cattle ranging in weight from 400 to 500 kg were used in the studies. Blood samples (approximately 20 mL) were collected in Vacutainer blood tubes containing heparin (BD Biosciences, Oxford, UK). The plasma was separated by centrifugation and analysed for PBZ residues using a supported liquid extraction technique before quantification and confirmation by a UHPLC-MS/MS method validated according to Commission Decision 2002/657/EC. The EU permits the analysis of unauthorised substances to be assessed by reference to the decision limit (CCα) (Commission Decision 2002/657/EC, 2002). The current method has a CCα of 0.28 ng mL^{-1} so a confirmed concentration greater than this is considered non-compliant with a concentration lower than this considered compliant.

Experiment 1: Contaminated feeding vessel

Two bullocks (T1 and B1), known to be free from PBZ residues, were housed in separate pens with *ad-lib* access to haylage and water. T1 was treated with Equipalazone Oral Powder (Veterinary Supplies Company, Lisburn, UK) containing 1 g of PBZ

per sachet as follows (manufacturer's recommended dosage for a horse of the same weight): Two sachets twice on day 1, one sachet twice on days 2, 3 and 4, one sachet on day five. For each treatment 500 g of course cattle ration was placed in a feeding bucket and the required sachets sprinkled over and given to T1. After the entire contents were consumed 500 g of course cattle ration was added to the same bucket without cleaning and given to bullock B1. Blood samples were taken from B1 before the trial started (pre-bleed), then on days 6, 9, 17, 20, 25, 28, 31, 39, 46, 49, 53, 62, 68 and 77 after last feeding from the contaminated vessel.

Experiment 2: Close contact with a treated animal
After 5 days of treatment with Equipalazone, as per manufacturers guidelines, bullock T1 was moved into a clean pen with fresh bedding along with three other bullocks (H1, H2 and H3), known to be free from PBZ residues for 28 days. All animals had *ad lib* access to haylage and fresh water for the duration of the trial. Blood samples were taken from H1, H2 and H3 on days 0 (pre-bleed), 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 20 and 28.

Experiment 3: Pasture occupied by a treated animal immediately before
One bullock (T2) housed individually received 5 days of treatment with Equipalazone, as per manufactures guidelines. After treatment, T2 was moved into a grass paddock (approximately 30 x 12 m) and allowed to graze for four days then removed. Three bullocks (P1, P2 and P3), known to be free from PBZ residues, were introduced to the paddock and allowed to graze for three days then removed. Blood samples were taken from P1, P2 and P3 on days 0 (pre-bleed), 1, 2, 3, 4, 5, 7, 10, 16 and 24.

Experiment 4: Pasture occupied by a treated animal three weeks before
After bullocks P1-P3 were removed from the paddock, it was left unoccupied for three weeks before three bullocks (E1, E2 and E3), known to be free from PBZ residues, were introduced and allowed to graze for one week then removed. Blood samples were taken from E1, E2 and E3 on days 0 (pre-bleed), 5 and 7.

Results

Experiment 1: Contaminated feeding vessel
This trial was designed to investigate if contamination can occur through feeding an animal not intended to receive PBZ with a vessel, previously used to treat an animal with PBZ, which had not been thoroughly cleaned. Six days after feeding from the contaminated vessel had finished, bullock B1 showed a concentration of 990 ng mL^{-1} in the plasma, representing substantial contamination considering the CCα of 0.28 ng mL^{-1} of the analytical method. It was not until day 49 that the concentration of PBZ in the plasma fell below the detection capability of the method. Table 1 shows the concentration of PBZ in the plasma of B1 at various times during the trial.

Table 1. PBZ concentrations of animal B1 plasma over a range of time intervals.

Days after last feeding from Contaminated vessel	Conc. (ng mL^{-1}) in plasma
(pre-bleed)	<0.28
6	990
9	758
17	42.94
20	22.44
25	8.20
28	16.65
31	12.70
39	3.44
46	0.62
49	<0.28
53	<0.28
62	<0.28
68	<0.28
77	<0.28

Experiment 2: Close contact with a treated animal

The aim of this trial was to investigate whether an animal treated with PBZ could potentially contaminate untreated animals. After 24 hours all three animals, H1-H3, displayed concentrations of PBZ in plasma above the CCα of the method. PBZ concentration in H2 was the highest of the three animals peaking after 2 days at 2.85 ng mL^{-1} and stayed above the CCα until day 20. Levels in H1 and H3 rose steadily and peaked after 6 days (1.55 ng mL^{-1}) and 5 days (2.12 ng mL^{-1}) respectively. Twenty days after T1 was introduced, concentrations of PBZ in H1-H3 all fell below the CCα of the analytical method. Table 2 shows the concentration of PBZ in the plasma of animals H1-H3 at different time intervals during the trial.

Table 2. Concentrations of PBZ in plasma samples collected from animals H1-H3 having been housed for four weeks with an animal (T1) treated with PBZ.

Days after T1 was introduced to the pen Containing H1-H3	Conc. (ng mL^{-1}) in plasma		
	H1	H2	H3
0 (pre-bleed)	<0.28	<0.28	<0.28
1	0.39	2.65	0.41
2	0.97	2.85	0.82
3	1.39	2.67	1.47
4	1.39	2.22	1.65
5	1.44	2.43	2.12
6	1.55	2.11	1.93
7	1.41	1.83	1.51
9	0.71	0.85	0.96
11	0.41	0.57	0.60
14	<0.28	0.29	0.34
20	<0.28	<0.28	<0.28
28	<0.28	<0.28	<0.28

Experiment 3: Pasture occupied by a treated animal immediately before

After T2 was removed from the grass paddock three animals, P1-P3, were immediately allowed to graze on it for three days to ascertain if an animal could be contaminated without direct contact of the PBZ treated animal. P1 showed the highest concentrations of PBZ in plasma peaking at 11.95 ng mL^{-1} on day two. P2 and P3 had lower concentrations of PBZ but were still above the CCα of the analytical method from day 1-5 and day 2-5 respectively. Table 3 shows the concentration of PBZ in the plasma of animals P1-P3 at different time intervals during the trial.

Table 3. Concentrations of PBZ in plasma samples collected from animals P1-P3 after grazing on a paddock immediately after being occupied by an animal treated with PBZ.

Days after P1-P3 were introduced to the pen previously occupied by T2	Conc. (ng mL^{-1}) in plasma		
	P1	P2	P3
0 (pre-bleed)	<0.28	<0.28	<0.28
1	8.98	0.29	<0.28
2	11.95	0.68	0.48
3	8.78	0.85	0.70
4	5.50	0.69	0.58
5	4.19	0.47	0.31
7	1.85	<0.28	<0.28
10	0.55	<0.28	<0.28
16	<0.28	<0.28	<0.28
24	<0.28	<0.28	<0.28

Experiment 4: Pasture occupied by a treated animal three weeks before

As a follow up to the previous trial, the paddock was left unoccupied for three weeks after P1-P3 were removed, to determine if an animal could become positive for PBZ by grazing on an area which had been unoccupied by a treated animal for a period of time. This trial was carried out in June so significant re-growth of grass in the paddock was observed. After five days

animals E1-E3 all displayed concentrations of PBZ above the CCα of the analytical method and all three showed a further increase in levels of PBZ in plasma after seven days. Table 4 shows concentrations of PBZ in plasma of animals E1-E3 on day five and seven.

Table 4. Concentrations of PBZ in plasma samples collected from animals E1-E3 after grazing on a paddock occupied by an animal treated with PBZ 24 days previously

Days after E1-E3 introduction to the paddock	Conc. (ng mL^{-1}) in plasma		
	E1	E2	E3
0 (pre-bleed)	<0.28	<0.28	<0.28
5	0.73	1.36	0.67
7	1.10	1.69	1.05

Discussion and conclusions

When results from the four experiments were taken into consideration it was not surprising that feeding an animal from a contaminated vessel led to a non-compliant result. This route of contamination could easily occur accidently and producers should be vigilant when using equipment between treated and non-treated animals. A treated animal can act as a source of PBZ contamination when in contact with untreated animals which could be due to body-to-body contact or possibly from behavioural tendencies such as grooming and intake of the treated animal's urine. Of the three animals placed in the paddock immediately after it was occupied by the treated animal, one had particularly high concentrations of PBZ in the plasma. This is probably down to the random grazing pattern of the animals and the random areas where waste from the treated animal was located in the paddock. There is evidence to suggest that waste from the treated animal leached into the soil and was still available after three weeks which again delivered non-compliant results from clean animals. It is not clear if this occurred through ingestion of grass, soil or a combination of both. If PBZ is to be used extreme care should be taken by producers as the risks of contamination are very real.

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QUANTIFICATION OF FIVE DIFFERENT CLASSES OF VETERINARY ANTIBIOTICS IN (TREATED) SWINE MANURE USING A VALIDATED UHPLC-MS/MS METHOD

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Abstract
This study was conducted to determine the presence of selected veterinary antibiotics in swine manure before and after biological treatment. In a previous study, a UHPLC-MS/MS method was developed and validated for the simultaneous detection and quantification of colistin (polymyxin E), sulfadiazine, trimethoprim, doxycycline, oxytetracycline and ceftiofur and for the detection of tylosin A. This method was used for the quantification of these selected antibiotic residues in swine manure from different manure pits. For some antibiotic residues, high concentrations were recovered from swine manure. The experiments conducted in this study will provide more insight into the presence of these antibiotic residues in biologically treated swine manure.

Introduction
In Belgium, pigs are the main production animal with a population of 6.3 million in Flanders, where the majority of the animals are reared. Every year, a large amount of manure is generated by pigs in Flanders. In 2013, this accounted for approximately 69 kilotons N and 32 kilotons of P_2O_5 (1). Due to fertilising restrictions, a percentage of this manure has to be treated. In 2013, the treatment of pig manure accounted for nearly 15 kilotons N which is 43% of the total amount of N treated (2). For pig manure, the most commonly used technique is biological treatment of the liquid fraction. The first step of this technique is to separate the raw pig manure into a solid and a liquid fraction by means of centrifugation. The solid fraction, which contains the main fraction of phosphorus, is exported or composted. The liquid fraction, which contains the main fraction of nitrogen, is further processed during biological treatment. This is a process of nitrification and denitrification using bacteria. During nitrification, nitrifying bacteria convert ammonia (NH_3) into nitrate (NO_3^-) in the presence of oxygen. During denitrification, heterotrophic bacteria convert nitrate into nitrogen gas (N_2) under anaerobic conditions. The products of this biological treatment can be used as fertilizers.

In addition, the use of antibiotics can be high in intensive pig production and it has been estimated that about 75% of the overall administered antibiotics are excreted in the urine or the faeces (3). These residues may enter the environment directly by spreading of manure and can either accumulate there, leach into surface- and groundwater or be taken up by plants intended for human or animal consumption (4, 5). The last years, concern about the occurrence and dissemination of antibiotic residues in the environment has emerged as antibiotic residues can increase the antibiotic resistance in microbial communities. This includes resistance in pathogens which can be induced directly or indirectly through horizontal transfer of resistance elements between microorganisms. However, little is known about the concentrations and fate of antibiotics in manure and soil and no regulations exist for concentrations.

In 2010, the most frequently used antibiotic classes in Belgian swine farming at group level were penicillins and polymyxins, followed by macrolides/lincosamides, sulphonamides in combination with trimethoprim, tetracyclines and cephalosporins (6). With regard to these results, Van den Meersche *et al*. (2016) (7) chose the most regularly used antibiotics in each of these classes for group treatments for the development of a multi-method for the quantification of five different classes of antibiotics, including polymyxins, in swine manure.

This method will be used to determine the presence of the selected antibiotic residues in swine manure before and after biological treatment.

Materials and Methods

Reagents and materials
Acetonitrile (MeCN, LC-MS grade), methanol absolute (MeOH, LC-MS grade), acetone and formic acid 99% (FA, ULC-MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Ammonium formate and trichloroacetic acid (TCA, analytical grade) were purchased from Sigma-Aldrich (Diegem, Belgium). Water (H_2O) was high-performance liquid chromatography (HPLC) grade (generated by a Milli-Q Gradient purification system, Millipore, Brussels, Belgium).

Different internal standards (IS) were used for each antibiotic class. The reference standards of amoxicillin trihydrate (1), oxacillin sodium salt monohydrate (IS) (2), ceftiofur (3), sulfadiazine (4), tylosin tartrate (5), roxithromycin (IS) (6), trimethoprim (7), doxycycline hyclate (8), oxytetracycline hydrochloride (9), 4-epidemeclocycline hydrochloride (IS) (10), colistin sulfate (11) and the polymyxin B solution (IS) were purchased from Sigma-Aldrich (Diegem, Belgium). Sulfadimethoxine-13C6 (IS) (12) and trimethoprim-d9 (IS) (13) were purchased from WITEGA Laboratorien Berlin-Adlershof GmbH (Berlin, Germany) and ceftiofur-d3 hydrochloride (IS) (14) was purchased from Toronto Research Chemicals (Toronto, Canada).

Individual stock solutions at a concentration of 1 mg mL^{-1} were prepared for each of the analytes, after correction for purity, and stored at -20°C. For compounds 1-4 and 12, stock solutions were prepared in H_2O/MeCN (50/50), for 5-10 and 13-14 in MeOH and for 11 in H_2O/MeCN (80/20). Working solutions were prepared freshly prior to each experiment by diluting the stock solutions in H_2O/MeCN (80/20).

Polyvinylidene fluoride (PVDF) filters were purchased from Merck-Millipore (Carrigtwohill, Ireland), polypropylene (PP) tubes and PP inserts were procured by Novolab (Geraardsbergen, Belgium) and Grace Alltech associates inc. (Lokeren, Belgium), respectively.

Liquid chromatography and mass spectrometry

The mass-spectrometric parameters and liquid chromatographic conditions were optimized on a Xevo TQ-S mass-spectrometer and an Acquity UPLC H-class system, respectively. A reversed-phase Kinetex C_{18} column (100 mm x 2.1 mm i.d., 1.7 µm) with a SecurityGuard Ultra quard cartridge system (Phenomenex) was used to achieve separation of the antibiotic residues. A gradient elution was performed with changing amounts of H_2O/MeCN (95/5) + 0.5% FA + 0.1% ammonium formate (solvent A) and MeCN + 0.1% FA (solvent B). Determination of the analytes was carried out with mass-spectrometry by a multiple reaction monitoring function (MRM). For the selected antibiotics two transitions were determined, for the internal standards only one transition was determined. This was described in detail by Van den Meersche *et al.* (2016) (7).

Sample collection

Blank swine manure samples were collected from three different manure pits in West Flanders (Belgium) on a swine farm where none of the antibiotics considered in this study were used for the last five years. For the analysis of swine manure samples, seven swine farms in East and West Flanders (Belgium) were selected based on their use of one or more of the antibiotics selected in this study. On three farms, two different manure pits were sampled. To investigate the use of antibiotics, the antibiotic registers were consulted for the three months preceding the sample collection.

For the analysis of treated manure samples, samples were taken at different stages of the biological treatment on a farm in East Flanders (Belgium). Samples were taken from the manure pit where the manure is gathered before treatment, the liquid fraction, the solid fraction and the effluent. The sampling was repeated in time. To investigate the use of antibiotics, the antibiotic register was consulted for the three months preceding the sample collection.

Two litres of swine manure were collected from the different sampling points. Subsequently, each sample was subsampled into sampling jars of approximately 60 mL each and stored at -80°C.

Sample preparation and extraction

Sample preparation and extraction are described in detail by Van den Meersche *et al.* (2016) (7). Briefly, the extraction was carried out by means of shaking and centrifugation using MeCN and trichloroacetic acid, followed by ultrasonication and filtration. Polypropylene materials were used to avoid the adsorption of the polymyxins to glass (8).

Results and Discussion

Detection and quantification of antibiotic residues in swine manure

Results for the detection and quantification of antibiotic residues in swine manure are presented in Table 1. These results indicate that there is a link between the antibiotics used and recovered with the exception of ceftiofur which is easily degraded in swine manure. Additionally, some antibiotic residues can be present at high concentrations in the manure and may be spread in the environment.

Detection and quantification of antibiotic residues before and after biological treatment

The results from this study will be presented during the conference.

Table 1: Overview of antibiotic residue concentrations (µg kg^{-1}) recovered from swine manure samples at different farms or from different manure pits on one farm and antibiotic use in the respective pens during the three months before sampling. (Source: Van den Meersche et al., 2016 (7))

Compound		Farm 1a	Farm 1b	Farm 2a	Farm 2b	Farm 3a	Farm 3b	Farm 4	Farm 5	Farm 6	Farm 7
Ceftiofur	detected	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	used					x	x				
Colistin A	detected	1 320	48 600	ND	ND	ND	ND	ND	1 982	1 140	ND
	used	x	x						x	X	
Colistin B	detected	627	40 800	ND	ND	ND	ND	ND	1 435	957	ND
	used	x	x						x	X	
Doxycycline	detected	1 336	ND	11 960	22 760	1 221	ND	ND	1 400	19 525	2 715
	used			x	x				x	x	x
Oxytetracycline	detected	ND	ND	ND	ND	ND	ND	ND	18	ND	2 029
	used										x
Sulfadiazine	detected	ND	ND	763	2 980	ND	23	21	ND	217	77
	used			x	x					x	
Trimethoprim	detected	ND	ND	<LOQ	6	ND	<LOQ	ND	ND	4	ND
	used			x	x					x	
Tylosin A	detected	ND	ND	ND	NQ	ND	ND	ND	ND	NQ	ND
	used							x			

NQ: compound detected but not quantifiable due to high measurement uncertainty; ND: compound not detected; <LOQ: below limit of quantification

Conclusions

The validated method can be applied to detect the selected antibiotics in swine manure and a link was found between the antibiotics used and detected. The presence of high concentrations of antibiotic residues indicates the possibility for selection of antibiotic resistant bacteria. Conclusions concerning the manure treatment will be presented during the conference.

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ION MOBILITY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY: THE POSSIBILITIES, THE LIMITATIONS

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Abstract
Ion mobility (IMS) provides complementary, orthogonal data to high resolution mass spectrometry (HRMS). Until very recently, the use of such coupled technology was the domain of research oriented laboratories. This has changed with the availability of recently introduced instrumentation which is intended to cater to the need of routine residue laboratories.

Introduction
High resolution mass spectrometry (HRMS) is increasingly competing with conventional tandem quadrupole technology (QQQ MS/MS) in the field of targeted residue analysis. In the past, most HRMS-based methods were limited to qualitative screening applications. Currently more and more quantitative methods have been validated and published. Instrument vendors are now positioning their HRMS technology as quantitative instruments which are equally suited for quantitative, targeted analysis as QQQ MS/MS instrumentation. In addition, there is little doubt that HRMS is the superior technology when used for non-targeted work. Limitations of HRMS were related to the sensitivity, selectivity and software. Progress has been made in both fields. The use of new interfaces and detectors significantly improved the sensitivity. The availability of higher mass resolving power and increased ruggedness addressed the selectivity issue. It has been reported that a mass resolving power of 50,000 full-width at half maximum (FWHM) permits the definition of narrow mass windows which result in a selectivity that is equivalent of that of unit mass resolving QQQ MS/MS technology (Kaufmann 2010). Hence, monitoring a precursor ion by HRMS is equivalent to recording a MS/MS transition. Yet, the confirmation of a positive finding requires a second transition. Because of that, full scan HRMS based findings still need an additional confirmatory dimension. Hence, there is still a need for additional confirmatory data like non-precursor or precursor selected product ions (Berendsen 2015). Alternatively, required confirmatory information may be obtained by including two dimensional chromatography or ion mobility spectrometry (IMS).

IMS is currently available as Field asymmetric waveform IMS or drift time IMS. Field asymmetric waveform is basically a filter technique which only permits ions of a particular ion mobility to reach the interface (cone or orifice) of the mass spectrometer. This methodology cleans up the acquired mass spectra. However, it is a targeted technique, basically just permitting the monitoring of a single compound. Ions beyond the selected mobility range are lost. Drift time IMS operates in a different way. Ions originating from the continuous ion beam are trapped and later released in the form of a single ion cloud. These ions are separated by their ion mobility. Unlike when relying on Field asymmetric waveform IMS, all drift time separated ions are forwarded to the MS. Such a drift time separation resembles a chromatogram. However, the separation time is much faster (few milliseconds). Hence it is possible to perform a complete ion mobility separation (mobilogram) for each chromatographic data point (*e.g*. every 0.25 seconds). Again, a high number of MS spectra have to be taken in order to sample a mobilogram. In the case of the utilized IMS instrument, 200 MS consecutive MS spectra are taken to monitor the mobilogram. This is sufficient to follow the peak width of the ion mobility separated ions. Time of flight mass spectrometry (TOF) is sufficiently fast to meet the required spectra acquisition speed. These technical capabilities permit the acquisition of truly comprehensive three dimensional data (chromatographic retention time; ion mobility and *m*/*z*).

Materials and Methods

LC-IMS-TOF Analysis
Samples were analysed with an Acquity I-Class UHPLC and the Vion IMS-Q-TOF (ion mobility followed by a quadrupole, collision chamber and time-of-flight mass spectrometry) instrument (both from Waters, Manchester, United Kingdom). Chromatography was based on a core shell column (C-18; 2.6 µm; 150 x 2.1 mm) from Phenomenex; Torrance; USA) and a reversed-phase gradient. Data was acquired and processed with the UNIFI software (Waters).

Results and Discussion
Drift time IMS coupled with TOF produces multi-dimensional data. A whole three-dimensional data space exists for every chromatographic data point (*e.g*. every 0.25 seconds). Figure 1 shows a chromatogram of a liver extract. The x-axis gives the retention time, the y-axis the drift time and the z-axis the intensity. IMS is capable in resolving co-eluting analytes. Yet the chromatographic resolving power is clearly higher than the IMS resolving power.

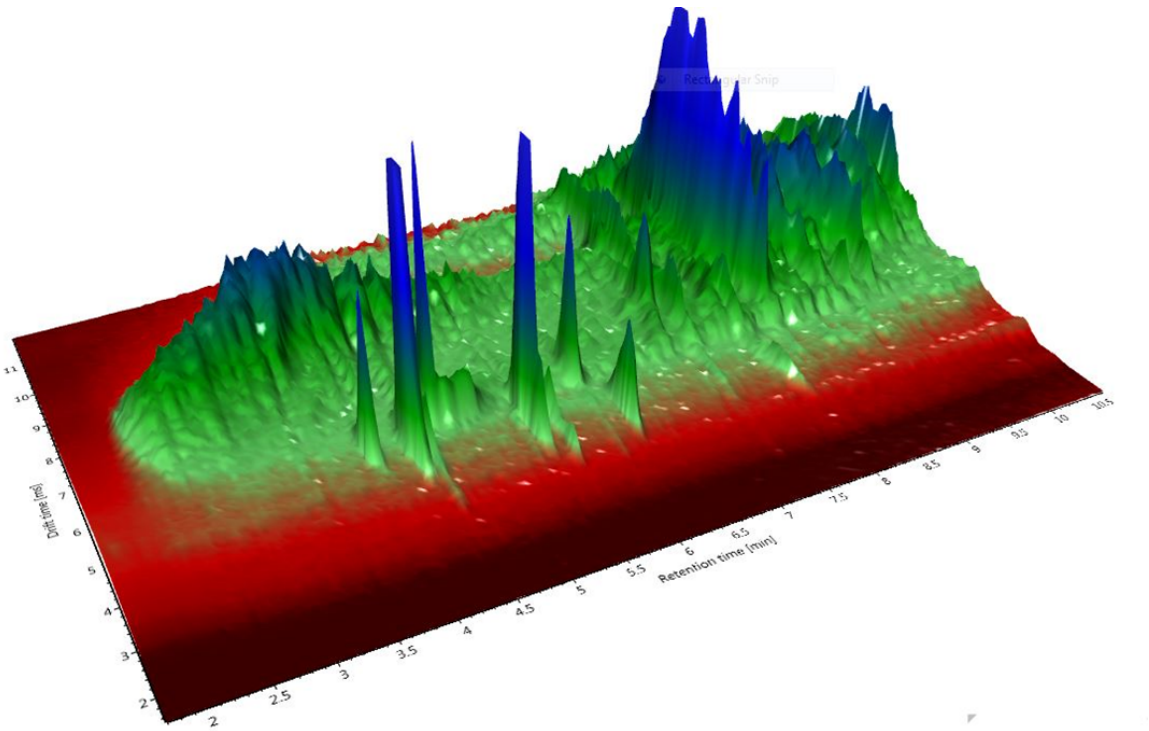

Figure 1: Liver extract (x-axis = retention time; y-axis= drift time)

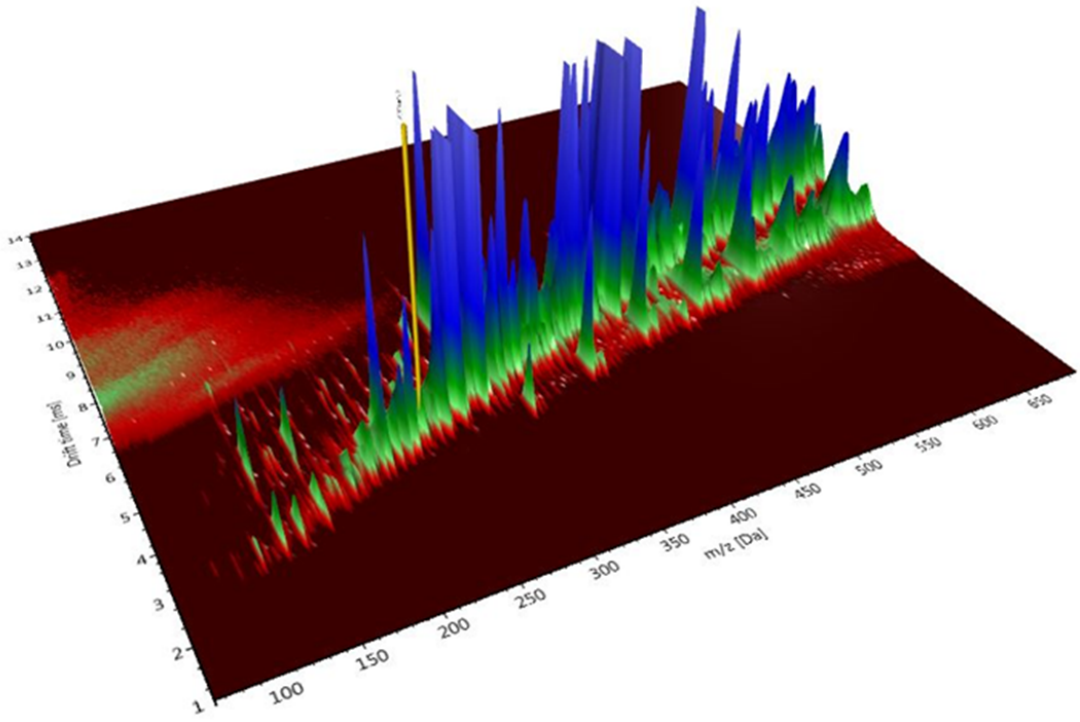

Figure 2: Liver extract at a selected retention time (x-axis = m/z; y-axis = drift time)

Figure 2 focuses on a single chromatographic data point and shows the observed 3-D space consisting of *m*/*z*; drift time and signal intensity. The data shows that IMS can resolve isobaric ions. However, IMS is not truly orthogonal to mass spectrometry, as there is a clear correlation between drift time and *m*/*z*. This is due to the fact that IMS separation is based on size and shape. Size is strongly correlated to *m*/*z*. This is similar to alternative techniques like 2-D LC where a similar degree of "orthogonality" is observed.

An interesting feature of the used IMS-Q-TOF is the capability to clean up the spectra of compounds present at trace concentration in complex matrices. This is first done by a process called componentization. Componentization extracts every signal out of the 4-D space. The software only extracts signals which meet the multi-dimensional criteria of a chromatographic peak, as well as the ion mobility and *m/z* shape of a true compound. Unlike conventional chromatographic software, the UNIFI software does not determine a two dimensional peak area but a four dimensional space which is used for quantitative purposes. Continuous signals (e.g. mobile phase or column bleed) as well as noise are not extracted. This can lead to a large number of peaks (*e.g.* > 1,000,000). Based on their chromatographic peak shapes, these signals are grouped into "candidate compounds". A "candidate compound" consist not only of a monoisotopic ion but also the isotopes, possible adducts (*e.g.* sodium) and fragments. Normally the instrument operates in the MS^E mode. In this mode each chromatographic data point consists of two MS acquisition (low and a high collision energy). Fragmentation of all incoming ions occurs after having undergone IMS separation. This leads to product ion spectra where all product ions of a given precursor show the same ion mobility. Hence, product ions of a particular compound are not scattered within the *m/z* versus IMS plot but form a horizontal line. The capability of the software to extract truly chromatographic peaks (componentization) and the linking of the low and high energy fragmentation regime with an ion mobility filtering step results in clean product ion spectra. This is visible in left two panels in Figure 3 (derivatised nitrofuran metabolite AOZ; 10 µg kg^{-1} in standard) and right two panels in Figure 3 (derivatised nitrofuran metabolite AOZ; 10 µg kg^{-1} in liver). Note that the masses 104; 134 and 149 are indeed product ions of the investigated precursor *m/z* = 236. Without the combined effects of componentization and IMS filtering, the product ion spectra would be dominated by matrix peaks. It is important to realize that a truly comprehensive multi-dimensional space is acquired across the whole chromatographic separation. Hence the technique permits new strategies for non-target approaches.

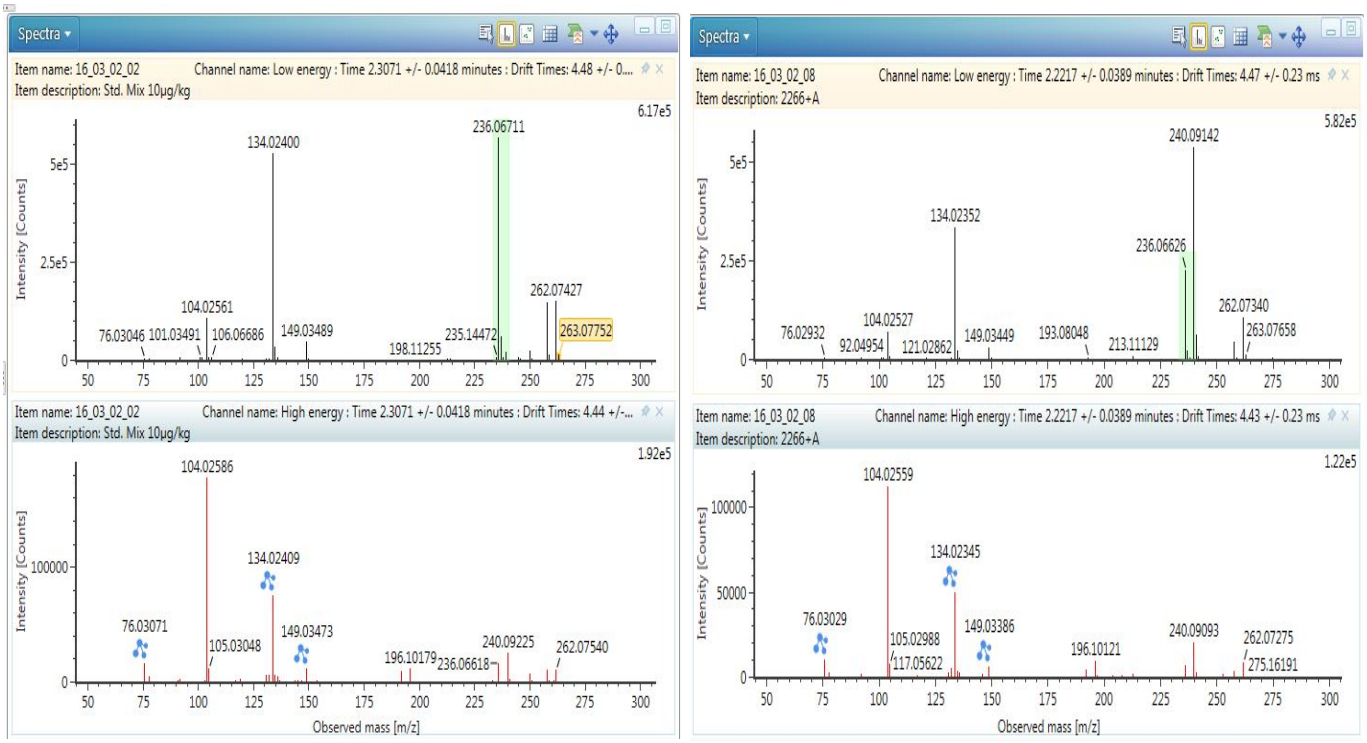

Figure 3. MS^E of derivatised AOZ 10 µg L^{-1} in standard (left two panels) and liver extract (right two panels). Top panel: low energy; bottom panel: high energy.

Conclusions

IMS-HRMS has been a high end research tool. There are currently two vendors (Agilent and Waters) who provide comprehensive IMS-HRMS separation instrumentation targeted for the routine residue analysis market (*e.g.* pesticides and veterinary drugs). IMS does not only provide an additional separation potential. Based on the measured IMS drift time, the compound specific cross collision section (CCS) becomes available as an additional degree of confirmation. CCS values are not only highly reproducible but even platform and vendor-independent. Hence, CCS have the potential of replacing retention times as a criterion for the correct assignment of compounds.

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DEVELOPMENT OF IMMUNOMAGNETIC PRECIPITATION METHODS FOR THE DETECTION OF RECOMBINANT *BOVINE* SOMATOTROPIN BY UHPLC-MS/MS

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Abstract
Recombinant *bovine* somatotropin (rbST), a synthetic growth hormone, is used to stimulate growth and to enhance milk production in dairy cows. Its use and commercialization of dairy products from treated animals are prohibited in the European Union, Japan, Australia, New-Zealand and Canada but is approved in several countries (*e.g*. USA, Brazil).
Screening methods aim at the analysis of anti-rbST antibodies (biomarkers) of treated cows. Confirmatory methods are required to pinpoint rbST abuse. Major challenges for analysis are its potential low level, the high homology with native bST and interferences in the tested matrix. To overcome these hindrances, we developed an immunomagnetic-precipitation method hyphenated to UHPLC-MS/MS for the detection of rbST. Briefly, protein-G magnetic beads pre-coated with in-house produced monoclonal antibodies were added to plasma. After incubation, magnetic beads were isolated by centrifugation, thoroughly washed (PBS-tween, PBS). Finally, rbST was released from beads by alkalinisation.
After isolation and concentration by immune-precipitation, samples were digested with trypsin prior to UHPLC-MS/MS analysis in MRM mode. Validation was done in accordance with the European Commission Decision 2002/657/CE. Matrix-matched calibration with internal standards were used. A CCα of 0.11 µg L^{-1} was reached with this approach.

Introduction
Bovine somatotropin (bST) is produced and secreted by the anterior pituitary gland. Through the somatotropic axis, this hormone regulates several physiological processes involved in metabolism, growth and reproduction. It is well-known that the exogenous administration of bST redirects nutritional partitioning towards milk synthesis in dairy cows, which is therefore translated into an increase in milk production ranging from 10 to 40%.

Genetically-engineered or recombinant isoforms of the *bovine* somatotropin have been developed and produced since the early 1990's. Recombinant methionyl bST (rbST), initially commercialized by Monsanto and then by Elanco (Animal Division of Eli Lilly and Company) under the trade name of Posilac®, is the only product approved by the Food and Drug Administration (FDA) in USA and by the corresponding competent authorities in Brazil, South Africa, Mexico and Korea. However, its marketing and utilization as well as the trade of dairy products obtained from rbST-treated animals, are prohibited within the EU, and other countries such as Japan, Australia, New Zealand or Canada. Several adverse effects reported for treated animals include diminished fertility and an increased occurrence of lameness and clinical mastitis, which requires additional antibiotic treatments. Extensive use of antibiotics in modern agricultural farming has been linked to the development and emergence of antibiotic resistance that is currently affecting both human and veterinary medicine worldwide.

In order to control illegal administration of rbST and to ensure high-quality and safe milk and consumer protection, reliable analytical methodologies capable of unambiguous identification of the presence of the synthetic methionyl growth hormone are required. Current analytical methods for determination of rbST rely on instrumental technologies such as LC-MS/MS. the direct analysis of rbST itself is highly preferable, in order to circumvent problems associated with inter and intra-individual variation of biomarkers expression levels, which can lead to misinterpretation of results.

Materials and Methods

Standards
Recombinant methionyl *bovine* somatotropin (rbST) was extracted from the formula of Lactotropin® syringes (Elanco, Greenfield, IN, USA). Pituitary *porcine* somatotropin (pST) from Sigma Aldrich was used as internal standard.

The N-term rbST ^{13}C-labelled (M-F-P-A13C-M-S-L13C-S-G-L13C-F-A13C -N-A13C-V-L13C–R) was provided by Eurogentec (Liège, Belgium). The monoclonal anti-rbST antibodies (mAB) were in-house produced (Marloie, Belgium). Magnetics beads Bio-Adembeads Protein G were supplied by Ademtech (Pessac, France). Modified *porcine* trypsin, sequencing grade, was commercialized by Promega (Madison, WI, USA).

Sample Preparation
The protocol used here was based on the methodology described by Dervilly-Pinel *et al*. (2012) with slight modifications. Plasma sample (5 mL) was first diluted two-fold with PBS + 0.2% Tween 20 (pH 7.4) and incubated under slight agitation at room temperature for 2 h with 50 µL magnetic beads coated with 5 µg mAB (MBs-mAB), the internal standard (pST) and an

appropriate amount of rbST (for QC and calibration curve). After centrifugation and elimination of the supernatant, beads were recovered and placed into a clean Eppendorf tube. For separating the beads from solution, a magnetic separation stand (MagneSphere, Promega, Mannheim, Germany) was employed.

Then, MBs-mAB were washed twice with 1 mL buffer PBS/Tween 0.2% (pH 7.4) and 1 mL PBS (pH 7.4) to remove nonspecifically bound proteins, and then with 1 mL ultrapure water to remove the remaining buffer. The supernatant was removed carefully and rbST was released by incubating MBs-mAB with 100 µL 20 mM NaOH for 15 min at room temperature. Subsequently, reconstituted trypsin solution (20 µg mL^{-1} in 50 mM ammonium bicarbonate) was added. The samples were allowed to digest overnight at 37°C. The digestion was quenched by adding 4 µL formic acid, and then 5 µL of rbST-^{13}C IS solution was added. The supernatant was subjected to LC–MS/MS analysis.

LC-MS/MS analysis

Chromatographic analyses were performed with an Acquity UPLC system (Waters, Milford, MA, USA) and separations were done on an Acquity UPLC HSS T3 column (150 × 2.1 mm, 1.7 µm particle size) from Waters. The column was equilibrated at 50°C and the injection volume was 50 µL. The mobile phases consisted of 0.05% formic acid in water (A) and 0.05% formic acid in ACN (B). The gradient used was as follows: 0-0.5 min: 10% B, 0.5-3.7 min: increase to 68 % B, 3.7-3.9 min: 100 % B; 3.90-4.90 min: 100 % B and finally, returned to 10% B for 1 min. The gradient was run at a flow rate of 0.5 mL min^{-1}. The column and autosampler were maintained, respectively, at 50°C and 15°C.

Mass-spectrometric analysis was carried out with a Waters Acquity TQ mass spectrometer (Waters, Manchester, UK). ESI-MS/MS conditions were optimised by individual direct injection of each compound at a concentration of 10 µg mL^{-1} and a flow-rate of 5 µL min^{-1}. The instrument was operated with an electrospray ionization source in the positive (ESI+). The ESI parameters were adjusted as follows: capillary voltage 3.0 kV, cone voltage 30 V, source temperature 150°C, desolvation temperature 400°C, cone gas (nitrogen) flow 50 L h^{-1}, desolvation gas (also nitrogen) flow 1,200 L h^{-1}. Collision-induced dissociation was done with argon as the collision gas at 4 10^{-3} mbar pressure in the collision cell. Data acquisition was done with the MassLynx 4.1 software and the TargetLynx 4.1 software (Waters).

Method validation

The validation study was performed according to the requirements of Commission Decision 2002/657/EC. The parameters taken into account were decision limit (CCα), detection capability (CCβ), specificity, linearity, trueness, precision (repeatability and within-laboratory reproducibility). Validation levels were set at 0.2, 0.5 and 2 µg L^{-1}. Plasma from 20 cows was used for the validation.

Eight points calibration curves were prepared and analysed within 3 days. Concentration levels were fixed at 0.2, 0.5, 1, 2, 5, 10 and 20 µg L^{-1}. pST was used as internal standard and was added at the beginning of the purification. Its use allows calculation of the content of rbST in each QC and calibration point whereas N-term rbST labelled with ^{13}C was used to calculate relative retention time. The CCα and CCβ limits were calculated from this dataset.

Table 1. Method performance characteristics obtained from the validation study.

Level (µg L^{-1})	Recovery (%)	Repeatability (%)	In-house reproducibility (%)	R^2 min	CCα (µg L^{-1})	CCβ (µg L^{-1})
0.2	96.4	14.3	16.4		0.11	0.15
0.5	94.3	14.9	13.3	0.989		
2.0	98.5	11.4	15.2			

Results and discussion

The results of the full validation study according to the criteria specified in Commission Decision 2002/675/EC for a quantitative confirmation method, are summarised in Table 1. The specificity of the method was tested by comparing the chromatograms of blank plasma and spiked plasma. A typical chromatogram of a blank plasma sample and a blank spiked at 0.2 µg L^{-1} rbST is depicted in Figure 1.

The calibration curves for the determination of rbST were prepared by analysing spiked plasma samples in the range of 0.2 to 20 µg L^{-1}. Calibration curves were calculated using weighed (1/*x*) linear regression of internal ratios (analyte/IS peak areas) versus analyte concentrations. A good linearity in the whole range of the tested concentrations was achieved for the three validation days (Figure 2) with a coefficient of determination (R^2) higher than 0.989. Addition of pST at the beginning of the extraction allows to correct for losses during the different steps of the immuno-precipitation purification.

Spiked quality control (QC) samples (n = 6) at three concentration levels (0.2, 0.5 and 2 µg L^{-1}) were assayed to determine recovery, precision and accuracy on the three different validation days by comparing calculated values against nominal concentration. The recoveries of the method, determined at three different concentrations (0.2, 0.5 and 2 µg L^{-1}) ranged between 94.3 and 98.5%. Precision (repeatability and within-laboratory reproducibility) expressed as % RSDs were all below the recommended limit (15.1 %).

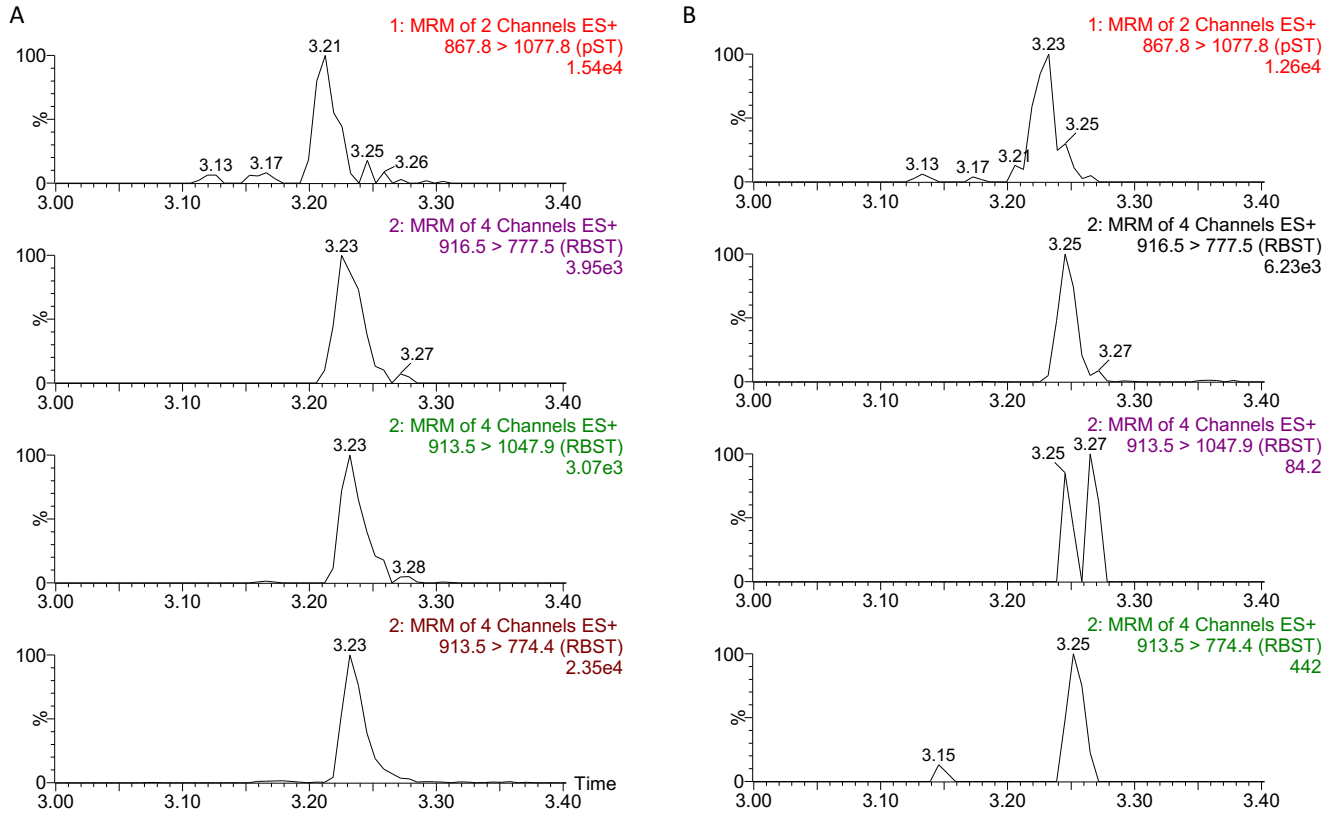

Figure 1. MRM chromatograms of a blank plasma sample spiked with a) rbST at 0.2 µg L^{-1} and internal standard, and b) internal standard only. As regards to the specificity, in all 20 different bovine plasma samples examined, no significant interferences at the retention time of rbST were observed.

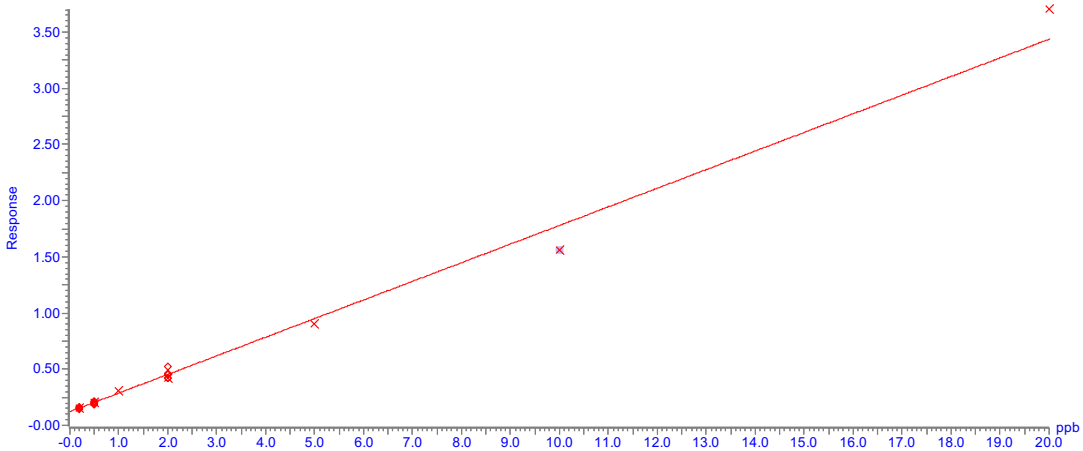

Figure 2. Example of a calibration curve of the N-terminal rbST peptide extracted from plasma.

The decision limit (CCα) and the detection capability (CCβ) were obtained by the calibration curve procedure detailed in the EU Decision 2002/657/EC. CCα and CCβ values of 0.11 µg L^{-1} and 0.15 µg L^{-1}, respectively, were achieved for rbST.

The method also fulfils the criteria for residue identification with four identification points through the measurement of two product ions plus the precursor ion. rbST was detected and identified in all samples spiked at the three levels. Acceptable ion ratio and retention times were obtained for all replicates.

Conclusions

The above method which was developed for the determination of rbST in *bovine* plasma based on immune-magnetic precipitation followed by LC-MS/MS and proved to be reliable. The method meets all the requirements for a confirmatory method according to 2002/657/EC.

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CONFIRMATORY MULTI-CLASS METHOD FOR RESIDUES OF ANTIMICROBIALS IN MILK BY LC-HRMS/MS

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Abstract

A multi-class method for screening and confirmatory analysis of antimicrobial residues in milk has been developed and validated. Sixty-two antibiotics belonging to ten different drug families (amphenicols, cephalosporins, lincosamides, macrolides, penicillins, pleuromutilins, quinolones, rifamycins, sulfonamides and tetracyclines) have been included. Following the addition of an aqueous solution of EDTA, the milk samples were extracted twice with acetonitrile, evaporated and dissolved in ammonium acetate. After centrifugation, 10 µL were analysed using LC-Q-Orbitrap operating in positive electrospray ionization mode. The method was validated in *bovine* milk in the range of 2 to 150 µg kg^{-1} for all antibiotics. For four compounds with Maximum Residue Limits (MRLs) higher than 100 µg kg^{-1}, the validation interval was extended to 333 µg kg^{-1}. The performance characteristics were satisfactory complying with the requirements of Commission Decision 2002/657/EC. Good accuracies were obtained also taking advantage from the versatility of the hybrid mass-analyser. Identification criteria were achieved verifying the mass accuracy and ion ratio of two ions, including the pseudo-molecular one, where possible.

Introduction

In the European Union, only officially registered and approved drugs are allowed in veterinary practice. For drugs, which are registered for use in food-producing animals, withdrawal periods are prescribed during which the concentration of residues in foodstuffs of animal origin (milk, meat, eggs, honey etc.) should be reduced to a level not threatening the consumer's health. This level is called Maximum Residue Limit (MRL). The relevant EU MRLs are listed in Regulation 37/2010 (Commission Regulation (EU) No 37/2010). The use of antibiotics on lactating cows for dry-cow therapy or to treat mastitis still presents a high risk of contamination of the milk supply. Long-acting antimicrobials are commonly used in dry-cow therapy. Antimicrobial agents administered to cows in the course of lactation can pass in milk at various levels.

During the last fifteen years, liquid chromatography coupled with tandem mass spectrometry technique (LC-MS/MS) has been more and more important in routine food analysis of residues. More recently, a great effort has been made to develop LC-MS/MS protocols, including more than one class (multi-class) of antimicrobials to carry out a more efficient and extensive control of residues in food. This has been possible thanks to the great improvement of both hardware and software of the traditional LC-MS/MS systems (triple quadrupoles). At the same time, a new generation of mass-spectrometers, *i.e*. the high-resolution mass analysers (LC-HRMS), allowing the acquisition of accurate mass in full scan mode, improved the possibility to arrange a theoretically unlimited number of analytes. The full scan acquisition, which does not involve the *a priori* selection of the MS/MS transitions, furnishes the (theoretical) possibility to carry out untargeted analysis, *i.e*. to detect unexpected compounds and retrospective analysis. Despite these tremendous technological advances, the development of quantitative multi-class methods for veterinary drugs remains a very challenging goal because, in this context, sample preparation protocols have to be inevitably generic, including as many analytes as possible with final extracts rich of interfering substances. As a consequence, large matrix effects are generally measured and great attention must be devoted to calibration practices, also considering the lack of suitable (labelled) internal standards for a dozen of molecules included in multi-class procedures.

The introduction at benchtop level of the hybrid high resolution mass-spectrometers (LC-HR-MS/MS), such as LC-Q-orbitrap or LC-Q-TOF systems, offered further advantages. Combining the mass selection/isolation capability of the quadrupole and the high resolving power, these machines have a great potential to avoid both false positive and false negative results giving new chances to improve both selectivity and quantitative performances, even in complex matrices.

The aim of this work was the development of a multi-class method for antibiotic determination in milk with the suitable performance characteristics required for a confirmatory quantitative method (Commission Decision 657/2002/EC). The procedure was based on high-performance liquid chromatography coupled with high-resolution hybrid tandem mass-spectrometry. Ten antibiotic families were included in the method scope: amphenicols, cephalosporins, lincosamides, macrolides, penicillin, pleuromutilins, quinolones, rifamycins, sulfonamides and tetracyclines. Applying a set of different MS experiments, the hybrid MS system allows overcoming the most of issues due to interfering substances. The price to pay was a partial decreasing of the potential of HR analysers to carry out analysis of untargeted compounds with retrospective data handling.

Materials and Methods

Individual stock standard solutions (100 µg mL^{-1}) were prepared in methanol (amphenicols, lincosamides, macrolides, pleuromutilins, sulfonamides, tetracyclines and trimethoprim) and in H2O/ACN 75/25 (v/v) for β-lactams except ceftiofur (DMF).

Quinolones were dissolved in MeOH/H_2O 80/20 (v/v) except ciprofloxacin, enrofloxacin and oxolinic acid (DMF). Finally, rifaximin was dissolved in MeOH/H_2O 50/50 (v/v). Stock solutions were stored in freezer and the storage times varied from 1 month (cefquinome) to 24 months (sulphonamides). Intermediate solutions at 1 µg mL^{-1} were prepared in H_2O/ACN 75/25 (v/v) and in methanol for β-lactams and for all other antibiotics, respectively, and stored for three months. The solutions of labelled internal standards were prepared in the same solvent or mixture of native compounds. Details about the preparation and the stability of analyte solutions were reported elsewhere (Moretti *et al*., 2016).

Chromatographic and MS conditions

Chromatography was performed on a Thermo Ultimate 3000 High Performance Liquid Chromatography system (Agilent Technologies, San Jose, CA, USA). Analytes were separated on a Poroshell 120 EC-C18 column (100 x 3.0 mm; 2.7 µm - Agilent Technologies) connected with a guard column (Poroshell, 5 x 3.0 mm). LC eluent A was an aqueous solution containing 0.1% (v/v) formic acid and eluent B was methanol. The gradient started with 5% eluent B for 1 min, continued with linear increase to 95% B in 19 min. This condition was maintained for 5 min. The system returned to 5% B in 1 min and was re-equilibrated for 4 min (run time: 30 min). The column temperature was 30°C and the sample temperature was kept at 16°C. The flow rate was 0.25 mL min^{-1} and the injection volume 10 µL.

The mass spectrometer Q-Orbitrap (Thermo Scientific) was equipped with heated electrospray ionization (HESI-II) source. The optimized HESI-II temperature was set at 320°C, the capillary temperature at 300°C, the electrospray voltage at 3.00 kV (positive mode). Sheath and auxiliary gas were 35 and 15 arbitrary units. Two different MS acquisition methods were developed: the first was for the "screening run" applied to all unknown samples to decide whether they were suspect or not (Table 1). Full MS and targeted-SIM (t-SIM) experiments were combined. Later, only the suspect samples were re-analysed using the same chromatographic conditions, but focusing on the confirmatory parameters ("confirmatory run"). In this run all extract mass traces were based on a 5 ppm mass window (accuracy) and the resolution set at 70,000 (FWHM at *m/z* 200).

Table 1. MS acquisition experiments: screening run.

Time (min)	Experiment	Resolution (FWHM at 200 *m/z*)	Scan range (*m/z*)	AGC	Injection time (ms)	Analytes in the inclusion list
2.5 - 4.5	Full MS	17,500	150-1200	3×10^6	100	-
	t-SIM[a]	70,000	-	1×10^6	75 ms x 4 (MSX)	sulfaguanidine, sulfanilamide^{13}C6, sulfanilamide, florfenicol amine
4.5 -14.5	Full MS	70,000	150-1200	3×10^6	100	-
6.3 – 7.3	SIM (in source CID)	35,000	347.9-350.3	1×10^6	200	-
14.5 - 20	Full MS	70,000	250-600	3×10^6	100	-
	t-SIM	35,000	-	1×10^6	200	3-O-acetyltylosin, erythromycin A, rifaximin, tylosin A, tylvalosin

Sample preparation

An amount of 0.5 g milk was spiked with 15 µL of a solution containing the two labelled β-lactams at 1 µg mL^{-1} (cefadroxil-d4 and penicillin G-d7) and 15 µL of a solution of all other antibiotics ISs at the same concentration. Later, 1 mL 0.1 M EDTA was added and the sample was extracted twice with 3 mL of acetonitrile. The reunited extracts were evaporated and dissolved in 1.5 mL 0.2 M ammonium acetate. After centrifugation (12,000 rpm, 10 min), 10 µL were injected in the LC system.

Method validation

To test selectivity, at least twenty blank *bovine* milk samples were analysed. The linearity in matrix has been evaluated in the range 2-150 µg kg^{-1} (2, 10, 33, 100 and 150 µg kg^{-1}). The matrix-matched solutions were prepared adding the analytes immediately prior to LC injection (post-extraction matrix-matched curves). The experimental plan was carried out to estimate the performance characteristics required by Commission Decision 2002/657/EC taking into the account the relevant MRLs (Commission Regulation 37/2010). Blank *bovine* milk was spiked at the beginning of the extraction procedure with the appropriate standard solutions. The spiking levels were five: 2, 10, 33, 100 and 150 µg kg^{-1}. In addition, for the four antibiotics with an MRL higher than 100 µg kg^{-1} (cephapirin, lincomycin, spiramycin and neospiramycin), the level 333 µg kg^{-1} was investigated too. For the spiking level 333 µg kg^{-1}, the final extract was diluted tenfold with 0.2 M ammonium acetate. Four replicates (n=4) for each spiking level were analysed during the same day along with a post-extraction matrix-matched calibration standard at the expected final concentration and a blank muscle sample. Each series was repeated on three different days

(60 or 72 spiked samples) varying time, milk, operator and calibration status of LC-HR-MS/MS equipment. The precision (repeatability and within-laboratory reproducibility), recovery (trueness), limit of detection (LOD) and limit of quantification (LOQ) were estimated for each antibiotic. Decision limit (CCα) and detection capability (CCβ) were calculated only for the compounds with a fixed MRL.

Results and Discussion

Optimization of mass-spectrometric conditions

The chromatographic conditions were the same described in our previous paper developed for a multi-class method for antibiotics in muscle (Moretti *et al.*, 2016). In contrast, very important changes have been carried out in MS conditions for milk considering the stringent MRLs, especially for some penicillins (4 µg kg^{-1}). In addition, in certain chromatographic zones, co-eluting interfering substances prevented the full scan acquisition of analytes, making this matrix more challenging than muscle. For laboratories involved in drug residue analysis, it is a common good practice to re-analyse the suspect samples with a second analysis (confirmatory) to avoid both false non-compliant results (laboratory contamination) and imprecise quantitative determination due to unpredictable incurred levels. Therefore, two-steps strategy was followed here: i) screening run devoted to minimize false compliant results in unknown samples; ii) confirmatory run devoted to minimize false non-compliant results in suspect samples and definitively confirm the identity and concentration of analyte(s).

Screening run - Full scan acquisition was performed along all the run time. In addition, in the first (2.5-4.5 min) and in the last part (14.5-20 min) of the chromatogram where the most interfering peaks eluted, target-SIM (t-SIM) experiments were necessary (Table 1). After about 14.5 min, multi-charged ions belonging to co-extracted endogenous milk substances (proteins) with *m/z* higher than 600 were predominant and, for this reason, the full MS acquisition was limited in the restricted range 250-600 *m/z*. This cutting was aimed to avoid the strong suppression of signals belonging to the analytes, which eluted in this retention time period. This suppression type (post-interface ion-suppression) was previously discussed by Kaufmann *et al.* (2010). Not the Orbitrap cell itself, but the C-trap, which is an integral part within the Orbitrap instrument, was the probable location. The description "post-interface suppression" indicates that this phenomenon is not due to the classical ion suppression in MS source (interface).

In Figure 1 the chromatograms of a methanolic solution of sulfamethazine (5,000 ng mL^{-1}) and sulfamethazine $^{13}C_6$ (2 ng mL^{-1}) gives a clear demonstration of post interface ion suppression. The signal of labelled sulfamethazine is highly suppressed (trace a2) in presence of 5,000 ng mL^{-1} of native sulfamethazine (trace a1), when the two compounds were acquired in the same full MS experiment with a mass range of 15 Da including both pseudo-molecular ions (range 275-290 *m/z*). Otherwise, in the same chromatographic run, both compounds were well detected (traces b and c), performing two separate full MS experiments with two narrower mass ranges specific for the pseudo-molecular ion of each compound (277.1-281.1 *m/z* and 283.1-287.1 *m/z* for sulfamethazine and sulfamethazine-13C6, respectively). Probably, post interface suppression is not necessarily connected only to the presence of multi-charged heavy ions and this phenomenon should be studied more in deep.

Since the pseudo-molecular ion of five antibiotics eluting after 14.5 min have *m/z* higher than 600 Th, t-SIM acquisition was achieved besides the full scan MS experiment (3-O-acetyltylosin, erythromycin A, rifaximin, tylosin A and tylvalosin). The zone of chromatogram from 2.5 to 4.5 min was rich of several polar interfering substances and a t-SIM experiment was needed to detect the antibiotics with the lower retention times. Finally, amoxicillin required a peculiar experiment because, at 2 µg kg^{-1} (0.5 MRL), this penicillin was not detectable both in full scan and in t-SIM when its pseudo-molecular ion was acquired.

Confirmatory run - The purpose of the confirmatory run was to reach the required number of Identification Points (IPs), *i.e.* at least three for permitted substances. The IP system was introduced by Commission Decision 2002/657/EC, which is the relevant regulation to validate analytical methods for veterinary drug residues. However, the identification criteria described in the Decision have not been updated to include the advances of mass-spectrometry analysers. Therefore "Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed" was considered too (SANTE /11945/2015). The considered criteria were: acquisition of two ions (preferably including the pseudo-molecular ion), their ion ratio and mass accuracy (≤5 ppm). The use of isotopic ratio as well as of non-specific fragmentations was avoided, because they were considered not characteristic enough. Theoretical *m/z* values for fragments were calculated using postulated fragmentation patterns from literature and/or from Mass Frontier® software allowing to check also the mass accuracy of fragments. This is an additional constraint even compared to SANTE /11945/2015, which requires only the mass accuracy of the pseudo-molecular ion.

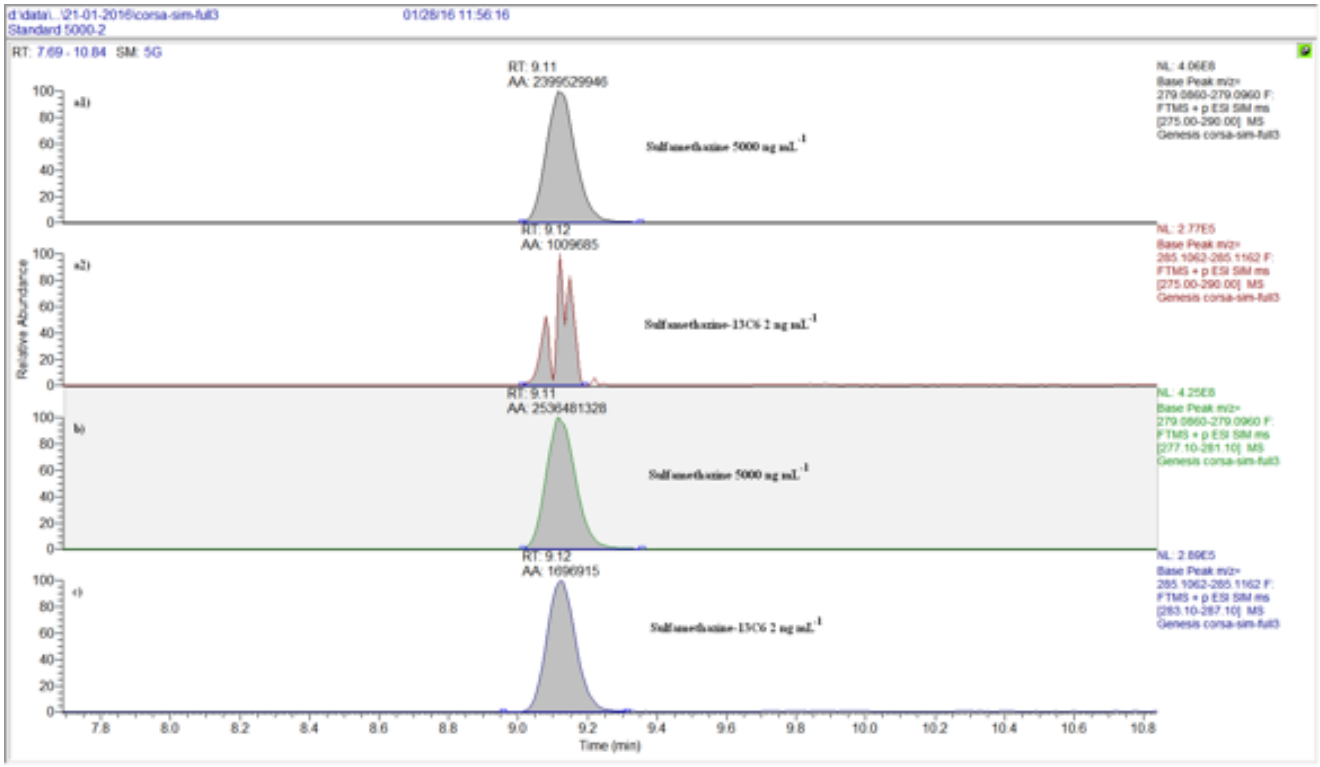

Figure 1. Chromatograms a1) and a2): sulfamethazine and sulfamethazine-^{13}C6, respectively, acquired in the range 275-290 m/z; Chromatogram b): sulfamethazine acquired in the range 277.1-281.1 m/z; Chromatogram c): sulfamethazine-^{13}C6 acquired in the range 283.1-287.1 m/z. All these experiments were carried out during the same chromatographic run.

Optimization of sample preparation

The most significant confirmatory multi-class methods in milk have been reviewed. Few sample preparation strategies were possible since for multi-class procedures the protocols have to be generic, designed to be as inclusive as possible. The published method approaches could be categorized as follows: 1) QuEChERS, 2) SOSLE (Salting-Out Supported Liquid Extraction) (Kaufmann *et al*., 2014; Wang *et al*., 2015), 3) reverse-phase solid phase extraction (SPE) and 4) Others, which are generally procedures involving only a deproteinization step. We decided to adopt this latter generic approach, since the literature data demonstrated that more specific sample preparation strategies failed to recover some important antibiotics classes (mainly, amphoteric quinolones, penicillins and tetracyclines). The extraction and purification of the milk sample was carried out only with acetonitrile in presence of EDTA. Since without EDTA or acids such as formic or acetic acid, the extraction rate of tetracyclines was low, we preferred adding EDTA since acids can compromise penicillin recoveries. Obviously, a low cleanliness of the injected extracts was expected, but the hybrid mass analyser could be used as a further "purification" step.

Method validation

The adopted alternative validation strategy allows to check accuracy (trueness and precision), decision limits and detection capabilities of each analyte without separate experiments (Kaufmann *et al.,* 2014; Moretti *et al*., 2016). Commission Decision 2002/657/EC requires spiking levels around the MRL of each analyte (0.5, 1 and 1.5 times the MRL). However, alternative validation schemes are possible, if the underlying strategy, with the respective prerequisites, assumptions and formulae is laid down in the validation protocol (paragraph 3.1.3. of the Decision). Multi-class procedures with dozens of compounds with and without MRLs fully justify different and more flexible plans such as that here proposed.

The main validation parameters are reported in Table 1. Method repeatability (CV_r) and within-laboratory reproducibility (CV_{wR}) were estimated applying the Analysis of Variance (ANOVA) on the available 12 experiments at each spiking level. The recoveries were obtained comparing the peak area of each compound in the spiked samples against its peak area in the post extraction matrix-matched curve. The raw data were always corrected for recovery. In Table 2 the accuracies obtained pooling the data at all validation levels are listed.

The mean recoveries of all the analytes were in the range from 70% to 120%, except for sulfaguanidine, sulfapyridine and sulfamethazine with recoveries from 60 to 70% Although all intra-lab reproducibility was always lower than or equal to 22%, these three sulfonamides, as well as cloxacillin and tylvalosin, demonstrated also the poorest intra-lab reproducibility ($CV_{wR,pooled}$: 15-22%). Unlike the similar procedure developed for muscle, for milk good recoveries were observed for all β-lactams and tetracyclines, which generally have the most critical performances in muscle tissue (Moretti *et al.*, 2016). A peculiar case was erythromycin A, of which the recovery (61%) was lower than the other macrolides. For this antibiotic, under acidic

conditions (pH<4), the loss of a water molecule forming anhydroerythromycin and other isomeric degradation products, such as erythromycin A enol ether and pseudo erythromycin A enol ether, is well documented in literature (Thompson and Van den Heever, 2012).

For substances with MRL, the decision limit and detection capability were then calculated as follows:

$$CC\alpha_{MRL} = MRL + 1.64 \cdot CV_{Rw,pooled} \cdot MRL \quad \text{and} \quad CC\beta_{MRL} = CC\alpha_{MRL} + 1.64 \cdot CV_{Rw,pooled} \cdot CC\alpha_{MRL}$$

The lowest limits (LOD and LOQ) were estimated evaluating the accuracies at the first and at second validation levels (2 and 10 µg kg^{-1}). Accordingly, operative LOD/LOQs were fixed at 2 µg kg^{-1} for all the antibiotics, except 3-O-acetyltylosin, cephacetrile, florfenicol, sulfaguanidine, sulphanilamide, tylosin A and tylvalosin (10 µg kg^{-1}).

Table 2. Precision and recovery.

Analyte	$CV_{r,pooled}$ (%)	$CV_{wR,\ pooled}$ (%)	Recovery (%)	Analyte	$CV_{r,pooled}$ (%)	$CV_{wR,\ pooled}$ (%)	Recovery (%)
Sulfaguanidine[a]	13	21	67	Difloxacin	5.1	6.7	102
Florfenicol Amine	10	12	108	Ampicillin	2.6	3.2	88
Sulfanilamide[a]	14	22	79	Sulfamonomethoxine	7.5	9.6	79
Desacetylceph.	2.6	3.8	94	Florfenicol[a]	5.9	7.1	98
Amoxicillin	4.6	5.9	87	Cefoperazone	10	14	91
Sulfadiazine	8.3	13	74	Sarafloxacin	5.4	6.1	101
Sulfathiazole	8.9	12	72	Epi-chlorotetracycline	11	12	101
Cephapirin	3.0	3.6	94	Neospiramycin	6.9	8.4	95
Sulfapyridine	9.8	16	69	Chlortetracycline	7.6	7.3	93
Tildipirosin	5.7	6.1	95	Spiramycin	7.4	7.7	96
Cefquinome	3.6	4.4	93	Sulfadimethoxine	6.5	8.0	86
Sulfamerazine	10	15	72	Sulfaquinoxaline	8.9	9.7	82
Cefacetrile[a]	5.6	7.5	92	Oxolinic Acid	5.6	6.7	102
Cefalonium	3.2	3.9	92	Ceftiofur	5.5	6.5	87
Lincomycin	5.5	5.5	96	Gamithromycin	5.5	6.0	98
4-Epitetracycline	6.3	6.8	102	Tilmicosin	5.7	6.6	100
Trimethoprim	4.8	5.2	98	Doxycycline	5.6	5.6	95
Thiamphenicol	6.4	7.0	98	Penicillin G	7.1	9.5	88
Tul. Marker (CP 60,300)	4.8	6.0	99	Tiamulin	6.0	7.1	95
Marbofloxacin	5.0	6.2	103	Flumequine	7.1	7.8	99
Sulfamethazine	9.6	16	69	Tylosin A[a]	12	14	92
Epi-oxytetracycline	9.3	9.1	99	Erythromycin A	10	14	61
Tetracycline	5.0	5.3	94	3-O-Acetyltylosin[a]	8.0	9.7	98
Cefalexin	7.5	7.7	87	Oxacillin	8.5	9.1	91
Oxytetracycline	6.1	6.0	94	Penicillin V	9.3	12	92
Ciprofloxacin	5.9	6.1	99	Cloxacillin	16	22	92
Enrofloxacin	5.5	5.9	101	Valnemulin	7.0	7.1	89
Tulathromycin	6.5	6.4	98	Dicloxacillin	6.1	9.2	88
Danofloxacin	5.3	7.1	103	Nafcillin	4.1	5.4	90
Cefazolin	4.3	4.6	94	Tylvalosin[a]	14	16	97
Sulfamethoxazole	7.2	8.6	86	Rifaximin	9.1	9.3	82

[a] *For these analytes the first validation level (2 µg kg^{-1}) was not included in the calculation of overall recovery and precision (<LOQ).*

Conclusions

After the long work devoted to method development, the final procedure is very rapid and simple. The quadrupole-orbitrap instrument has a unique configuration and its features enable a wide range of experiments. High resolution, trapping capability and versatility of the instrument have allowed satisfactory selectivity and quantitative results for all antibiotics included in this study. Probably, the initial applications of hybrid equipment in the field of multi-class analysis of drug residues in food (Kaufmann *et al*., 2014; Wang *et al*., 2015; this work) should be further improved, enriching the range of targeted MS experiments. The proteomic studies have better explored this topic (Gallien *et al*., 2012) and some of the published refined approaches can suggest new strategies to analytical chemists involved in food residues analysis. The price to pay is a lesser chance of performing untargeted analysis of unexpected contaminants, which is one of the most celebrated characteristics of HR analysers, but at present not so applicable in complex matrices without more powerful software resources and MS/MS databases.

Acknowledgements
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HAIR AND FEATHERS: THE MATRIX OF CHOICE FOR ANTEDATING THE USE OF ANTIBIOTICS, B-AGONISTS AND STEROIDESTERS?

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Abstract
For forbidden compounds like β-agonists and hormonal growth-promoters such as steroid esters, the use of non-invasive sample materials like hair is common practice as only the confirmation of the presence of such banned drugs is sufficient for enforcement. For registered veterinary drugs, *e.g*. antibiotics, analysis has focused on the enforcement of the maximum residue limits in edible products. However, in the fight against bacterial resistance new policies aim for the reduction of antibiotic usage in animal husbandry. Nowadays in The Netherlands the registration of all treatments with veterinary drugs is required. Furthermore, disputes over analysis results sometimes cause discussions on if a treatment was done by the farmer or by a previous owner of the animal. Therefore, antedating of the treatment is important to indicate the time the animals were treated.
To check farmer's registration of veterinary drug treatments and/or the time of administration of forbidden and registered compounds new strategies have to be developed. Due to fast depletion/secretion of most veterinary drugs from edible matrices and excreta, only recent treatments can be detected. Clearly, other strategies are needed allowing a longer detection window to detect misuse of antibiotics, β-agonists and steroids (off label, sub-therapeutic, illegal use etc.). The use of hairs and feathers as a non-invasive sample matrix proved to be a promising tool.
Examples and the pros and cons of using hair and feathers as a matrix in residue analysis for banned and registered substances will be presented, subdivided in three parts: detection, antedating and the discrimination between different treatments.

Introduction
The use of substances having hormonal or thyreostatic action as well as β-agonists is banned in the European Union through Directive 96/22/EC. Hormones may be illegally administered to farm animals for promotion of muscle development or increase water retention (thyrostats), aiming to obtain an economical benefit. Residues of hormones or their metabolites can remain in products of animal origin (like meat, eggs, milk) which may pose a threat to the consumer's health through exposure to the residues (Reig *et al.*, 2008). For these banned compounds, the use of non-invasive sample materials like hair is common practice as only the confirmation of the presence of such banned drugs is sufficient for enforcement.

For registered substances, the use of non-edible matrices is not so obvious as they are allowed for use in veterinary practices. However, in the fight against bacterial resistance, new policies aim for the reduction of antibiotic usage in animal husbandry. Nowadays in The Netherlands the registration of all treatments is required. To check farmer registration, new strategies have to be developed to detect antibiotic usage (preferably covering the whole lifetime of an animal). Due to fast depletion/secretion of most veterinary drugs from edible matrices and excreta, only recent treatments can be detected. Clearly, other strategies are needed allowing a longer detection window to detect misuse of antibiotics (off label, sub-therapeutic etc.). The use of hairs and feathers as a non-invasive sample matrix proved to be a promising tool.

To enforce the new policies and monitor risks on the use of antibiotics, substances having hormonal or thyreostatic action and β-agonists, methods are needed not only for the detection of these drugs in products of animal origin to enforce the law but also for the detection of these drugs in general. Here, the benefits of using hair and feather samples in residue analysis are presented. These include the detection of drugs with a large time window, the determination of the time of administration, and the discrimination of oral treatments and external contaminations.

Detection of veterinary drugs in hair and feathers
Hair has been suggested as an alternative sample material for banned drugs having hormonal action and β-agonistic action. A well-know example is the detection of steroid-esters in hair (Nielen *et al.*, 2006). Active compounds can be retained for a longer time than in urine or blood due to the low metabolic activity of hair (Dunnett *et al.*, 2003; Durant *et al.*, 2002; Gaillard *et al.*, 1999; Duvivier *et al.*, 2015; Nielen *et al.*, 2006; Nielen *et al.*, 2008; Stolker *et al.*, 2007). Furthermore, hair can be easily collected, transported, stored, and extracted. The collection method is non-invasive and does not cause any damage or pain to the animal (Gratacós-Cubarsí *et al.*, 2006). Furthermore, Duvivier *et al.* (2016a) provided detailed scientific evidence for forensic hair testing in support of the recommendations of the Society of Hair testing. However, external contamination can cause false positive results in forensic hair testing for drugs of abuse and is therefore a major concern when hair evidence is used in court. A protocol is suggested as the preferred decontamination protocol to remove external cannabis contamination

from hair while preserving the incorporated compounds (Duvivier *et al.*, 2016a). Furthermore, hair pigmentation can have an influence on the incorporation of compounds such as clenbuterol in hair (Gleixner *et al.*, 1996).

Research demonstrated that all sorts of drugs are deposited in feathers and hair. Feathers have been suggested as an alternative sample material for monitoring antibiotic use (San Martin *et al.*, 2007; Grove *et al.*, 2008; Conejo *et al.*, 2011; Berendsen *et al.*, 2013; Jansen *et al.*, 2016). A high transfer of the fluoroquinolones, ciprofloxacin and enrofloxacin to feathers in combination with a low secretion rate compared to tissues was reported by San Martin *et al.* (2007). The transfer to feathers was attributed to the secretion through the uropygial gland and the successive transfer to feathers by grooming behaviour. The mode of transfer remains uncertain as Jansen *et al.* (2016) demonstrated that only relatively low concentrations of enrofloxacin were present in the uropygial gland after treatment. The high transfer of antibiotics to feathers was confirmed by Cornejo *et al.* (2011) in a study by using different formulations of flumequine, another quinolone antibiotic. They showed a slow secretion of the drugs from feathers and suggested that this was caused by reabsorption of the vascularized pulp that fills the shaft of the feather throughout the maturation process. A study on the disposition of oxytetracycline to feathers after poultry treatment was reported by Berendsen *et al.* (2013) and also this antibiotic was deposited in feathers. Later these findings were again confirmed by Jansen *et al.* (2016) in a study on enrofloxacin.

A major disadvantage of feather analysis compared to tissues is that the extraction procedures for feather are more extensive in order to extract all antibiotics from the matrix. Different types of extraction solvents were tested and the use of organic solvents proved to be mandatory (Berendsen *et al.*, 2013; Jansen *et al.*, 2016). Depending on the research question, before analysis, external contamination has to be removed by extensively washing the feathers.

Berendsen *et al.* (2013) compared the concentration of OTC in feathers with the concentrations determined in muscle and liver of chicken that underwent different treatments (A: therapeutic treatment from day 7-11, B: therapeutic treatment from day 20-22 and C: sub-therapeutic treatment from day 7-30). The results are presented in Table 1. Although a higher level of OTC was administered in treatment A compared to treatment B (five days versus three days), levels in all three tested matrices are similar. This is explained by the different withdrawal period for both treatments (19 days for treatment A vs. 8 days for treatment B). Clearly, the sub-therapeutic treatment resulted in the highest levels in all matrices, which is expected because treatment was continued until slaughter.

Notable is the difference between the levels found in tissue and in feathers: for all treatments, the OTC concentration in feathers is in the mg kg^{-1} range. The relatively high concentration of OTC in feathers compared to tissues is in accordance with the results previously reported for quinolone antibiotics (San Martin *et al.* 2007; Cornejo *et al.* 2011). Apparently, the elimination of OTC from feathers is slow, and thus it is concluded that feathers are a very informative and easy-to-obtain matrix for the detection of the use of antibiotics in poultry having a long detection window.

Table 1. OTC concentrations in liver, muscle and feathers from OTC-treated chickens, slaughtered at day 31 (reproduced from Berendsen et al*. 2013).*

Treatment	OTC in muscle (RSD*) (mg kg^{-1})	OTC in liver (RSD*) (mg kg^{-1})	OTC in feather (RSD**) (mg kg^{-1})
A	0.01 (141%)	0.04 (89%)	6.8 (23%)
B	0.020 (17%)	0.04 (24%)	6.8 (3%)
C	0.21 (13%)	0.35 (3%)	21 (8%)

*Notes: A, 1200 mg L^{-1} during days 7 to 11; B, 300 mg L^{-1} during days 20 to 22; and C, 156 mg L^{-1} during days 7 to 30. OTC, oxytetracycline. *; Duplicate analysis of two different animals (analytical and biological variation). **; Duplicate analysis of homogenised feathers obtained from different animals (analytical variation only). ***; No OTC was detected in one of the duplicates resulting in a high RSD.*

Antedating

The retrospective assessment of the time of clenbuterol administration in agriforensics using analysis of segmented calf hair was demonstrated (Duvivier *et al.* 2015). Calf tail hair samples (two different animals) were taken at different days, before and at the end of the clenbuterol administration for a period of 14 days. The sampled locks of calf tail hair were analysed after cutting Segments (1 cm), each corresponding to approximately 17.5 days of hair growth. The resulting clenbuterol concentration profiles along the hair locks, prior to and up to 108 days after clenbuterol administration, are shown in Figure 1. No clenbuterol was detected in the samples taken 5 days prior to clenbuterol administration, indicating that the calves did not come in contact with clenbuterol prior to the controlled animal study. The experimental data of hair segments containing clenbuterol peak concentrations were in good correlation with calculated theoretical positions of the clenbuterol administration. Segmented hair analysis is reproducible in the assignment of the relevant segment position and, therefore, results in a retrospective estimation of the time of drug administration.

Hair analysis has a few disadvantages. Firstly, the preparation of the samples is a very extensive process and includes washing to remove external contaminations. Furthermore, the mincing procedure is much more elaborate compared to homogenizing tissue, urine or faeces. Secondly, the sample intake is usually low (< 200 mg) and therefore, sample homogeneity is of

prime importance. This can also compromise the limits of detection. Thirdly, for the extraction organic solvents are needed and in some cases a hydrolysis procedure is mandatory.

Another complicating factor in hair analysis is that sampling is a very critical step. Note, that inaccurate hair sampling can cause differences in the segmentation position, even within a single lock of hair. To reach the highest certainty about time of drug administration, multiple locks of hair should be sampled by cutting as close as possible to the skin and as straight as possible. Then individual hairs should be kept in place by fixating them using a rubber band or between glass plates. This issue might be solved by emerging techniques, like Direct Analysis in Real Time (DART) mass spectrometry, which might facilitate the analysis of only a single intact hair (Duvivier *et al.*,2016b).

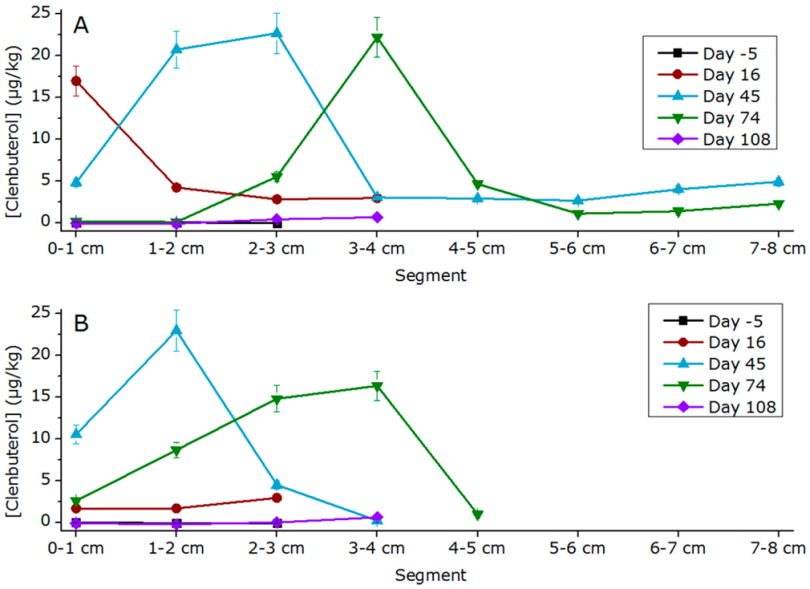

Figure 1. Clenbuterol concentration profiles versus hair segments of calf tail hair samples, taken at different time points (days before or after the end of the clenbuterol administration period of 14 days), of two different calves (A and B). Error bars represent the analytical reproducibility of the method (10.6%). (reproduced from Duvivier et al., 2015)

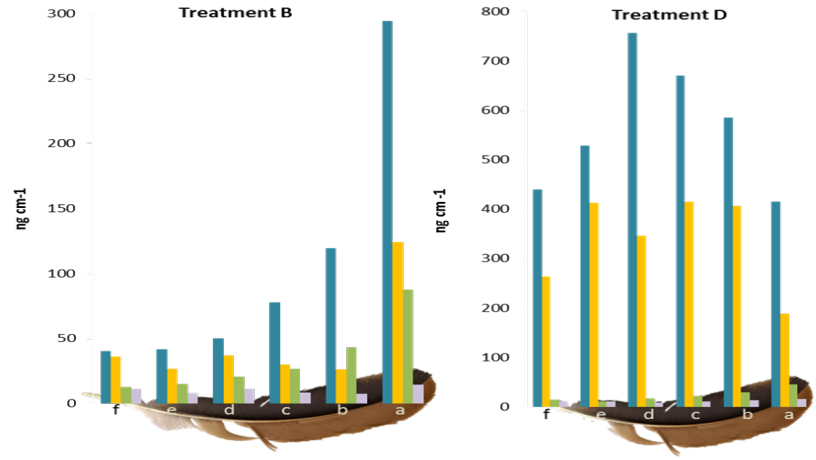

Figure 2. Enrofloxacin concentrations (blue bars) in feather segments after (B) therapeutic oral treatment and (D) sub-therapeutic oral treatment (reproduced from Jansen et al.*, 2016). Other results presented are extensively discussed in Jansen* et al*. (2016).*

Jansen *et al.* (2016) proved that antedating antibiotic treatments using feather analysis by segmentation is possible. Feathers of 34-day-old chickens which were treated with enrofloxacin (B: a therapeutic oral treatment from days 6-10 and D: a sub-therapeutic continuous oral treatment) were analysed. Feathers were segmented in six parts of approximately equal length. Figure 2 shows the results of the analysis of antibiotic in the feather segments after treatments B and D. For oral therapeutic treatment from days 6–10, it is hypothesized that the antibiotics are built into the feather during treatment and subsequently grow with the feather, ending up in the top segment at day 34.

As expected for treatment B, concentrations of enrofloxacin (ERF) are highest in the top segment. After sub-therapeutic continuous oral treatment (treatment D), chickens were daily exposed to low concentrations of enrofloxacin until slaughter. The enrofloxacin pattern shows that in these feathers, residues are far more equally distributed throughout the whole feather. Based on these results, it is suggested that it is possible to estimate the approximate date and length of treatment based on the distribution of the antibiotics throughout the different feather segments. Further research is needed to determine the reproducibility of the patterns for other antibiotics and the precision of antedating. Results of this study will be presented at EuroResidue VIII.

Discriminating treatments

From the same animal study (Jansen *et al., 2016)* the possibility to discriminate between oral treatments and external contact was examined. Feathers from 34-day-old chickens, which were treated with enrofloxacin orally (B) or with a spray (C) from days 6-10, were analysed. With and without removing freely extractable residues by washing with trifluoroacetic acid in methanol, feathers were segmented in six parts of approximately equal length. Figure 3 shows the antibiotic concentrations in the different feather segments after treatment B or C. Here, a difference is made between the total amount of ERF and ciprofloxacin (CPF) in the feather (blue respectively green bars) and non-freely extractable ENR and CPF (yellow respectively grey bars). For the spray treatment (C), a similar pattern for total enrofloxacin (ERF) among the different segments compared to oral treatment (B) is observed. Therefore, the analysis of only the segments cannot discriminate between oral treatment and external exposure. However, a clear difference is observed in the non-freely extractable ERF and total ERF ratio between the oral (B) and spray (C) treatment. Clearly, when external exposure occurs, the levels of the freely extractable ERF are much higher compared to the non-freely extractable ERF, which is present inside the feather. Furthermore, a difference in the CPF/ERF ratio is observed. As CPF is metabolised in the liver, it is hypothesised that its levels are much higher after oral intake compared to external contamination. This is indeed the case, and thus, also based on this metabolite (CPF), both treatments can be distinguished.

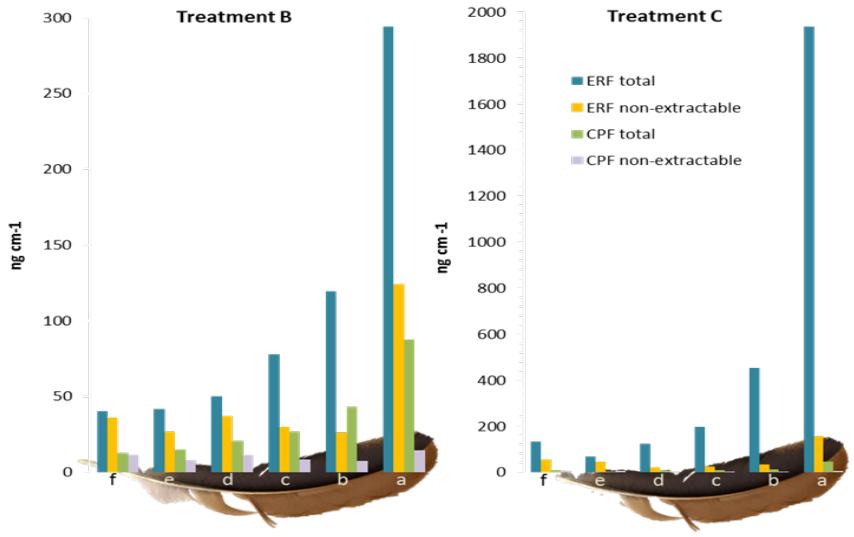

Figure 3. amount of total ERF (blue bar), non-freely extractable ERF (yellow bar), total CPF (green bar) and non-freely extractable CPF (grey bar) in feather segments for therapeutic oral treatment B and spray treatment C (reproduced from Jansen et al*., 2016).*

Conclusion

Detection of drugs in hair and feather seems to be very promising in the fight against unintended use of registered and forbidden drugs. Some examples of methods in which antibiotics, substances having hormonal action as well as β-agonistic are analysed in feathers or hair are presented. The main advantage is that feathers and hairs show a long detection window and thus misuse of drugs can be detected for a long period of time. Another advantage is that by the analysis of segmented feathers and hairs, it is possible to determine the approximate time and length of administration. Last, in feather analysis it is also demonstrated that it is possible to distinguish between oral treatment and external exposure.

Further research is needed to determine the reproducibility of the patterns for other drugs and the precision of antedating and treatment distinction.

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TESTING OF SALIVA AS *ANTE*-MORTEM SCREENING FOR ANTIMICROBIALS IN PIGS

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Abstract
The Belgian meat industry is interested in ante-mortem screening of pigs for antimicrobials in order to prevent positive carcasses after slaughtering. Sample preparation was adapted in order to check urine or saliva on the presence of residues of β-lactams, tetracyclines, sulfonamides and tylosin by means of fast lateral flow assays originally developed for milk testing. Monitoring was set up in order to evaluate the possibility of saliva testing at the pig farm prior to loading. In such a protocol, positive test results result in a delay of slaughtering and the testing of drinking water and feed in order to try to find the origin of the contamination. In the meantime, ante-mortem screening based on saliva testing is becoming operational, protocols were developed to check the carcasses on the presence of antimicrobials by rapid dipstick tests performed in the slaughterhouse before the carcasses or the meat are distributed and exported.

Introduction
The presence of residues of antibiotics above the Maximum Residue Limit (MRL, Commission Regulation 37/2010) in pork could lead to a recall and destruction of significant quantities of meat since pigs are mostly mass-treated by medicated feed or drinking water and hence a single sample is representing a group of animals. According to the Food and Feed Safety Alerts - RASFF data (*Anon*., 2016) in the last five years there were eight alarms and one information due to residues above MRL in pork of Belgian origin. It concerned six times the presence of sulfadiazine, once sulfadimethoxine, once oxytetracycline, and once doxycycline.

To prevent positive pig carcasses after slaughtering, alternatives for the post-mortem monitoring of carcasses were investigated at ILVO-T&V (Technology & Food Science Unit of the Institute for Agricultural and Fisheries Research of the Flemish Community). The research was focussed on the analysis of blood, urine and saliva of pigs by rapid dipstick tests. Antibiotics of interest were the families of β-lactams (penicillins and cephalosporins), tetracyclines, sulfonamides and tylosin.

Materials and methods

Reagents
Charm QUAD-KIWI test (Charm Sciences Inc., Lawrence, MA) is a lateral flow one step test developed for screening of milk for β-lactams, tetracyclines, sulfonamides and tylosin in 5 min. Sample pre-treatment is needed to test urine or meat.

Trisensor Milk and Tylosensor Milk (Unisensor s.a., Ougrée, BE) are dipstick tests allowing the detection of β-lactams, tetracyclines and sulfonamides, and tylosin in 6 to 7 min, respectively. Sample pre-treatment procedures for blood, urine, saliva, meat, drinking water and feed were developed at ILVO.

For the sampling of saliva, TEGO swine oral fluids (ITL Corporation, Melbourne, AU) or similar unbleached cotton ropes were used.

Liquid chromatography and tandem mass spectrometry
The liquid chromatographic system consisted of an Acquity UPLC® system (Waters, Milford, MA). For the saliva, separation was achieved on a Kinetex® C_{18} 2.1 x 100 mm, 1.7 µm column protected by a guard column of the same material. The column was held at 35 °C, the injection volume was 5 µL and the eluent flow was at 0.4 mL min^{-1}. The elution was performed gradually with changing amounts of H_2O/ACN (95/5) + 0.3 % acetic acid and H_2O/ACN (5/95) + 0.3 % acetic acid. For the meat samples, separation was achieved on an Acquity UHPLC C_{18} 2.1 x 100 mm, 1.7 µm column protected by a guard column of the same material. The column was held at 35°C, the injection volume was 5 µL and the eluent flow was at 0.45 mL min^{-1}. The elution was performed gradually with changing amounts of H_2O + 0.05 % acetic acid and ACN/MeOH (50/50) + 0.05 % acetic acid.

The mass-spectrometric equipment consisted of a Xevo TQ-MS® (Waters) equipped with a Z-spray system. The analytes were determined with tandem electrospray positive mass-spectrometry with one transition in screening mode and at least two transitions for the confirmation mode. Following compounds were measured: amoxicillin, ampicillin, benzylpenicillin, cloxacillin, dicloxacillin, oxacillin, nafcillin, cefalexin, cefazolin, cefapirin, desacetylcefapirin, cefoperazone, cefquinome, ceftiofur, desfuroylceftiofur, cefacetrile, cefalonium, danofloxacin, difloxacin, ciprofloxacin, enrofloxacin, flumequine, marbofloxacin,

oxolinic acid, erythromycin, spiramycin, tilmicosin, tylosin, lincomycin, pirlimycin, sulfadimethoxine, sulfadiazine, sulfmethoxazole, sulfamethazine, sulfadoxine, sulfamerazine, sulfapyridine, sulfachloropyridazine, sulfaquinoxaline, sulfaclozine, sulfamethoxypyridazine, sulfathiazole, trimethoprim, oxytetraycline (+ 4 epimer), tetracycline (+ 4 epimer), chlortetracycline (+ 4 epimer), doxycycline, florfenicol and thiamphenicol.

Methods

Sample pre-treatment protocols were developed and the detection capabilities for the antimicrobials of interest were determined for the different dipstick tests.

LC-MS/MS analysis: 3 mL milk was added to 1 mL saliva. After addition of the internal standards the sample was equilibrated for 10 min and 6 mL acetonitrile was added. The tube was vortexed for 30 s and then centrifuged for 10 min at 1,912 *g*. The upper solution of the extract was transferred to a graduated glass tube. The tube was placed in a water bath at 40°C and the extract was evaporated under nitrogen until 4 mL. The extract was filtered over a 0.22 µm filter and 5 µL of the extract was injected into the LC-MS/MS instrument.

Internal standards were added to 5 g meat in a falcon tube. After 10 min of equilibration, 15 g sodium sulphate was added. The sample was dried with a spatula. Fifteen mL of a mixture of ACN/MEOH (95/5) was added and the tube was placed on a shaker for 30 min. The tube was centrifuged for 15 min at 4,000 *g*. The supernatant was transferred in another falcon tube. A second extraction with 15 mL was performed as described before. Of the combined supernatant, 15 mL was transferred in a fresh falcon tube. The supernatant was evaporated at 40°C under nitrogen. The extract was dissolved in 1 mL of H_2O/MeOH/ACN (50/25/25) + 0.05 % acetic acid and vortexed. The extract was filtered over a 0.22 µm filter and 5 µL of the extract was injected into the LC-MS/MS instrument.

The detection capabilities of Trisensor Milk and Tylosensor Milk for the different β-lactams, tetracyclines and sulfonamides with a registration in Belgium for use in pigs and for tylosin were determined. Each compound was spiked in saliva and meat in different concentrations, namely at 0.5×MRL, 1xMRL, 2×MRL, 5×MRL and 10xMRL where the MRL values are the MRLs fixed for pork.

Based on results, methods were chosen for further experiments.

Two samplings were organised. A first sampling of saliva at 108 different pig farms (101 farms with no antimicrobial treatment of the pigs; 7 farms with application of antibiotics to the pigs) was performed in order to check if sampling of saliva of pigs could be performed in an easy way. At the different pig farms, also samples of drinking water and feed were gathered to have an indication about the origin of the contamination in case of a positive saliva test result. To collect saliva, ropes were fixed in the piggery at pig's shoulder height to allow the pigs to chew on the rope for 15-30 min. The oral fluids were collected by squeezing the rope in a plastic bag and collecting the saliva in a small sample tube. All samples were refrigerated during transport.

The samples were tested by means of the rapid assays described above. Samples giving a positive result were further analysed by means of LC-MS/MS in order to have a qualitative and quantitative result of the residue(s) present. These results allow comparison with the data about antimicrobial treatments.

A second sampling was performed at 23 farms (26 compartments) with pigs under antimicrobial treatment in order to have an idea about the distribution of the residues among the different parts of the animal and the ratio of the concentration of veterinary drug residues in the saliva compared to the concentration in the other animal matrices. Involved in the study were farms with treatment of pigs with amoxicillin, benzylpenicillin, doxycycline, lincomycin, spectinomycin, sulfadiazine, sulfadoxine, tilmicosin, trimethoprim or tylosin. At some farms, pigs were treated with a combination of veterinary drugs. Here again saliva was sampled together with drinking water and feed and at the same time at each farm an animal was killed to sample blood, meat, kidney and liver. All samples were examined with the rapid tests and all animal tissues (saliva, meat, blood serum, kidney and liver) were analysed quantitatively with LC-MS/MS.

Results and Discussion

Suitability of the tests for different matrices

Two different sample preparation protocols were tested for **blood serum** on Trisensor and Tylosensor Milk. Regarding the difficult sampling of blood from living pigs and the occurrence of false positive results, it was decided not to focus on blood serum for the ante-mortem screening of pigs on antimicrobials.

With a suitable sample dilution, **urine** could be tested using a rapid test (Charm QUAD-KIWI test, Trisensor Milk and Tylosensor Milk). However, urine is also very difficult to be sampled from living pigs and since the link between antimicrobial residues in the urine and the tissue (meat) is not clear, we cancelled further research on this matrix.

Different sample preparation protocols were tested to make testing of **saliva** possible with milk testing reagents. Dilution with blank milk was compared to dilution with sample buffer. The use of Charm QUAD-KIWI reagents to test saliva remained problematic. For the use of Trisensor and Tylosensor Milk, a 1+4 dilution in blank milk showed the best sample preparation

to allow flow over the dipstick and not losing too much detection capability. Also the impact of a heating step was tested but this heat-treatment influenced thermo-labile compounds like doxycycline. About 60% of doxycycline was destructed by a heating step at 60°C for 5 min. Finally, it was decided not to heat the saliva but the second incubation step was increased with one min to 4 min in order to get a decent colour formation of test and reference lines even when the sample is flowing slower over the dipstick.

Charm QUAD-KIWI test, Trisensor Milk and Tylosensor Milk could be used for testing of antimicrobials in pork tissue (**meat**). Charm Sciences Inc. developed an extraction protocol including addition of Butterfields buffer to ground meat, homogenization, addition of a tablet containing an antibody to prevent matrix interference, centrifugation and addition of supernatant to lyophilized creamed milk. The reconstituted milk solution could be used in the test. The extraction protocol for tissue for testing with Trisensor Milk and Tylosensor Milk involves an extraction with tissue buffer, centrifugation and the use of the supernatant in the test. Compared to the testing of milk, the second incubation step is prolonged to 4 min for meat.

Detection capability of Trisensor Milk and Tylosensor Milk

The detection capability of the Trisensor Milk and Tylosensor Milk for β-lactams, tetracyclines, sulfonamides and tylosin in saliva is shown in Figure 1. The detection capability is expressed relatively to the MRL (EU-Regulation 37/2010 and amendments) in pork tissue since no MRLs are fixed in saliva. Of the respective families only the compounds with a MRL in pork and with a registration in Belgium for use in pigs were tested. It concerned the β-lactams benzylpenicillin, ampicillin, amoxicillin, ceftiofur and cefquinome; the sulfonamides sulfadiazine, sulfadoxine and sulfamethoxazole; the tetracyclines chlortetracycline (+ 4-epimer), oxytetracycline (+ 4-epimer) and doxycycline and tylosin A. All tested β-lactams could be detected in saliva at the MRL fixed for pork (EU-Regulation 37/2010 and amendments) by the Trisensor Milk except for cefquinome that is detected at 2×MRL. Of the 3 sulfonamides only sulfadiazine could be detected at MRL and also all tested tetracyclines except the 4-epimers could be detected at MRL. Finally, tylosin A is also detected in saliva at MRL for pork.

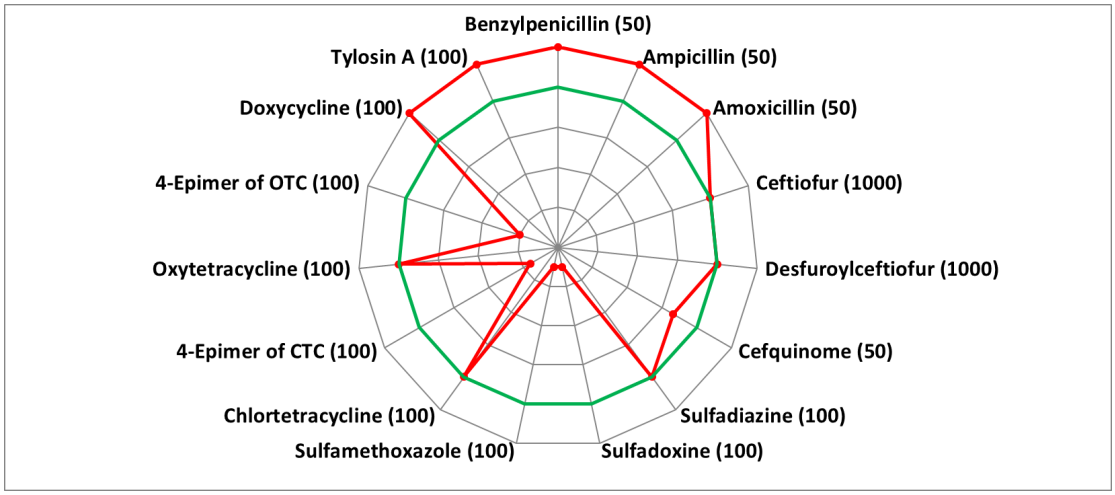

*Figure 1. Detection capability of Trisensor Milk and Tylosensor Milk for β-lactams, tetracyclines, sulfonamides and tylosin in saliva related to their respective MRL in pork meat (Commission Regulation (EU) N° 37/2010 (situation 01/01/2016)). Inner circle = 10 × MRL; circle 2 = 5 × MRL circle 3 = 2 × MRL; circle 4 = **MRL**; circle 5 = 0.5 × MRL. MRL (µg kg^{-1}) in pork meat in between brackets after the name of each substance. Results obtained with ReadSensor and cut-off = 1.10. Notes: CTC: chlortetracycline; OTC: oxytetracycline.*

The detection capability of the Trisensor Milk and Tylosensor Milk in pork is rather similar (no details shown). All β-lactams with a registration in Belgium for use in pigs could be detected in meat at MRL. Of the 3 registered sulfonamides only sulfadiazine could be detected at MRL. The registered tetracyclines (parent drugs) could also be detected at MRL but not their 4-epimers. Finally, tylosin A is not detected at MRL, but from 1.5×MRL on.

Results of the first sampling

There were no problems encountered with the collection of saliva at the different piggeries. In most cases, 15 min were sufficient to obtain enough saliva for testing. In 13 out of 101 samples collected at farms with no antimicrobial treatment during the sampling, at least one positive saliva result was encountered. It concerned 6× sulfonamides, 3× tetracyclines, 3× tylosin and 1× the combination of sulfonamides and tylosin. LC-MS/MS analysis confirmed these data and revealed the identity of the compounds: 1× sulfamethazine, 4× sulfadiazine + sulfadoxine, 1× sulfadiazine + sulfamethoxazole, 3× doxycycline, 3× tylosin and 1× the combination of sulfadiazine and tylosin. Eleven feed samples and four drinking water samples were contaminated with β-lactams, 2 feed samples with sulfonamides and 1 feed sample with tetracyclines. These results proof that problems with carry-over from medicated feed to non-medicated feed still exist. In some cases, the contaminated drinking water

was caused by an error made by the farmer. At the farms with pigs under treatment with sulfadiazine, tylosin or doxycycline, positive saliva samples were found for the respective antibiotic group. However, the oral treatment with amoxicillin was not detected in the saliva. Further experiments showed that β-lactam antibiotics are neutralised by different enzymes present in the saliva. On the other hand, the saliva sample from the farm with pigs treated with amoxicillin was positive for sulfadiazine due to contamination by drinking water. Some false positive results for Tylosensor in saliva and feed were encountered.

Results of the second sampling

No data were found in literature about the ratio between the concentration of residues in the saliva and the concentration in the meat or other animal matrices (blood, liver, kidney). From our results, it can be concluded that for animals with oral treatment of antimicrobials the concentration in the saliva is in general much higher than the concentration in meat. Only in two cases of intramuscular treatment (florfenicol and lincomycin), the concentration was higher in the meat than in the saliva. Following saliva/meat ratios were measured (mean (min-max, number of comparisons)): sulfadiazine, 30 (6-40, n= 10); trimethoprim, 21 (2-146, n=10); doxycycline, 69 (5-199, n= 5); tylosin, 413 (2-1696, n= 5); tilmicosin, 66 (41-85, n=4); florfenicol, 13 (0.5-43, n=4) and lincomycin, 57 (0.06-160, n=3). Very large variations were obtained which could be explained by so many interfering factors like the quantity and the time of consumption of medicated feed or drinking water, the resorption, the size and health status of the animal.

Also in this sampling many cross-contaminations were stated, even the presence of antibiotics (e.g. thiamphenicol) claimed to be never used on the farms.

The analyses of blood serum, liver and kidney samples are in progress.

Conclusions

Our results indicate that sampling and checking of saliva of pigs on antimicrobials is feasible and could be a good alternative for the post-mortem carcass monitoring. Sampling and analysis with rapid tests could be performed at the farm by the driver of the truck collecting the pigs for transport to the slaughterhouse. In case of a positive saliva test result, the animals will remain at the farm and the slaughtering will be delayed. In this way the costs for recall and destruction of non-compliant pig meat due to antimicrobials above the legal concentration could be avoided. The same rapid tests could also be used to search for the origin of the contamination by testing the feed and the drinking water.

At the same time, ante-mortem monitoring of residues of antimicrobials in saliva of pigs will motivate farmers and the feed industry to handle the use of antimicrobials in piggeries with more care. A stricter respect of the withholding time, a special attention to the correct use of antimicrobial drugs (oral treatment by medicated feed or drinking water and intramuscular application) and more prevention of carry-over of antimicrobials in the production of residue-free feed should lead to a significant drop in pork carcasses with presence of antibiotic residues.

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INSECTS ON YOUR PLATE: MONITORING CHEMICAL CONTAMINANTS AND RESIDUES

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Abstract
A growing world population together with ecological as well as economical concerns related to livestock industry enhances the quest towards alternative protein sources of which insects are acknowledged to have great potential. However, health hazards, such as (organic) chemical contaminants possibly related to the consumption of insects or insect-derived ingredients are not yet sufficiently identified nor monitored to guarantee health-safe end products. To achieve the latter purpose, the development of a broad-range extraction and detection technique, specific for insect tissue as well as their feed/substrate is mandatory. Detection of a broad range of pesticides, relevant (veterinary) drugs and coccidiostats, a bacterial toxin and a large spectrum of mycotoxins was achieved by UHPLC-Q-Orbitrap™-HRMS. Several liquid chromatographic (column type, column oven performance, flow rate, solvent types and gradient) and mass spectrometric (sweep gas, auxiliary gas, S lens RF level and resolution) parameters were optimized by injecting analytical standards of the target compounds. Additionally, generic extraction techniques for several insect species (such as *Hermetia illucens*, *Tenebrio molitor*, *Locusta migratoria* and *Acheta domesticus*) and their potential substrates (*e.g*. wheat-bran, grass, waste products (supermarket), chick-flour, chicken blood and faeces) are currently under development, assisted by a fractional factorial design set-up. Finally, these techniques will be validated and applied for the targeted and untargeted detection of chemical contaminants possibly threatening the food safety of edible insects.

Introduction
Recently, edible insects experience an increasing popularity in Central Europe as ingredients in food and feed (Belluco *et al.*, 2013; Mleck *et al.*, 2014, Verbeke *et al.*, 2015). Consuming edible insects as a meat substitute was already intensively explored in non-European countries (*e.g*. Congo, Thailand, Mexico) (Belluco *et al.*, 2013; Dzerefos *et al.*, 2013; Van Huis *et al.*, 2013). The growing world population together with ecological as well as economic concerns related to livestock industry (Mleck *et al.*, 2014), have led to increasing interests for potential alternative food chains. Insects may hereby provide in the daily needs (human and animal) for protein, vitamins, carbohydrates, minerals and fatty acids (Belluco *et al.*, 2013; Rumpold and Schluter, 2013; Mleck *et al.*, 2014).

In Europe, edible insects are considered as a novel food. Ten insect species are meanwhile allowed for human consumption in Belgium (FAVV, 2014). A European Novel Food legislation (Regulation 2015/2283) was introduced in January 2016 requesting the submission of an application for authorization before January 1, 2018 for the market-release of any (products of) insects for human consumption (European Union, 2015). This regulation highlights the lack of information on the potential microbiological, chemical and physical hazards related to the consumption of edible insects.

Scientific data on the extraction of chemical compounds from insect tissue and related substrates are indeed rare and the existing studies mostly consist of targeted analysis of only a few compounds, predominantly applying liquid-chromatography coupled to mass-spectrometry (Wood *et al.*, 2003; Bily *et al.*, 2004; Kamel *et al.*, 2010; Bushby *et al.*, 2012). To ensure health-safe end products of edible insects, we aimed to develop a broad range screening method for the detection of relevant (organic) chemical contaminants and residues such as pesticides, (veterinary) drugs, coccidiostats and mycotoxins after optimized extraction from insects and their substrates.

Materials and Methods

Chemicals and materials
Analytical standards of 25 pesticides, 29 (veterinary) drugs and coccidiostats and 25 mycotoxins were purchased (Table 1) (Sigma Aldrich, Diegem, Belgium; Federal Office of Consumer Protection and Food Safety, EU and National Reference Laboratory, Berlin, Germany; Fermentek, Jerusalem, Israel; Abcam, Cambridge, UK; Chiralix, Nijmegen, The Netherlands; TRC, Toronto, Canada).

Solvents of LC/MS grade (acetonitrile, methanol, ammonium formate (99.995%) and formic acid (98-100%)) were used (Sigma Aldrich, Diegem, Belgium). Ultra-pure water was derived in-house (Arium 611 UV system). Disposable Chromafil AO-45/25 syringe filters (Machery-Nagel, Düren, Germany) were applied during extraction.

Table 1. Overview of the compounds included in the targeted list.

Pesticides				
atrazine	atrazine-desethyl	atrazine-desisopropyl	bromacil	chlortoluron
chloridazon	chlorpyrifos	cyanazine	diazinon	dichlorobenzamide
dichlorvos	dimethoate	diuron	isoproturon	kepone
linuron	metamitron	metazachlor	methabenzthiazuron	metobromuron
metalachlor	metoxuron	pirimicarb	simazine	terbutylazine
(Veterinary) drugs and coccidiostats				
acetylsalicyl acid	atenolol	bezafibrate	bisoprolol	carbamazepine
chloramphenicol	clofibric acid	decoquinate	diclazuril	diclofenac
erythromycin	fenofibrate	halofuginone	ibuprofen	ketoprofen
lasalocid	maduramicin	metoprolol	monensin	narasin
nicarbazin	ofloxancin	paracetamol	propranolol	robenidine
salinomycin	semduramicine	sulfamethoxazole	trimethoprim	
Mycotoxins				
15-acetyldeoxynivalenol	3-acetyldeoxynivalenol	aflatoxin B1	aflatoxin B2	aflatoxin G1
aflatoxin G2	altenuene	alternariol	alternariol methyl ether	cereulide
deoxynivalenol	diacetoxyscirprenol	fumonisin B1	fumonisin B2	fumonisin B3
fusarenon-X	HT2-toxin	neosolaniol	nivalenol	ochratoxin A
roquefortine C	sterigmatocystin	T2-toxin	tenuazonic acid	zearalenone

Extraction

The goal of the experiment was to develop a generic extraction method applicable for four insect species (*Hermetia illucens* (black soldier fly), *Tenebrio molitor* (yellow mealworm), *Locusta migratoria* (field grasshopper) and *Acheta domesticus* (house cricket)) and for 6 substrates (supermarket waste (vegetable), chicken blood and faeces, wheat-bran, grass and chick-flour). A generic extraction method for the insect species was developed for two insect-species initially. This species-selection was based on the different fat percentages in the different metamorphic stages. Insects in the larval stage (*e.g*. yellow meal-worm) contain a higher fat percentage (30-45 %) as compared to the adult stage with a fat percentage of (19-25%) (*e.g*. field grasshopper). Such properties can have an influence on the extraction method and may require the inclusion of a de-lipifica-tion step.

Prior to freeze-drying, the insects and substrates were stored at -80°C during minimal 24 h. Subsequently, the insects were freeze-dried during 48 h at -55°C (Alpha 1-4 LSC plus (Christ, Germany)). As a first extraction step, each dried sample was ground into fine powder using a pestle and mortar.

During the optimization of the extraction, two extraction methods were explored: liquid-liquid extraction (LLE) and acceler-ated solvent extraction (ASE). During the optimization of the liquid-liquid extraction, the following parameters were opti-mized: sample weight, spike volume and concentration, solvent type and volume, number of liquid-liquid steps, evaporation temperature. The alternative ASE method is an automated system for extracting organic compounds from a variety of solid and semi-solid samples. Following parameters were varied and evaluated: the quantity of aluminium oxide, sample and dia-tomaceous earth, the concentration and volume of the mix-standard solution, the solvent type and volume, the temperature of extraction and the number of extraction cycles. The evaporation temperature was also optimized.

Detection

An ultra-high performance liquid chromatograph (UHPLC), equipped with a degasser, autosampler, LC pump and column oven (Dionex Ultimate 3000, Thermo Fisher Scientific) was used for detection of the pesticides, (veterinary) drugs, coccidio-stats and mycotoxins. During the optimization five parameters of the liquid chromatographic part were altered. Five different C18 column types were tested: Acquity UPLC BEH (2.1 x 100 mm, 1.7 μm)(Waters), HSS T3 (2.1 x 100 mm, 1.8 μm) (waters), Hypersil Gold (2.1 x 100 mm, 1.9 μm)(Thermo Scientific), Nucleodor Pyramid (2.1 x 100 mm, 1.8 μm) and Nucleodor Isis (2.1 x 100 mm, 1.8 μm)(Machery-Nagel). For the optimization of the mobile phase, more than 20 different (supplemented) solvent combinations were tested, *e.g*. methanol or acetonitrile acidified with formic acid (0 to 0.15%) and ammonium formate (0 to 1 mM). The gradient of the mobile phase was further optimized in multi-step experiments. The optimal flow rate and column temperature were screened between 0.300-0.450 mL min^{-1} and 35-55°C, respectively.

The detection of the listed contaminants was carried out on a Q-Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific) with heated electrospray ionization (HESI-II) source operated in the positive and negative ion mode. The instrumental setting parameters (Table 2) were optimized to maximize the specific signal of the selected analytes.

Table 2. HESI-II working parameters for ionization of the targeted compounds.

Setting parameters	Range
Scan Range (Da)	100-1200
Resolution (FWHM)	17500-140000
Polarity	pos/neg
Spray voltage (kV)	0-8
Flow 'sheat' gas (au)	0-80
Flow 'sweep' gas (au)	0-10
Flow 'auxillary) gas (au)	0-40
Capillary temperature (°C)	0-450
Vaporizer temperature (°C)	0-600
S-lens (RF level)	0-100

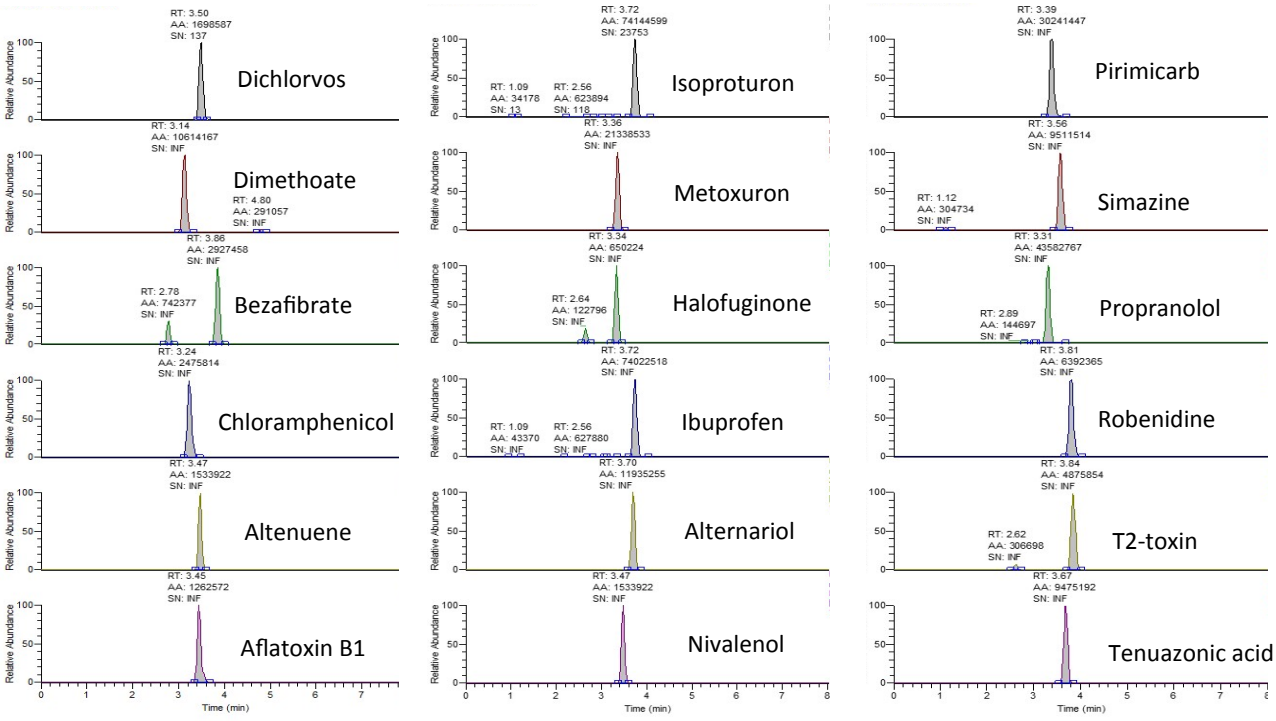

Figure 1. Chromatograms of spiked mealworms with 6 pesticides, 6 (veterinary) drugs and coccidiostats and six mycotoxins at a concentration of 0.25 μg L^{-1} in mealworms.

Results and Discussion

For the separation of almost 80 pesticides, (veterinary) drugs, coccidiostats and mycotoxins an ultra-high performance liquid chromatography was applied. The best chromatographic separation was achieved on a HSS T3 column (2.1 x 100 mm, 1.8 μm)(Waters). The most suitable mobile phase-combinations consisted of water (A) and methanol (B) both containing ammonium formate and formic acid. Ammonium formate and formic acid were added to prevent that components with a pKa-value between 4 to 5 ionize prior to elution from the column. When components ionize, they will retain on the column and hardly elute. The sample injection volume was 10 μL. The column oven and tray temperature were optimized at 45°C and 10°C, respectively. A solvent-gradient of 10 min was fine-tuned, starting with 90% A and 10% B. Within 7.5 min the percentage of B increased (through a multi-step gradient) to 100% which was maintained during 0.5 min. Equilibration to initial con-

ditions was completed in 2 min. The detection of the compounds was carried out on a Q-exactive™ benchtop mass spectrometer (Thermo Fisher Scientific) with heated electrospray ionization (HESI-II) source operated in the positive and negative ion mode. The detection was performed at a scan range of 100-1,200 Da and a resolution of 140,000 FWHM.

The development of an extraction method for a broad range of compounds from insect tissues requires a range of parameters to be challenged. In a first attempt, liquid-liquid extraction (LLE) was explored for the extraction of (organic) chemical contaminants from the yellow mealworm, whereby different solvent combinations were tested. LLE was performed with methanol, isopropanol, diethylether and acetonitrile and with or without acidification. This resulted in the successful extraction of more than 50 targeted compounds. Currently, other solvent combinations are explored for LLE in order to increase the extraction efficiency. In a second parallel attempt for method optimization, an ASE extraction method is explored.

Conclusions

Current UHPLC-Q-HRMS method is capable of detecting a broad-range of almost 80 pesticides, (veterinary) drugs, coccidiostats and mycotoxins within 10 min. The optimization of the extraction method for insects and substrates is still ongoing.

Acknowledgements

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DEPLETION STUDY OF OXYTETRACYCLINE (OTC) AND ITS METABOLITE 4-EPI-OXYTETRACYCLINE RESIDUES (4-EPI-OTC) IN CLAWS OF BROILER CHICKENS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

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Abstract
The use of antimicrobials in poultry production systems is an effective tool for the control of infectious diseases. However, antimicrobial residues may remain in products and by-products destined for human consumption or for animal feeding. Broiler claws, due to their anatomical characteristics, would promote the persistence of tetracyclines at higher concentrations for longer periods than in other edible tissues of these animals.
To assess depletion of oxytetracycline (OTC) and its active metabolite (epimer) in this matrix, an LC-MS/MS method was developed to detect and quantify the analytes of interest. Results showed that the methodology is valid and suitable for its purpose according to European Commission Decision 2002/657/EC criteria. All calibration curves showed a coefficient of determination (R^2) in excess of 0.95 and a recovery better than 90% was obtained. The method was able to detect and quantify OTC and its epimer at and above 20 µg kg^{-1}, which is the calculated detection limit.
A drug depletion study was conducted with a group of 40 broiler chickens raised under controlled conditions. Birds were treated with an oral commercial formulation of OTC 10% for seven days. A withdrawal time of 39 days for OTC and 54 days for its metabolite 4-epi-OTC was established in claws when considering a 95% confidence in the 95th percentile.

Introduction
Oxytetracycline (OTC) is an antimicrobial from the tetracyclines family, authorized for therapeutic use in poultry to combat infectious diseases (Alvarado *et al*., 2008). It is often administered orally through medicated water or feed in the case of outbreaks (Stolker and Brinkman, 2005). It is quickly absorbed by the gastrointestinal tract, being widely distributed throughout the organism and reaching high levels in kidneys and liver (Botsoglou and Fletouris, 2001). Its therapeutic use has decreased the incidence of infectious diseases and mortality associated to them. Also, it has allowed the consumer, to purchase meat and eggs from these productive systems at a reasonable price, of good quality and safe (Donoghue, 2003). However, when antibiotics are not used in the appropriate form surpassing the withdrawal times (WDTs) established for each formulation, they may persist as chemical residues in food affecting their safety standards. In this way, the presence of antibiotics in food products become a risk to consumers' health.

One of the risks of tetracycline residues is ossification and teething disorders. This has been reported especially in children, due to the chelating action of tetracyclines on the calcium ion. Moreover, it could potentially cause allergic reactions in individuals who have been previously sensitized (Turnidge, 2004; Anadón and Martínez-Larrañaga, 2012). Besides, the introduction of these antimicrobial agents in production systems has created favourable conditions for the selection, propagation and persistence of antimicrobial resistant bacteria, capable of causing infections in animals and humans (EFSA, 2008).

In the case of edible tissues of broiler chickens, such as muscle and liver, there is enough information to evidence that antimicrobials after therapy completion may remain for varying times depending on the drug's elimination kinetics and on its pharmaceutical formulation (Schneider and Donoghue 2003; San Martin *et al*. 2009; Cornejo *et al.,* 2011; Anadon *et al.,* 2012). Several authors have studied the OTC depletion profile and its metabolite 4-epi-OTC to establish the post-treatment residue levels in chickens' edible tissues, such as muscle, liver and kidneys (Capolongo *et al.,* 2002; Bogialli *et al.,* 2006; Yu *et al.,* 2011).

However, there is scarce information in the literature about the behaviour of antimicrobial concentrations in poultry by-products. The case of chicken feathers is still under investigation, although some authors have already established that antimicrobial residues are found at higher concentrations when compared to edible tissues, even after therapy completion (San Martín *et al.,* 2007; Cornejo *et al.,* 2011 and 2012; Heinrich *et al.,* 2013; Berendsen *et al.,* 2013). Consequently, antimicrobials may re-enter the food chain through feather meal used for animal feeding (Love *et al*., 2012). Hitherto, there are no studies on chicken claws. This by-product may be destined to human consumption directly or indirectly through claw-meal.

During the last few years, the market for this by-product has grown. In 2007, frozen broiler claws were among the fifteen main agrarian products imported by China, with over US$ 519 million; 106% higher than the previous year (Odarda, 2008). During 2013, exports of this by-product increased with 5.6% in the United States, reaching 2.72 million metric tons, (USA PEEC, 2014). Chile exported a total of 14,990 tons of chicken claws valuated at US$ 19.194 million in 2014 (APA, 2015a).

Chicken claws are basically bone and cartilage, so they may accumulate at higher tetracyclines concentrations compared to other tissues due to their affinity for calcium. Medina *et al.* (2008) analysed the tetracycline content in bones from slaughtered swine and observed that 81% of the analysed samples were positive while 50% of the edible tissues samples were positive. The incidence of tetracycline residues in commercial bone and meat meal samples was studied by Körner *et al*. (2001). They found tetracyclines in all samples with concentrations of up to 2,295 µg kg^{-1}. In a more recent study, Odore *et al*. (2015) analysed bones samples (breastbone, femur, tibia and fibula) from broilers treated with a therapeutic dose of OTC, by LC MS/MS. They found OTC and 4-epi-OTC levels as high as 1,286 µg kg^{-1} at day 10 post-treatment. These results show the persistence of this drug in the studied tissues, exceeding notoriously the corresponding MRLs for edible tissues. It also gives evidence for the possible re-entry of veterinary drug residues into the food chain. Moreover, some authors state that due to OTC thermostability allows this drug is not affected by conventional cooking for human consumption, or by the industrial thermal processes performed in meals, including by-products (Kühne *et al.,* 2001; Love *et al*., 2012).

Currently, there are no specific studies on broiler claws assessing information about the presence of antimicrobial residues. A study carried out by Heinrich *et al.* (2013) researched on the presence of ceftiofur in whole chickens. In order to accomplish this, they analysed a pool of matrices including claws, detecting residues of this antimicrobial, but no specific by-product was studied. This indicated that OTC residues may remain in this broiler claws evidencing the need of carrying out specific studies to detect OTC in this matrix. The pharmacokinetic characteristics of this antimicrobial would promote the accumulation of residues at higher concentrations during longer periods of time. Thus, considering the potential risk of a re-entrance of antimicrobials into the food chain through this by-product, OTC residue depletion in broiler claws has been studied for the first time in birds treated therapeutically.

Materials and Methods

Controlled treatment study

Following ethical approval and animal welfare guidelines based on the Directive 2010/63/EU recommendations for the protection of animals used for scientific purposes, 40 male one-day-old broiler chickens (Ross 308 genetic) were kept in individual cages (25 ± 5°C and 50–60% relative humidity) with *ad libitum* access to water and non-medicated feed. The cages had an elevated wire floor in order to avoid faecal contamination of claws.

After 15 days of breeding, chickens were randomly allocated in two experimental groups A and B containing 32 and 8 birds, respectively. In order to define the size of the experimental groups, criteria established by the European Medicines Agency guide were considered (Approach towards harmonization of withdrawal periods EMA/CVMP7036795 1996). Group A was treated with 50 mg kg^{-1} bw of OTC 10%. The drug was administrated orally once a day for seven consecutive days through premix powder and a WDT of 7 days was respected. Group B as the control group was not treated. Eight birds of group B and two of control group A were euthanized via cervical dislocation at day 3, 9, 15 and 19 post-treatment (4 sampling points). Day 19 post-treatment corresponded to an age of 40 days of the bird. The claws were collected immediately after euthanasia and individually stored at -20°C in properly identified plastic bags.

Sample processing

All samples were chopped with scissors, homogenized in a food processor and stored in fresh bags at -20° C until extraction.

Analytical methodology

The method for the extraction of OTC and 4-epi-OTC developed for broiler claws was based on the methods described by Reveurs and Díaz (1994), Khong *et al.* (2005) and Castellari *et al.* (2009). Samples for the implemented analytical method were obtained from previously analysed commercial broiler claws.

For the analysis and quantification of OTC and 4-epi-OTC in broiler claws, certified standard of purity were used (Dr. Ehrenstorfer Gmbh). As internal standard (IS) an isotopically labelled tetracycline (TC-d6) of certified purity was obtained from the Toronto Research Chemicals (Canada). The following reagents and solvents were used: HPLC-grade water (Milipore OR equivalent), HPLC-grade acetonitrile (Fisher or equivalent), HPLC-grade methanol (Fisher or equivalent), P.A.-grade oxalic acid (J.T. Baker or equivalent), citric acid monohydrate (Merck of equivalent), disodium hydrogen phosphate dehydrate (Merck or equivalent), ethylenediamine tetra-acetic acid buffer (EDTA) (Merck or equivalent) and a solid phase column extraction (SPE) sep-pak C18 (Waters or equivalent).

For the extraction of OTC and 4-EPI-OTC from the matrix, five 0.05-g samples were weighed and then spiked with TC-d6. EDTA-McIlvaine buffer was used as extraction solvent. Samples were agitated and centrifuged, obtaining a supernatant which was afterwards purified in a glass wool column. The supernatant was completely filtered by a SPE sep-pak C18 column,

conditioned with HPLC water and acetonitrile. For the column elution, 1.0 M methanolic oxalic acid was used. Solvents were evaporated under a mild nitrogen flux at 40-50°C. Finally, reconstitution was carried out with 250 µL of mobile phase (oxalic acid 0.01 M/acetonitrile, 5:1).

LC-MS/MS analysis was carried with a liquid chromatograph equipped with a quaternary pump, autosampler and a column oven (Agilent series 3200) coupled to a triple quadrupole mass spectrometer (API 4000, SCIEX). Chromatographic separation was performed using a mobile phase gradient of 0.1% formic acid in water (Phase A) and 0.1% formic acid in methanol (Phase B). The flow-rate was 0.2 mL min^{-1}, the injection volume was 25 µL and the column temperature was 30°C. The MS detector was operated according to the parameters listed in Table 1. Mass ions listed in Table 2 were monitored.

Table 1. Parameters of MS/MS detector.

Ionization	Negative ion mode with a Turbo Ion Spray ™
Scan type	MRM
Source temp.	450ºC
Nebulizer	40
Turbo ion	40
Curtain gas	12
Collision gas	6
Ion spray voltage	-3,000V
Entrance potential	-10
Dwell time	250.0 ms

Table 2. Monitored ion masses.

Ions	Precursor ion	Fragment ion
OTC y 4-epi-OTC	461.0	426.0
	461.0	381.0
TC d-6 (IS)	451.0	416.0

Table 3. Changes made for the improvement of analyte recovery

Changes in methodology	Results
Reduction of sample size	Improves matrix clean up
Increase in centrifugation time	Increases recovery
Addition of reagent tris (2-carbaxyethyl) phosphine hydrochloride 98% (TCEP-HCL)	No changes in extraction
Centrifugation at 0° C	Improves sediment decanting
Hexane addition	Decreases recovery
SPE OASIS HLB cartridge	Decreases recovery
SPE sep-pak C18 cartridge	Increases recovery

Validation of the analytical method

An internal protocol was used for the in-house validation. Estimation of linearity, repeatability, reproducibility, specificity and recovery was performed in accordance with Commission Decision 2002/657/EC. Limit of detection (LOD), limit of quantification (LOQ), was performed according the FDA (Food and Drug Administration) VICH GL49 validation of analytical methods used in residue depletion studies.

Determination of WDTs

In order to determine the WDTs for OTC and 4-epi-OTC in claws, samples were taken on day 3, 9, 15 and 19 post-treatment.

With the concentrations obtained for each sampling period, linear regression analysis was performed, building afterwards a depletion curve on a semi-logarithmic scale, through the use of the K Stange equation, considering a 95% confidence level to determine WDTs. As cut-point, the calculated LoD was used, as no MRLs have been set for chicken claws. With the aim of eliminating outliers, which may interfere with an appropriate interpretation and analysis of the results, interquartile ranges

were determined expressing the obtained results as natural logarithms (NL), for each analyte in the studied matrix excluding the values from the estimated range.

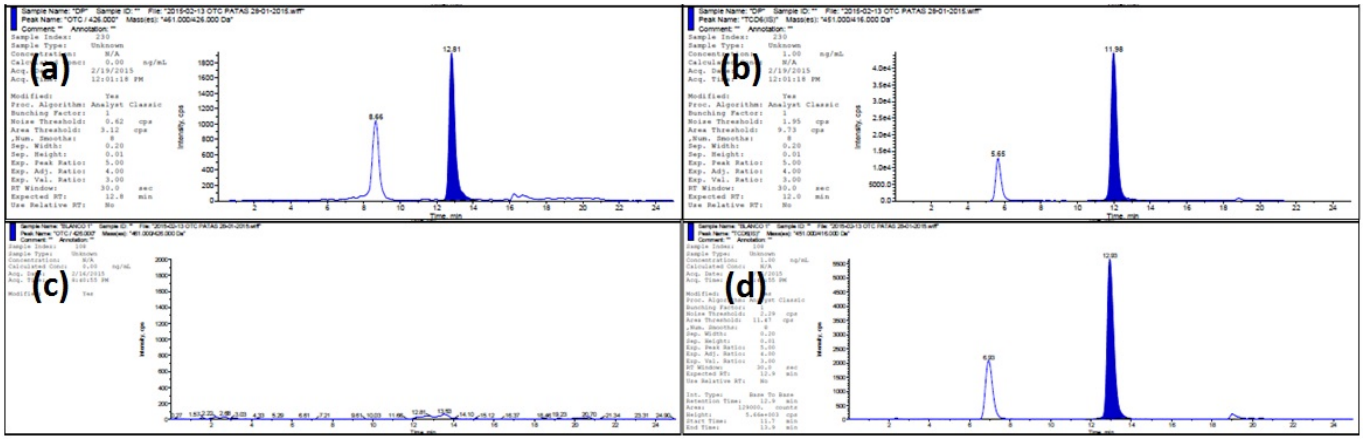

*Figure 1. Chromatograms representing six pure drug injections in panel (**a**) OTC retention time (461.0/426.0) and in panel (**b**) IS TC-d6 retention time (451.0/416.0). Chromatograms of claw blank samples injections representing 20 analyses: (**c**) no interferences at the OTC retention time (461.0/426.0) and (**d**) IS TC-d6 injection (451.0/416.0).*

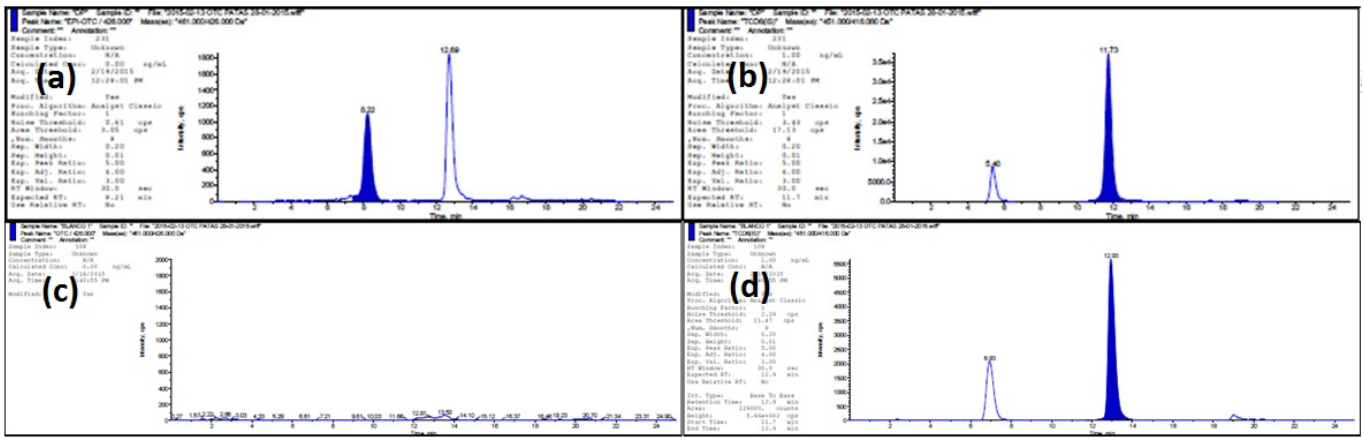

*Figure 2. **(a) and (b)*** Chromatograms representative of six pure drug injections. *(a) 4-epi-OTC retention time (461.0/426.0). (b)* IS TC-d6 retention time (451.0/416.0). ***(c) and (d)*** *Chromatogram of claw blank samples injections, representative of 20 analyses. (c)* No interferences in *4-epi-OTC (461.0/426.0). (d) IS TC-d6 injection (451.0/416.0).*

Table 4. Summary of validation data for OTC and 4-epi-OTC in claws.

Parameter	Retention time CV -5%	Linearity	Recovery (%) at LoD level	Repeatability (%) at LoD CV > Repro.	Reproducibility (%) at LOD CV < 23%	LoD (µg kg^{-1})	LoQ (µg kg^{-1})
Criteria		R^2≥0.95					
OTC	0.32%	0.9608	97%	13.8	16	20	22
		0.9932					
		0.9930					
4-epi-OTC	2.41%	0.9611	116%	10.3	15	20	22
		0.9927					
		0.9801					

Results

The analytical method was optimized with the aim to improve the analyte recovery. Changes made for the improvement are summarized in Table 1. Increasing centrifugation time allowed improvement of the recoveries of both analytes. The use of SPE sep-pak C18 instead of the SPE OASIS HLB® gave a better linearity of the calibration curves. Using SPE sep-pak 18 average

R^2 in the three curves was 0.991 for OTC and 0.983 for 4-epi-OTC. when the OASIS HLB® SPE columns was used average R^2 were 0.869 and 0.908, respectively.

The method fulfils the acceptance criteria for the studied parameters. The method can be considered specific, since no interferences were observed at the retention times of the analytes (Figures 1 and 2). Summary of the validation in claws is given in Table 4.

The concentrations of OTC and 4-epi-OTC found in claws from broilers treated therapeutically were evaluated and quantified. Table 5 describes the average concentrations for OTC and 4-epi-OTC at each sampling moment. The withdrawal time for claws was established on the basis of the statistical method stipulated by the EMA Guidelines (95% tolerance and 95% confidence). The moment (day) at which concentrations are equal to or lower than the LoD (20 µg kg^{-1}) was determined. Analytes concentrations were lower the LoD at 39 (38.6) days for OTC and 54 (53.5) days for 4-epi-OTC (Figures 3 and 4).

Table 5. OTC and 4-epi-OTC depletion in the claws of broiler treated with OTC.

Sample	Post-treatment day	Days of life	OTC average concentration (µg kg^{-1})	4-epi-OTC average concentration (µg kg^{-1})
M1	3	24	1835 (8)[a]	1162 (8)[a]
M2	9	30	984 (6)	370 (7)
M3	15	36	157 (7)	154 (7)
M4	19	40	20 (7)	84 (7)

[a] *number of observations*

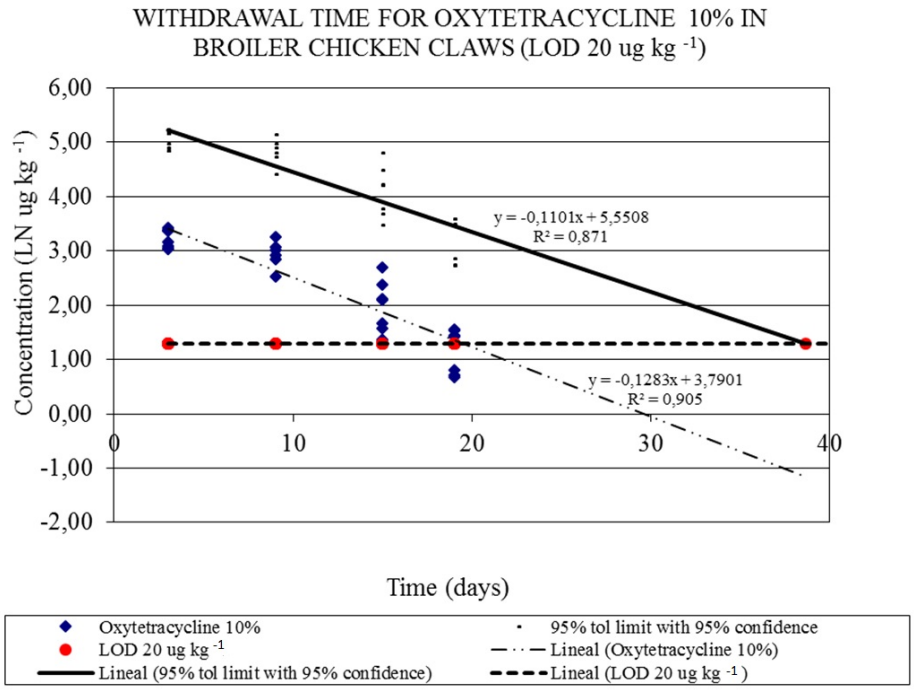

Figure 3. Depletion of OTC concentrations, showing a withdrawal time of 39 days in broiler claws.

Discussion and Conclusions

Chicken claws are a matrix in which the antimicrobial presence has not been studied until now. Thus, it was necessary to implement and validate an analytical method for OTC and its metabolite appropriate for its purpose. Main challenges found during the development of the method were the great amount of fat, cartilage and bone hindering the extraction and sample cleaning. Decreasing sample size and increasing centrifugation time improved analyte recovery. Hexane addition decreased the recovery of both molecules. For the same purpose, the TCEP-HCl hydrolysis reagent was also tested for its capacity to break disulphide bonds. No significant differences were, however, found regarding analyte recovery. Similar results were obtained by other researchers (Berendsen *et al.* 2013) in feather matrix, in which the use of this reagent did not show significant improvement of OTC recovery. During sample clean up, SPE columns sep-pak C18 and OASIS HLB were tested. SPE sep-pak C18 resulted in a better recovery and selectivity.

An in-house validation study was carried out The method met the acceptance criteria for the validation parameters, and thereby it is valid to quantify these analytes in claws in a reliable and precise manner.

Depletion study in chicken claws evidenced that this matrix can be a source of antimicrobial residues entry into food chain. OTC and its epimer residues were found in claws for long periods of time, which can even surpass the average life of a broiler chicken (42 days approximately). This highlights the fact that if the birds are treated with a commercial pharmaceutical formulation, OTC and its metabolite may remain in claws at high concentrations.

In order to observe the behaviour in the matrix, sampling started at day 3 post-treatment. Concentrations were under the method LoD on day 39 days for OTC and day 54 for 4-epi-OTC.S imilar results were obtained in the study by Odore *et al.* (2015). This group analysed bone and muscle from broilers treated with a therapeutic dose of 40 mg kg^{-1} OTC 20% through water by LC MS/MS. They found levels of this drug in muscle below 100 µg kg^{-1} on day 10 post-treatment. However, when analysing bone samples (breast-bone, femur, tibia and fibula), OTC reached an average concentrations of 1.286 µg kg^{-1} and a standard deviation ±26. These results agree with the tendency described in the present study, since at day 9 post-treatment, OTC plus 4-epi-OTC reached 1.354 µg kg^{-1}. This proves that OTC is capable to accumulate and remain in bones, since it binds to the matrix directly through a complex between the calcium ions in the bone and the four rings of the basic tetracycline structure. Thus, residues of this antimicrobial can remain in the claw for longer periods of time.

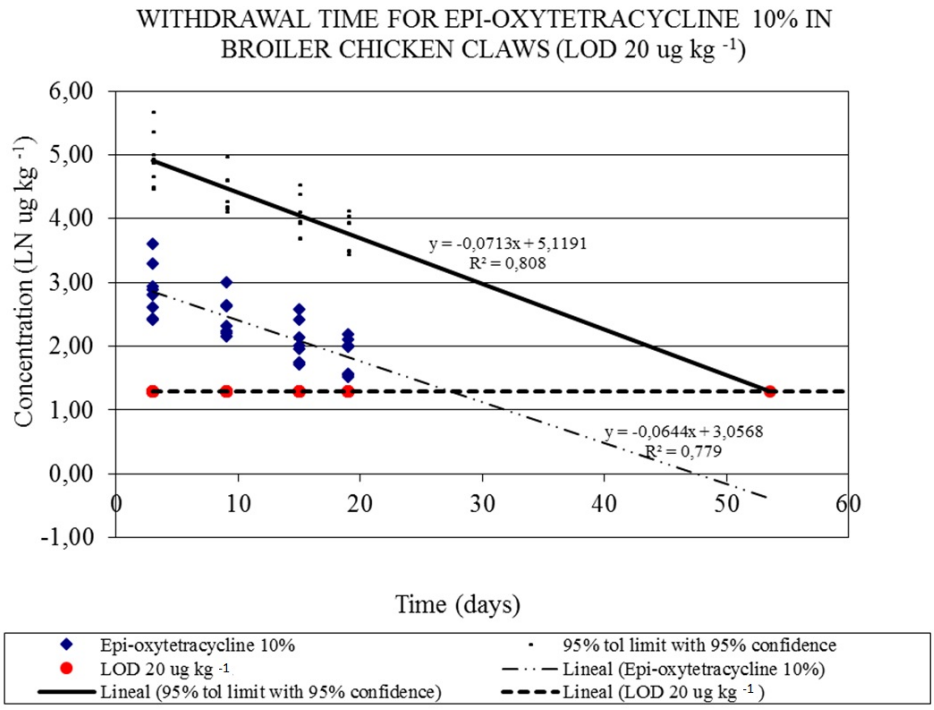

Figure 4. Depletion of 4-epi-OTC concentrations, showing a withdrawal time of 54 days in broiler claws.

The presence of residues from these antimicrobials may become a risk for public health due to their direct effects, such as ossification disorders and possible allergic reactions, also through the development of antimicrobial resistance (Botsoglou and Fletouris, 2001). For this reason, research in the antimicrobial re-entrance paths into the food chain by this or other alternative matrices is needed. The present study provides scientific foundations for establishing future regulations and monitoring residues in this matrix.

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O16
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VALIDATION OF MULTIPLEX BEAD-BASED ASSAYS FOR THE SIMULTANEOUS (ON-SITE) DETECTION OF COCCIDIOSTATS AND ANTIMICROBIALS

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Abstract

For monitoring purposes, multiplex (combined) microsphere (bead) based assays were developed for the rapid and simultaneous detection of coccidiostats and antimicrobials in different matrixes. These assays use paramagnetic carboxylated polystyrene beads (MagPlex^{TM}), internally dyed with two different red fluorophores. For antimicrobial detection, it was possible to select binders with a broad detection spectrum for an antimicrobial group, but the binders for coccidiostats were more specific, *i.e*. those for coccidiostats were only able to bind and detect 1 or 2 coccidiostats. R-phycoerythrin labelled secondary antibodies or proteins were used to quantify the binding to the beads. The beads were analysed by a transportable planar imaging detector (MAGPIX). The coccidiostat assay can simultaneous detect six coccidiostats in feed and eggs. The antimicrobial assay can detect at least forty-two frequently used antimicrobials in drinking water. The coccidiostat assay was single-laboratory validated for narasin/salinomycin, lasalocid, diclazuril, nicarbazin (DNC) and monensin in egg. Calculated percentages of inhibition (% B/B0) at the cut-off levels were 60, 32, 76, 80 and 84, respectively. In feed, the cut-off levels for narasin/salinomycin, lasalocid, nicarbazin (DNC) and monensin were 70, 64, 72 and 78%, respectively, but could not be determined for diclazuril. The preliminary validation results of the multiplex bead-based antimicrobial assay indicated that the assay is very sensitive, *e.g*. for some antimicrobials a detection limit in water below 1 µg L^{-1} is achieved. The preliminary calculated cut-off levels were 36, 53, 10, 36, 26 and 32% for gentamicin, ampicillin, sulfamethoxazole, norfloxacin, neomycin, and streptomycin when the level of interest was set at 100 ng mL^{-1}.

Introduction

The emerging problems caused by the dissemination of antimicrobial resistance were the reason for an EU-wide ban on the use of antimicrobials as growth-promoters in animal feed in 2006 (EC Regulation, 2003). Currently any use of antimicrobials must be authorized by a veterinarian and documented at the production site. Coccidiostats are still authorized to be used as poultry feed additives. Maximum levels (MLs) for feed have been set within the EU to prevent unintended carry-over from feed produced with the highest authorized dose of the coccidiostats into the afterwards produced non-target feed. To protect consumers for unwanted exposure to these compounds, maximum residue levels (MRLs) for antimicrobials and coccidiostats in food have been set.

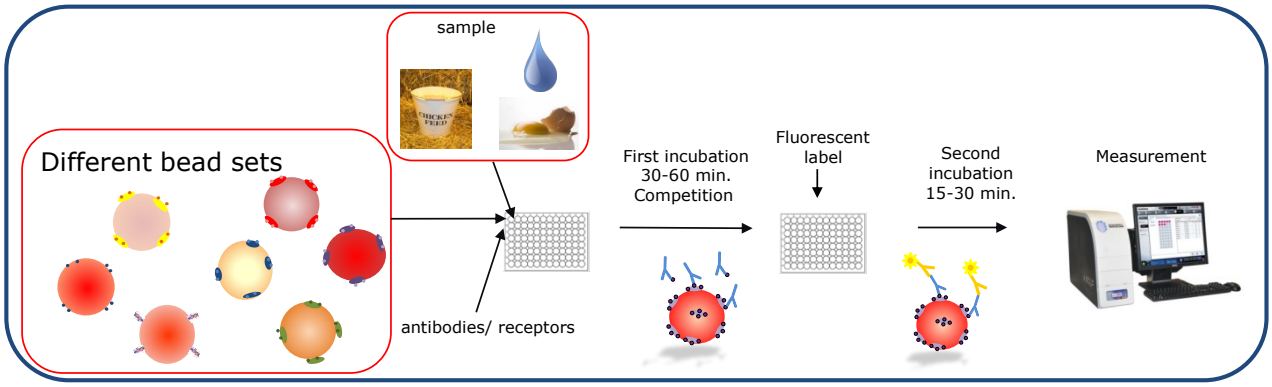

Figure 1. Overview of the general principle and procedural steps of the bead-based multiplex assays. For each compound to be tested a different bead set together with a specific binder was combined. The incubation times applied ranged from 45 min to about 2 h, depending on the assay.

Reliable and rapid screening methods for a broad spectrum detection of these kind of drugs are of great value, but are still very rare. For the broad, rapid and sensitive detection of antimicrobials and coccidiostats a microsphere (bead)-based technology was selected in combination with binders capable of binding an as wide as possible range of compounds. This technology is based on fifty paramagnetic beads sets that can be chemically coupled with different antigens/binders/ biomolecules (antibodies, receptors, aptamers, drugs, drug-protein conjugates and DNA) and analysed with the MAGPIX. In the presented

tests, for the detection of coccidiostats and antimicrobials, different numbers of bead sets have been used: 6 and 7 respectively. These bead sets are used in combination with antibodies or receptors that preferably have broad binding capabilities.

For all assays the first incubation step is a competition between the bead-bound drugs and the free drugs in the sample for the binding sites on the antibodies or receptors. The bead-bound antibody or receptor are measured after the second incubation step with fluorescent protein (R-phycoerythrin (PE)) coated labels. The planar imaging detector measured mean fluorescent intensities (MFIs) on the beads correspond with the amount of bound fluorescent bioreagents and are thus inversely related to the drug concentrations in the sample (Figure 1). The responses (MFIs) were corrected for daily inter-plate fluctuations by calculating the relative responses (%B/B0) from the maximum response (B0) obtained from a blank sample.

Coccidiostats and antimicrobials are relative small molecules and therefore the competition assay format was selected, resulting in lower responses with higher concentrations of the (target) analyte. The developed coccidiostat multiplex assay is capable of detecting narasin/salinomycin, lasalocid, nicarbazin (DNC), diclazuril and monensin. The antimicrobial multiplex assay has a broad spectrum and detects 42 antimicrobials that belong to the aminoglycosides, sulfonamides, tetracyclines, (fluoro)quinolones and β-lactams.

Efficient monitoring is stimulated by rapid screening methods that establish the use and abuse of drugs on-site and support inspections to reach regulatory goals. In order to apply these assays in routine monitoring, validation is needed. The coccidiostat multiplex assay has been validated and the antimicrobial assays are being validated at the moment. The key requirement for these validations is to prevent false-negative screening results. According to the Commission Decision 2002/657/EC, the β-error at the level of interest should be lower than or equal to 5 %. Different approaches were applied to select a rapid and reliable method to calculate the cut-off levels and the rate of false-negatives from blank samples and samples spiked at the level of interest or half the level of interest.

Materials and Methods

Materials

Three different multiplex assays were tested. The used drug-protein conjugates, derivatives, antibodies, and receptors in all these assays were used according to De Keizer *et al*. (2008), Bienenmann-Ploum *et al.* (2012), Bovee *et al.* (2016) and Bienenmann-Ploum *et al.* (2016).

Bead-based immunoassay protocol

Sample preparation, extraction and bead-based immunoassay protocols for the different multiplex assays were described before (De Keizer *et al*., 2008, Bienenmann-Ploum *et al.*, 2012, Bovee *et al.*, 2016 and Bienenmann-Ploum *et al.*, 2016).

In short, depending on the drugs and matrix to be tested, serial dilutions of a mixture of the tested drugs were prepared in buffer. Bead suspensions and antibody dilutions, according to the drugs to be tested, were made in 0.1% BSA blocking buffer (Luminex, 2012). Subsequently, 10 µL of the bead suspension, 100 µL of sample, positive or negative control, and 10 µL of antibody solution were added to a well of a flat-bottom 96-well plate and the plate was incubated on a microplate shaker at RT in the dark for 30 min to 1 h, depending on the assay. After washing thrice with wash buffer (PBS containing 0.05 % Tween-20 and 0.004% antifoam; by using a magnet), 100 µL of diluted SAPE, GAR-PE, DAS-PE and/or RAM-PE (or a mixture) were added. After 15 - 30 min (depending on the assay) incubation with the fluorescent label on the microplate shaker at RT in the dark, the beads were washed again. After adding 100 µL 0.1% BSA blocking buffer the samples were ready to be analysed in the MAGPIX. The antibody or receptor bindings were quantified by the responses (MFI) obtained from the amount of bound fluorescent reporter molecules (PE). The responses of the samples were compared with responses of positive and negative controls and used to classify the samples as compliant or suspect positive.

Results and Discussion

Before applying these assays for routine monitoring purposes, the assays have to be validated. In Commission Decision 2002/657 no real practical approach on how to determine the β-error is given, but only for a screening method the β-error should be lower than or equal to 5% at the level/concentration of interest.

The European Union Reference Laboratory (EURL) guidelines for validation of screening methods (2010) described two practical different methods. In this framework, and using blank and spiked samples, we compared both methods described in annexes 1 and 2 with the validation results of the coccidiostat and antimicrobial assay taking into account that the responses are inversely proportional to the concentrations in the samples. The cut-off level is the signal from a screening assay which indicates that a sample might contain a drug at or above the level of interest, for example the M(R)L. If the cut-off level is exceeded, the sample is classified as suspect and a subsequent confirmatory test has to be carried out. If the cut-off level is not exceeded, the sample is classified as negative (compliant) and no further action is needed.

Method 1 (annex 1) is a rapid approach to determine the cut-off level. The responses in the 20 blank samples and those spiked at 0.5 M(R)L, 1.0 M(R)Ls or the level of interest were examined. The cut-off levels could be set at the highest value (%

B/B0) of the spiked samples (at M(R)Ls or the level of interest) if there was no overlap with the lowest value of the blank. Meaning that all 20 spiked samples can be classified as suspect ($\beta < 5\%$). The 0.5 M(R)L level was also examined because of the low number (n =20) of analysed samples and to comply with the approach described in the guidelines.

Method 2 (annex 2) uses a statistical approach. The threshold values (T) and the cut-off values (FM) were calculated for each coccidiostat and antimicrobial using Eqs. (3) and (4).

Cut-off value = mean (spiked samples) + 1,64 x standard deviation (spiked samples) (3)
Threshold value = mean (blanks) − 1,64 x standard deviation (blanks) (4)

In case the cut-off value is below the threshold value than all 20 spiked samples are classified as suspect ($\beta < 5\%$).

For the 6-plex coccidiostat assay, 20 different blank egg samples and 20 different blank feed samples were used. These samples were analysed as blanks but also spiked at 0.5 M(R)L and 1.0 M(R)L. Five assays detect only a single coccidiostat. The narasin/salinomycin assay detects 2 coccidiostats, and therefore the samples were spiked with a mixture of narasin, lasalocid, nicarbazin (DNC), diclazuril and monensin or with salinomycin.

When using method 1, the cut-off values were below the thresholds of all assays, except for diclazuril in feed. Comparable results were obtained with the second method for both levels tested (Figure 1). Cut-off levels could be set for narasin/salinomycin, lasalocid, diclazuril, nicarbazin (DNC) and monensin in egg, calculated as percentages of inhibition (% B/B0) these cut-off levels were 60, 32, 76, 80 and 84, respectively. In feed, the cut-off levels for narasin/salinomycin, lasalocid, nicarbazin (DNC) and monensin were 70, 64, 72 and 78%, respectively. For salinomycin and monensin in feed, one false-negative result was obtained for each, which is still in accordance with the EURL guidelines. The diclazuril assay for feed did not fulfil the validation criteria (Bienenmann-Ploum *et al.,* 2013).

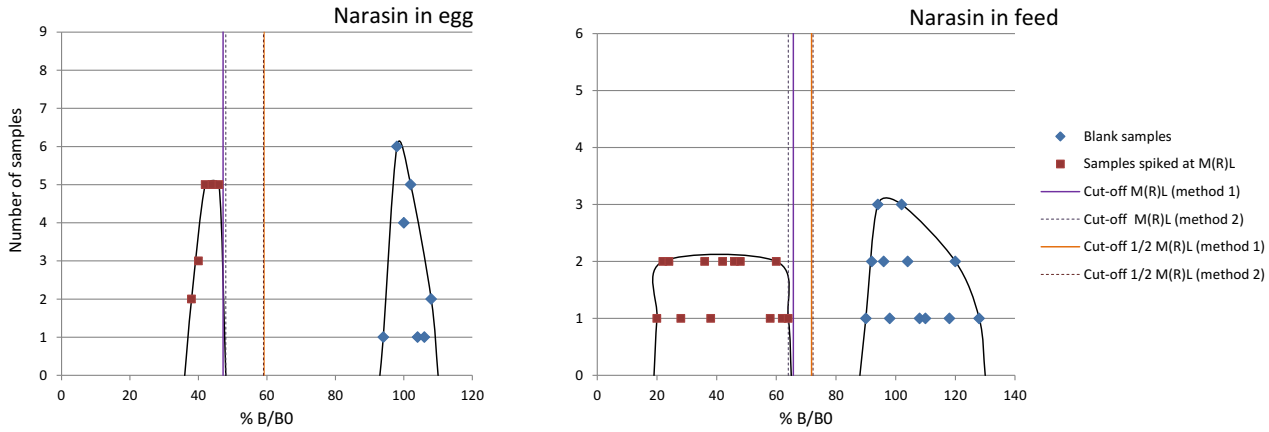

Figure 2. Comparison of the different calculated cut-off values and the measurements of the blank and samples spiked at M(R)L (Bienenmann-Ploum et al., 2013).

The newly developed bead-based antimicrobial screening assay consists of five multi compound assays detecting more than one antimicrobial within an antimicrobial group. The two other assays, neomycin and gentamicin, detected a single antimicrobial. It is important that the antimicrobials relevant for legislation/enforcement are detected and therefore the sensitivity and specificity are important. The antibodies and the receptor used were able to bind multiple antimicrobials from the same group but unfortunately the affinity of the antibody or receptor varies between different antimicrobials in one group.

There are no MRLs for animal drinking water and therefore the MRL for foodstuff from animal origin was taken as a reference. The sulfonamide assay turned out to have the broad detection range and was tested using 14 different standard solutions of sulfonamides at 1 and 100 µg L^{-1} in water (levels well below that of medicated water). Different sensitivities were observed towards different sulfonamides as shown in Figure 3B.

To validate the multiplex assay with forty-four antimicrobials may not be necessary because a number of antimicrobials representative for the antimicrobial group in question can be selected. The sulfonamide assay is again taken as an example. To demonstrate the robustness, repeatability and sensitivity, a single tap water sample was spiked at 1, 10 and 100 µg L^{-1} with sulfamethoxazole and was 19 times analysed spread over three days (Figure 3). The preliminary cut-off level for sulfamethoxazole could be set at 13%. If the cut-off would be set at 13 %, as calculated for 10 µg L^{-1} sulfamethoxazole, a sample containing 100 µg L^{-1} sulfadimidine would give a false-negative result (Figure 3A). For an assay for broad detection, it is important that the cut-off level is set with the antimicrobial with the lowest cross-reaction. Sulfadimidine showed the lowest inhibition in the sulfonamide assay, meaning that this assay is the most critical sulfonamide in the developed multiplex (Figure 3B). One

tap water sample was also spiked at 1 and 100 µg L^{-1} with gentamicin, ampicillin, norfloxacin, neomycin, and streptomycin and was 19 times analysed spread over three days. The preliminary calculated threshold value indicated that the antimicrobial assays were very sensitive, even able to detect some antimicrobials below 1 µg L^{-1}. However, a too sensitive screening method can lead to an increased number of "false-positive" outcomes, as several antimicrobials cannot be confirmed below this level. The preliminary cut-off values were determined according to method 1 and 2, and both methods resulted in about the same cut-off levels, i.e. B/B0 36, 53, 13, 36, 26 and 32 % for gentamicin, ampicillin, sulfamethoxazole, norfloxacin, neomycin, and streptomycin respectively.

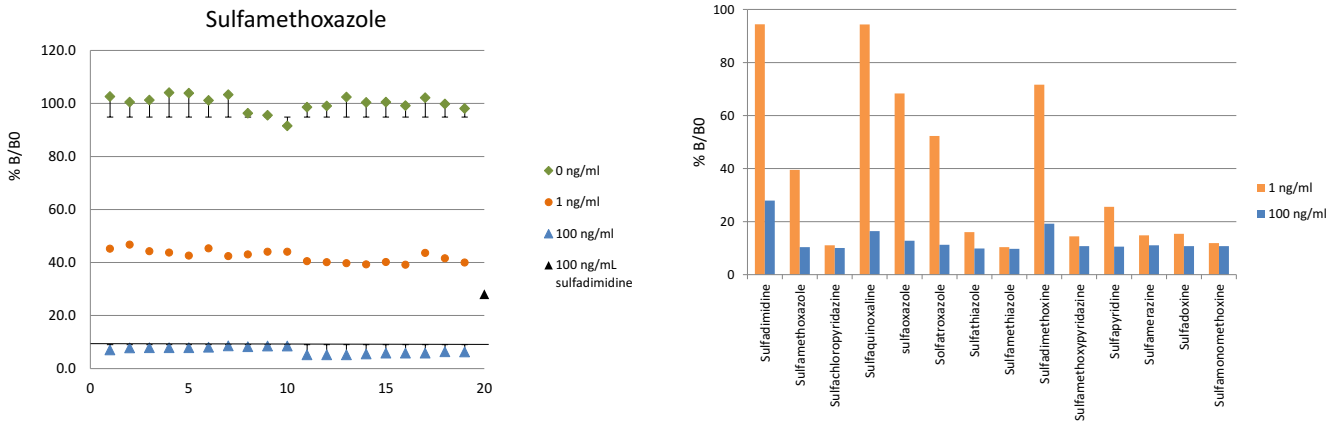

Figure 3. Graphical representation of threshold value and cut-off factor with the relative responses (% B/B0) obtained with blank tap water and the same tap water spike at the 1 and 100 µg L^{-1} (A). The relative responses of tap water spiked with different sulfonamides spike at the 1 and 100 µg L^{-1} (B).

Conclusions

The coccidiostat multiplex assay has been single-laboratory validated for narasin/salinomycin, lasalocid, diclazuril, nicarbazin (DNC) and monensin in egg. Cut-off levels, calculated as percentages of inhibition (% B/B0) at the MRL, were 60, 32, 76, 80 and 84%, respectively. In feed, the cut-off levels for narasin/salinomycin, lasalocid, nicarbazin (DNC) and monensin were B/B0 70, 64, 72 and 78%, respectively, but could not be determined for diclazuril.

A multiplex bead-based assay has been developed for the detection of antimicrobials and an initial validation with water samples was performed. The preliminary cut-off values are B/B0 36, 53, 10, 36, 26 and 32% for gentamicin, ampicillin, sulfamethoxazole, norfloxacin, neomycin, and streptomycin respectively, if the level of interest was set at 100 µg L^{-1}. Finally, the method has to be fully validated to be used as a screening assay for enforcement or monitoring purposes. The first results for robustness and repeatability were very hopeful. Moreover, the multiplex antimicrobial assay can be combined with the multiplex assay for β-agonist (in progress).

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APPROACHES TO VALIDATION OF METHODS FOR REGULATORY USE

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Abstract
Most countries, large and small, are engaged in global trade in today's global economy. To facilitate trade and remove any potential trade barriers, the Codex *Alimentarius* Commission has established guidelines for countries involved in global trade to follow. The Codex Committee on Residues of Veterinary Drugs in Foods has provided details of the requirements of the Codex *Alimentarius* Commission guidelines in its Guideline document CAC/GL-71-2009. These guidelines include establishing laboratory testing facilities which use methods that have been suitably validated and operate under international accreditation. Such validated methods are used by competent authorities to monitor the proper use of approved products to support enforcement programmes. They are also used by the Joint WHO/FAO Expert Committee on Food Additives to inform decisions when evaluating drugs in order to establish maximum residue limits. This presentation will review the validation procedures currently available and the experimental design considerations that should be taken into account in validating analytical methods for use in today's regulatory laboratories.

Introduction
There are currently many validation approaches and/or guidelines that are available in the open literature. These include those developed by the Association of Official Analytical Community (AOAC) International, the American Society for Testing and Material (ASTM) the Codex Committee on Methods of Analysis and Sampling (CCMAS), the European Committee for Normalization (CEN), the Cooperation on International Traceability in Analytical Chemistry (CITAC), the European Cooperation for Accreditation (EA), the United States Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), the International Conference on harmonization (ICH), the International Organization for Standardization (ISO), the International Union of Pure and Applied Chemistry (IUPAC), the United States Pharmacopeia (USP), the European Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, etc.

All these guidance documents begin with the basic premise that method validation is a process of establishing documented evidence that provides a high degree of assurance that a specific method and the ancillary instruments included in the method will consistently provide results that accurately reflect the quality characteristics of the product tested and demonstrate that the method is fit-for-purpose. For example, Clause 5.4.2 in ISO/IEC 17025:2005 states that: “The laboratory shall use test and/or calibration methods, including methods for sampling, which meet the needs of the customer and which are appropriate for the tests and/or calibrations it undertakes…” and further: “When the customer does not specify the method to be used, the laboratory shall select appropriate methods…”.

While most of the guidance documents deal with validation of methods applicable to single analyte methods, recent initiatives in regulatory laboratories have resulted in updates to such methods towards multi-residue analytical methods, where a multi-residue method is considered to be one for which three or analytes in the same drug class or family are included in the scope of the method (Appendix C; CAC/GL 71-2009 rev 2012, 2014).

Methods may be validated either to meet the requirements for single laboratory validation (SLV) also referred to as “in-house” validation or for a multi-laboratory/collaborative (inter-laboratory) validation. In this presentation, the focus will be on single laboratory validation. The single laboratory validation approach may be adopted if other laboratories are perceived as competitors, there is no general interest in the method by other laboratories and/or it will be too expensive to subject the method to a collaborative study. If the validated method is also to be used for routine analysis, there are additional validation requirements that it must meet. While the scope of applicability of validation methods can be expansive, in this 20-min presentation, I will strategically limit the focus to the validation of bio-analytical methods that can be used for the regulatory analysis of veterinary drugs in foods of animal origin either for generating PK and residue depletion data or for compliance monitoring. I am also going to limit this to quantitative analytical methods. To also ensure that we are consistently using appropriate terminology and acronyms in accordance with recommended word use, the words selectivity and ruggedness will be used instead of “specificity and robustness” as they are the preferred terms recommended by IUPAC.

A veterinary drug is any substance applied or administered to a food-producing animal, such as meat- or milk- producing animals, poultry, fish or bees, whether used for therapeutic, prophylactic or diagnostic purposes, or for modification of physiological functions or behaviour. When animal drug manufacturing companies formulate veterinary drugs for use in food animal production, extensive PK and pharmacodynamics (PD) as well as toxicological studies are conducted to provide evidence that the drugs and/or their metabolites are not carcinogenic, teratogenic, or genotoxic to the target animal and to human

consumers, and used to evaluate the efficacy of the drug in the food animal to which it would be administered. Depletion and disposition studies are also conducted to establish the absorption, distribution, metabolism and elimination (ADME) of the drug from the target animal and provide additional residue data to demonstrate that when administered according to Good Veterinary Practice (GVP) at the label concentration, the drug and its metabolites will not leave undue residues in edible tissues and milk of the food animal designated for human consumption. Residues of veterinary drugs include the parent compounds and/or their metabolites in any edible portion of the animal product, and include residues of associated impurities of the veterinary drug concerned.

Foods of animal origin and products derived from them are traded extensively. The Codex Alimentarius is a collection of internationally adopted food standards and related texts presented in a uniform manner to protect consumers' health and to ensure fair practices in the food trade. Codex Alimentarius publishes these standards, therefore, to guide and promote the elaboration and establishment of definitions and requirements for foods to assist in their harmonization and in doing so facilitate international trade. These Codex Standards and related text also contain requirements for food aimed at ensuring for the consumer a safe, wholesome food product free from adulteration, correctly labelled and presented. They are not a substitute for, or alternative to national legislation. They are intended to encourage the development, design and management of modern food production systems that will ensure that exposure of the food producing animal to veterinary drugs does not pose a risk to human health. Under these conditions, commercial entities involved in the production and marketing of food have the primary responsibility to ensure food safety.

Every country's laws and administrative procedures contain provisions with which it is essential to comply. For example, in the United States, the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) is responsible for the safety of meat, poultry and egg products. In the European Union, the European Commission for Health and Consumer Protection Directorate General (DG SANCO, now DG SANTE) is the competent authority for the safety of food chain, chemicals, contaminants, and pesticides and relies on expert scientific advice and recommendations provided by the European Food Safety Authority (EFSA) as its risk assessment body. These competent authorities control and regulate the use of veterinary drugs in food animal production and are responsible for developing appropriate practices that can be applied by the food industry; they also ensure that effective measures are in place within the veterinary drug distribution and food production systems to provide effective protection for consumer health and ensure fair trade in food consistent with the goals of the Codex Alimentarius.

These goals have been achieved to a large extent by the application of programmes based on risk management across all food groups and hazard classes to allow a more focussed application of resources to those areas which are most likely to generate real human health protection gains. The application of a control and verification programme based on risk should provide the basis for exporting countries to certify the safety of their exported food, and for importing countries to have the assurance/confidence to accept those commodities.

For veterinary drugs that have been toxicologically evaluated and found to have no carcinogenic, teratogenic or genotoxic effects on the food animal and the consumer, the general practice is for the risk assessment expert body such as the Joint WHO/FAO Expert Committee on Food Additives (JECFA) established through the United Nations World Health Organization (WHO) and the Food and Agriculture Organization (FAO), or EFSA for the European Commission to establish an acceptable daily intake (ADI) from which a maximum residue limit (MRL) can be derived to estimate the effects of short term (acute) and/or long term (chronic) dietary exposure of consumers to the drug of interest. The ADI is the type and amount of residue of the drug considered to be without any toxicological hazard for human health. On the basis of the ADI, a MRL considered to be the maximum concentration of residue resulting from the use of a veterinary drug (expressed in mg kg^{-1} or μg kg^{-1} on a fresh weight basis), can be derived by JECFA or ESFA or a risk assessment expert team from a competent authority. The derived MRL is then recommended by the JECFA (to the Codex Alimentarius Commission as the risk manager) to be the legally permitted or recognized concentration acceptable in or on food.

It is impractical to expect that every single food item is tested for compliance through end-product testing before it gets to the consumer or to the market. The application of a risk based approach across all food groups and hazard classes should allow a more focussed application of resources to those areas which are most likely to generate real human health protection gains. This is why competent authorities have developed food inspection programmes that include an integrated risk-based inspection system that promote the implementation and adoption of hazard analysis and critical control points (HACCP) at the slaughter and meat processing establishments. One effective approach to measuring the effectiveness of such a regulatory enforcement programme for compliance is by end point product testing. Such testing to measure the level/concentration of residues in the product at slaughter just before the meat or meat products enters the market and/or becomes exposed to the consumer has been integrated into all regulatory monitoring programs.

To also ensure that the rules of engagement for testing laboratories engaged in regulatory analysis are harmonized globally in order to reduce barriers and facilitate trade, the CAC has issued a guideline for laboratories involved in the import/export testing of foods that recommends that such laboratories should:

(a) comply with the general criteria for testing laboratories laid down in ISO/IEC 17025:2005 "General requirements for the competence of calibration and testing laboratories; (b) use internal quality control procedures, such as those described in the "Harmonised Guidelines for Internal Quality Control in Analytical Chemistry laboratories"; (c) participate in appropriate proficiency testing schemes for food analysis which conform to the requirement laid down in "The International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical laboratories"; and whenever available, use methods which have been validated according to the principles laid down by the Codex Alimentarius Committee. Such methods should have been validated in accordance with accepted criteria for single laboratory validation carried out by a laboratory accredited to the applicable international standard for testing laboratories or operating an equivalent system of quality management and exhibiting equivalent technical performance. For laboratories complying with and operating under internationally accredited the ISO/IEC 17025:2005 requirements which also incorporate the relevant elements of Good Laboratory Practice (GLP), they will operate under a Quality Management System (QMS) for their testing and calibration activities that also meets the quality management principles of ISO 9000.

Analytical methods for risk assessment and MRL evaluation

When analytical methods are developed and validated solely for generating data such as PK, PD, disposition and depletion studies for drug registration and licensing in different countries, every suitable analytical method is acceptable. For analytical methods used to generate PK and residue depletion data for drug registration and licensing that will ultimately also be submitted to the JECFA for MRL evaluation and recommendation, however, must have been validated in accordance with accepted criteria of at least a single laboratory validation carried out by a laboratory accredited according to the applicable international standard for testing laboratories and/or operating an equivalent system of quality management and exhibiting equivalent competencies. The method must have been demonstrated to have performed satisfactorily and reliably in the hands of the user involved in generating the data for that particular study. The data to be reviewed by the JECFA must be accompanied by a complete validation report for the method especially where the method has not been published in a peer-reviewed scientific literature. Because JECFA reviews the suitability of the method based on available validation data, the quantitative/determinative method should be described in an internationally recognized format (e.g. ISO Format) and the information on method validation should include the data generated in the process of determining the following characteristics performance parameters: *selectivity, recovery, limit of quantification, limit of detection, accuracy and precision (repeatability within laboratory).* A mathematical and/or statistical description of the calibration curves should also be given if such curves form the basis for the quantification of the drugs of interest. Details of the method requirements and the assessment process followed by the JECFA in recommending MRLs for compounds with ADIs are provided in detail in Chapter 8 "Maximum Residue Limits for Pesticides and Veterinary Drugs" published in the "Principles and Methods for the Risk Assessment of Chemicals in Food" Environmental Health Criteria 240 (EHC 240). In its assessment, JECFA also requires that a validated analytical method(s) suitable for use in the regulatory monitoring of compliance of commodities against the recommended MRL is available.

The disposition and depletion data generated by a drug manufacturer for drug registration and licensing consideration must provide residue depletion data in the four traditional edible tissues – kidney, liver, muscle and fat. Where lactating cattle and other lactating food animals are examined, depletion data should also be generated for milk. In some jurisdictions, additional residue data may be generated for injection sites (when the drug is administered intramuscularly or subcutaneously to the food animal) and offal where these are considered edible. In all of these pre-registration/licensing studies, it is important to identify a marker residue and a target tissue.

A *marker residue* is usually the parent drug or a metabolite of the parent drug which found at the highest concentration for the longest period of time in the target tissue. The *target tissue*, which is usually used for routine monitoring purposes, is the tissue in which the highest residues of the marker residue are found. Because it is important to know the total residue burden in the food animal, the relationship between the concentrations of residues of the marker residue to the total residue of the drug in the food animal should be determined. The MRL is expressed in terms of the marker residue which may be the parent drug, a major metabolite, a sum of parent drug and/or metabolites or a reaction product formed from the drug residues during analysis. In some cases, the parent drug or the metabolite may be bound to tissue proteins and will require chemical or enzymatic treatment or incubation to free them for chemical analysis. As far as practicable, the marker residue must retain its structure throughout the extraction/analysis procedure to provide unequivocal evidence of exposure to the drug.

Analytical methods for regulatory monitoring and compliance for domestic and import programmes

Methods developed and validated for monitoring of compliance of commodities with MRLs should meet the *selectivity, recovery, limit of quantification, limit of detection, accuracy and precision (repeatability within laboratory)* and additional criteria such as applicability, practicability and ruggedness. For these methods the validation study must also include the analysis of incurred residues in a number of independent tissues or commodities. The target tissue identified in disposition and deple-

tion studies and the marker residue are the two most important parameters that national residue control programmes monitor for compliance and surveillance actions. For lipophilic substances, the usual target tissue is fat. For most other drugs, the kidney or liver will be the target tissue depending on the route of drug elimination from the food animal. Generally, kidney or liver will be the designated target tissue for testing domestically produced foods. However, since organ tissues are typically not traded globally (except under very specialized export/import arrangements), muscle tissue is the target tissue that is traditionally available for testing imported meat and meat products.

Performance Characteristic Parameter Evaluation - The Single Laboratory Validation Criteria
In 2002, Thomson *et al*. published a guidance document describing the single laboratory validation approach under the auspices of IUPAC. In most cases, methods developed and validated for drug registration will be a single analyte method optimized for the drug of interest and would normally be the one also recommended for regulatory monitoring at the MRL. For validation of these single analyte methods, the CAC has published a guidance document CAC/GL 71-2009 that describes in detail the attributes expected of a method for use in a regulatory monitoring programme. These attributes that need to be characterized and validated through a single laboratory validation approach are (i) selectivity of the method; (ii) the calibration function which includes the limit of detection, limit of quantification (iii) the reliability of the results (precision and accuracy); (iv) stability of the analytes and extracts; (v) incurred residue studies (where possible). Since it is very unlikely to find single analyte methods being used in today's regulatory laboratories because most laboratories have resorted to developing and validating multi-residue laboratory methods on the sophisticated highly sensitive and highly selective analytical instrumentation platforms available to them in their facilities to increase efficiencies and reduce laboratory operation costs and generate results in shorter times than ever before.

The Codex Alimentarius Commission procedural manual recommends that in the absence of inter-laboratory validated methods, single laboratory validated methods can be used for regulatory analysis as long as:
(i) the method is validated according to an internationally recognized protocol such as the IUPAC guidelines for single laboratory validation; (ii) the use of the method is embedded in a quality management system in compliance with ISO/IEC 17025:2005 standard or with the principles of good laboratory practice (GLP); (iii) the method should be complemented with information on accuracy demonstrated for instance by regular participation in proficiency schemes, where available; (iv) calibration using certified reference materials, where applicable; (v) verification of results with other validated methods where available; and (vi) recovery studies performed at the expected concentrations of the analytes.

The most efficient process to completing a validation study is to use the design of experiments (DOE) approach. DOE for method validation seeks to validate the analytical method for a range of concentrations so that changes in formulation or concentration will not require additional validation as they are changes within a characterized design space. Once methods have been developed, qualified, and validated, the impact they have on out-of-specification rates and process capability needs to be quantified and evaluated to determine their fitness for use.

For linearity, LOD, LOQ, analytical range matrix effect, recovery:

1. Prepare neat standard solutions at six equally spaced concentrations that span the anticipated calibration range. Label these 'Chemical standards'.
2. Prepare six individually weighed negative control samples of edible tissue, fat or milk (including an internal standard where applicable) according to the described procedure. To the extract generated from the negative control samples fortify with the drug or drugs of interest at the same equivalent concentrations as in the neat standards, just before re-constitution for instrumental analysis. Label these Matrix-matched calibration standards.
3. Prepare six individually weighed negative control samples of edible tissue, fat or milk fortified with the drug or drugs of interest (with internal standard if available) at six equally spaced concentrations that span the anticipated calibration range. Label these samples Matrix-fortified calibration standards.

Replicates of these standards can be injected into an optimized instrument which has been determined using system suitability standards to be ready and suitable for analysis.

The results of the analysis from this sample set can be used to verify the estimated LODs and LOQs, the absolute and apparent recovery, the matrix effect and the linear regression function for quantitative analysis.

Once these data have been interpreted from the sample set and the system confirms all the characteristic operational parameters verified so far, the next stage will be to prepare a sample set to verify the selectivity, accuracy and precision of the method.

4. Use the described method to prepare negative control samples obtained from at least six independent geographical locations in the country in which the analysis is being conducted [This is to accommodate regional differences in veterinary practice].
5. Using negative control samples, independently weigh and fortify three samples at each of three concentrations spaced (together with internal standards if applicable) as follows wherever possible over the calibration range:

(i) 0.5 x MRL (ii) 1.0 x MRL and (iii) 1.5 x MRL
[The assumption here is that the drug is on the country's approved veterinary medicinal list]

6. Prepare an appropriate set of matrix-matched, neat chemical standard or matrix fortified calibration standards that bracket the sample concentrations prepared in 5.
7. Prepare an appropriate quality control sample and a negative control sample to be analysed under the same instrumental conditions as the samples prepared in 4, 5 and 6.
   After ensuring that the instrument has been qualified to be used for analysis, the samples prepared in 4-7 can all be analysed on the first day.
8. The sample sets prepared in 5, 6 and 7 can be freshly made and analysed on a 2nd day and then on a 3rd day and analysed.

The samples analysed in experiments 4, 5, 6, 7 and 8 will permit the estimation of the selectivity (false negative/false positive rates), within-day and between-day precision and accuracy, and measurement uncertainty.

9. In the absence of certified material, at least 15 samples must be prepared by the Quality Manager in triplicate using individually weighed samples at three concentrations over the calibration range of the analytical method. Negative control samples must be included in the sample set. The prepared samples must be labelled, randomized and provided blank to an experienced analyst for analysis.

Results from experiment 9 will provide some assurance that in the absence of incurred residue material as well as certified reference materials (CRMs), the method will be able to correctly and accurately identify and quantify the concentrations of the drug (s) in the prepared samples and demonstrate if it is fit-for-purpose.

Once the method has been validated and demonstrated to be fit-for-purpose, the next step is to transfer the method for use in routine analysis. At that point, other experienced analysts will follow their usual laboratory format for method transfer and method familiarization up to and until they are fully qualified as proficient to run the method independently.

Conclusions

When a fully developed and optimized method is subjected to the validation process, the time from the moment the idea is conceived to the completion of the validation can be very short. At validation, on the assumption that the instrument on which the analysis is to be conducted has been qualified to be suitable for use, and that all the critical control parameters that should be known of the method have been identified through a ruggedness study, then the minimum number of experiments that need to conducted in the laboratory by one analyst to complete the validation study for a single analyte method are labelled from 1-9 in the text above: *Performance Characteristic Parameter Evaluation - The Single Laboratory Validation Criteria*.

Since the essence for validating the method is to use it to support regulatory actions, the method has to be transferred to the routine diagnostic analysis laboratory where it can be implemented into the testing programme. In our laboratory, the beauty of this arrangement is that because the method development/method validation team operates in the same laboratory as the diagnostic staff, the characteristic parameters of the validated method can be further monitored while it is in the hands of the routine diagnostic laboratory.

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INVESTIGATIONS ON THE INFLUENCE OF HYDROLYSIS ON THE TOTAL AMOUNT OF MARKER RESIDUE AND CONSEQUENCES

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Abstract
The presented study focussed on (i) the investigation of the influence of enzymatic hydrolysis on the marker residue amount of selected veterinary drugs to be quantified in incurred materials and (ii) the consequences of the results in the control practice. The study showed that enzymatic hydrolysis often led to significantly increased amounts of residues compared to samples which had not been treated with these enzymes. Hence, dependent on the method applied for veterinary drug residue analysis, *e.g*. the assessment of sample compliance, the results of proficiency tests and the comparability of results for reference material are influenced. Therefore, harmonised procedures are required in order to guarantee comparability of analytical results of veterinary drug residues.

Introduction
As a consequence of animal drug treatment, residues of veterinary medicines can be found in various tissues or body fluids. Their maximum residue limits (MRLs) were established on the basis of studies on the acceptable daily intake for humans. To ensure human food safety, the MRLs for authorised substances, but also the potential misuse of prohibited or non-authorised substances, are subject to controls.

According to the rules governing medicinal products in the European Union (Volume 8), the adaption of the methods provided in the framework of the MRL procedure by official laboratories is not obligatory, but the authors assumed it to be beneficial [2]. However, the use of single-analyte methods is not practicable since an enormous amount of time would be required for validation and for residue control of the MRL compounds listed in Commission Regulation (EU) No 37/2010 [3] and of prohibited or non-authorised substances. Hence, the usual approach of the official residue control laboratories is the application of multi-methods, which simultaneously determine a wide range of residues of MRL compounds and furthermore of forbidden or non-authorised substances.

Residues can be present in samples as extractable or bound residues. Since routine methods normally not detect bound residues, this study is focussed on extractable residues. The extractable residue comprises the free drug as well as the metabolised drug, *e.g*. drug glucuronides or sulphates.

Difficulties in residue control may arise when it is not clear whether only free residues or, in addition, drug residue conjugates are determined by the analytical methods used in the drug registration process as it is the case for *e.g*. tolfenamic acid or vedaprofen. In terms of consumer protection, the analytical multi-methods applied for residue control should be characterised by high extraction efficiency for the free and the conjugated marker residues. As described in the reflection paper of the European Medicines Agency on the assessment of the bioavailability of bound residues in food commodities of animal origin, many different solvents and extraction conditions, *e.g*. pH, temperature and enzymes, can be applied to extract the free and the conjugated residues from tissues or biological fluids [4].

Since marker residues do not only occur in their free form, but can also be found conjugated or linked to cellular components, a hydrolysis step may influence the measurable analyte content of a sample. Hydrolysis of conjugates can happen spontaneously when *e.g*. the sample ages or under the influence of environment-related factors like temperature during handling of the sample and sample pre-treatment [1,5]. In many cases, method validation is conducted with spiked blank material due to the limited availability of incurred reference materials. Therefore, effects on analyte recovery in incurred material might be underestimated, if the extent to which a method determines conjugated residues is not clear. By means of practical experiments the influence of a systematic hydrolysis by enzymes on analyte recovery is shown in examples, the possible consequences are outlined and recommendations are given.

Experimental set-up and findings
In the past years in the EURL, different animal studies were conducted and several incurred milk samples were obtained from the treatment of cows with non-steroidal anti-inflammatory drugs (NSAIDs) and treatments of pigs with chloramphenicol (CAP). For the investigation of the effect of the enzymatic hydrolysis on these materials, β glucuronidase /arylsulfatase from *H*. *pomatia* was applied.

Investigations on non-steroidal anti-inflammatory drugs

The enzymes were added at the beginning of the incubation process for the samples with hydrolysis. The samples without hydrolysis were treated in the same way as the samples with hydrolysis, except that the enzymes were added after quenching the enzyme reaction by the addition of acetonitrile in order to get similar compositions of both sample extracts. The results are presented in Figure 1. The complete sample preparation procedure is described in the proceeding entitled "Confirmatory method for the determination of acidic and basic NSAIDs in milk by UPLC-MS/MS".

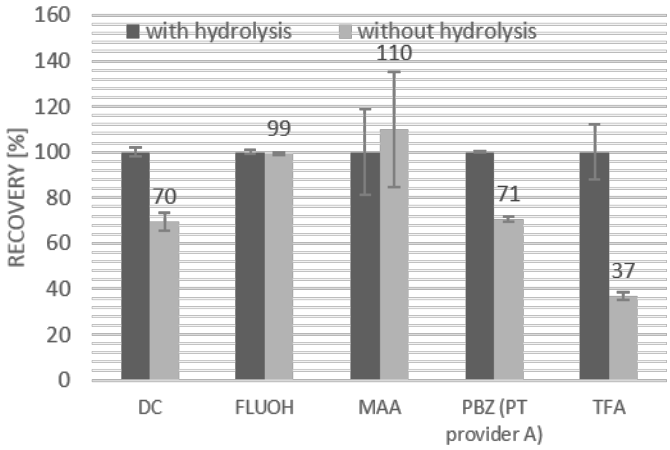

Figure 1: Recoveries of some selected NSAIDs in milk in laboratory test material and in a sample of a proficiency test (PT) of provider A, including the MRL compounds diclofenac (DC), 5-hydroxyflunixin (FLUOH), 4-methylaminoantipyrine (MAA) and tolfenamic acid (TFA) as well as phenylbutazone (PBZ), a compound with recommended concentration. The dark grey bars display the recoveries of samples with hydrolysis. The light grey bars show the recoveries of samples without hydrolysis. The recoveries of the samples with hydrolysis were set as 100%.

Conjugated residues contributed to the measurable analyte content in three of the incurred milk samples analysed. A significantly higher amount by a factor of 1.4 and 2.7 was observed for the MRL-compounds DC and TFA, respectively. Furthermore, the hydrolysis led to a 1.4-fold higher PBZ amount in a raw milk sample of a proficiency test.

Investigations on chloramphenicol

The enzymes were added at the beginning of the incubation process for the samples with hydrolysis. The samples without hydrolysis were treated in the same way as the samples with hydrolysis, except that no enzymes were added. The results are displayed in Figure 2.

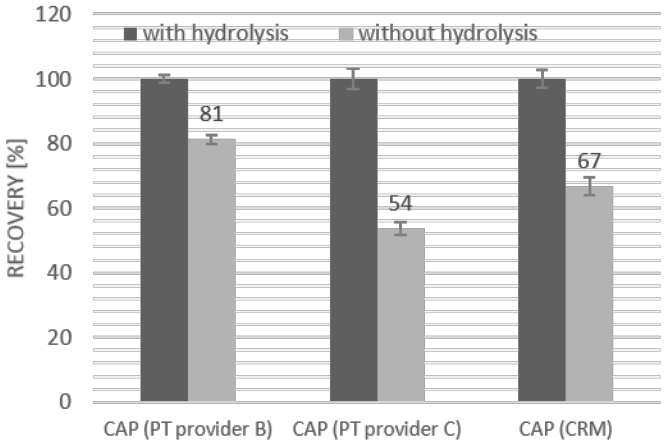

Figure 2: Recoveries of chloramphenicol (CAP) in samples from proficiency tests (PT) of provider B (pig muscle) and C (rabbit muscle) as well as in a certified pig muscle reference material (CRM). The dark grey bars display the recoveries of samples with hydrolysis. The light grey bars show the recoveries of samples without hydrolysis. The recoveries of the samples with hydrolysis were set as 100%.

A hydrolysis step included in the method always led to significantly increased CAP amounts for all muscle samples analysed. The muscle samples originated from different sources. Dependent of the material, the quantifiable amount was increased by a factor 1.2 to 1.9. Aspects like intra-species variations or different medication conditions can lead to changes in the metabolite pattern. Therefore, no general conclusions can be drawn from the given examples.

Discussion

Hydrolysis may lead to an increase of the quantifiable analyte amounts as proven for DC, PBZ, TFA and CAP in the incurred samples analysed. As a consequence, random or systematic hydrolysis could make a difference in the assessment of sample compliance. For compounds with reference points of action, samples are assed as compliant if the residue amount is below the limits laid down. Furthermore, for MRL compounds compliance is given when the quantified mass fractions are below the decision limit CCα. Assuming that a CAP content of 0.25 µg kg^{-1} was determined without the application of *H*. *pomatia* for enzymatic hydrolysis in a sample similar to the material of PT provider C, that sample would be assed as compliant. However, a CAP content of 0.46 µg kg^{-1} would be determined if an enzymatic hydrolysis step was included in the analysis of the same sample, so that the sample would not be compliant.

In proficiency tests (PTs), a possible higher uncertainty of the assigned value of a particular compound might even lead to non-exploitable results, and it might be impossible to evaluate the proficiency of the PT participants. There is also the risk for the participant of not passing the PT depending on how many of the other participants analyse the sample with or without hydrolysis.

A further consequence might be a deviation of own measurements from the assigned values of a reference material. The quantified analyte amount might be too low or too high, dependent on the methods (+/- hydrolysis) used for the determination of the assigned values. Similar to PTs, an unambiguous definition of the analyte is necessary for reference material.

Conclusions

In general, during method validation the effect of hydrolysis on the analyte amount determined in incurred material is not routinely checked, since usually spiked blank material is analysed in validation studies. However, it is recommended to include the analysis of incurred samples for representative analytes in the method validation process. This way the applicability of the method is determined, either for the free residue or for the sum of the extractable residues.

Investigations on potential contributions of extractable residues to the quantifiable analyte amount could be carried out by *e.g*. EURLs, PT/ RM providers and applicants for veterinary medicines to allow recommendations on methods to be used for official control, the measurement of PT samples or RM. Also inter-laboratory comparison with a free choice of analytical methods could be of interest if the evaluation of the data would not only cover the evaluation of z-scores but also would include an evaluation of potential bias caused by different methods or sample preparation steps.

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CHLORAMPHENICOL PROFICIENCY TESTS ON A GLOBAL SCALE – UNFORESEEN CONSEQUENCES

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Abstract
Although the use of chloramphenicol (CAP) as a veterinary medicine is banned in the EU and in many countries outside the EU, monitoring for its residue in food is routine. Positive detections are few but taken extremely seriously. Within the EU laboratories analysing for CAP should validate methods according to European Commission Decision 2002/657/EC and be accredited to ISO 17025, and will generally participate in proficiency testing (PT) schemes, such as those offered by FAPAS. The FAPAS PTs aim to cover a wide range of relevant matrices including honey, prawns, fish, milk and kidney. The test materials are prepared either by animal dosing studies or spiking raw matrix. The most common method reported by FAPAS participants used to screen for CAP residues is LC-MS/MS but ELISA kits are increasingly being used. A recent PT highlighted that the result obtained might be correlated to the type of analytical method being employed. Follow-up investigations have demonstrated that some of these variations in data are a function of the different stereo-isomeric forms of CAP. This paper will discuss the implication of this research on method validation requirements and EU legislation.

Introduction
Residues of CAP still occur in a variety of animal derived products, despite its banned use in the EU and elsewhere. Hence, its analysis is routine across the globe to ensure that international trade regulations are adhered to. Laboratories carrying out the analysis need to ensure that their own quality assurance (EU) is of a very high standard. Generally, this takes the form of accreditation (ISOa) to ISO 17025 but even laboratories without accreditation still need to demonstrate quality assurance to their stakeholders. The most common and effective demonstration of quality assurance is taking part in an external proficiency testing scheme.

The principal advantage of a proficiency testing (proficiency test, PT) scheme is that it provides a characterised reference sample external to the participants' control. Internal quality control measures provide a measure of repeatability precision but not of bias. It follows that the preparation and characterisation of the test material by the PT provider has to follow an equally rigid protocol (Thompson *et al*.) and/or accreditation (ISOb). The timescale of the PT process dictates that the test material preparation occurs several months before the planned start of the PT. In the case of veterinary medicine residues PT, this may take the form of an animal dosing study or spiking of the analyte into the test matrix. Either type of preparation requires the use of a reference standard of the medicine in question from a traceable and reputable source.

The PT itself involves the distribution of identical units of test sample to the participants at the same time. The definition of 'identical' means that each unit is an aliquot of the bulk test sample and that between-unit variation will not affect the outcome of the PT. The PT provider is required to demonstrate sufficient homogeneity (Thompson *et al*.; ISOb) before the PT commences, using an approved testing facility. Participants then have a time period typically of a few weeks in which to analyse the PT sample and return the results. In most cases, the PT provider does not dictate which method participants should use. In fact, the purpose of PT is to demonstrate laboratory performance to the established routine methods and procedures. It is helpful, but not obligatory, if participants also submit details of the method that they have used. Method dependency is occasionally observed (Sykes *et al.,* 2011) but, in general, PT results fit a normal distribution within pre-defined acceptance criteria.

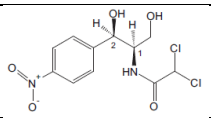
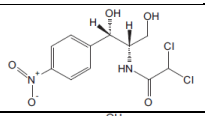
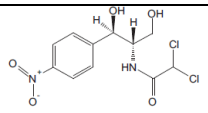
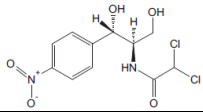
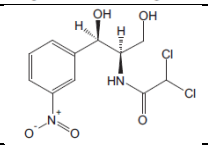
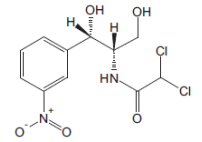
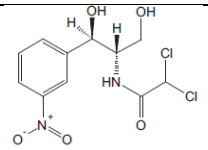
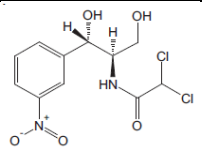
This paper provides a recent example of a CAP PT that did not fit the expected normal distribution. We provide evidence of the cause of the observed distribution and suggest a discussion topic for the future direction of veterinary medicines regulation.

Results of PT 02256
The PT in question was FAPAS PT 02256 (FAPAS), which took place in February-March 2015. The test material was prepared from prawn flesh that had been minced in bulk and screened for the presence of any incurred CAP. No CAP was detected and the material was spiked with a commercially-available reference solution of CAP at an intended concentration of 0.5 µg kg^{-1}. Homogeneity testing was carried out using an accredited method with LC-MS/MS determination. The sample was shown to be sufficiently homogeneous with a mean value of 0.47 µg kg^{-1}. Participants were instructed to analyse the sample according to their routine method, to report the recovery-corrected result together with the method of recovery correction and the CCβ (or LOQ if CCβ was not estimated) (EU; ISOc).

The distribution of z-scores in a PT would normally be symmetrical about the assigned value (corresponding to a z-score of zero). A laboratory performing normally would expect to receive a z-score between -2 and +2. A laboratory not performing normally would receive a z-score outside ±3. A |z|-score in-between 2 and 3 might indicate a problem or might be a statistical anomaly. In PT 02256, 84 laboratories submitted results. The unusual observation here is the large number of laboratories receiving a z-score of about -4.5. This indicates that they have been unable to detect the analyte (or quantified it at an extremely low level).

The set of extreme low z-scores was investigated for any trends according to the method used by the participants. The majority of participants in the PT as a whole used LC-MS/MS. The set of extreme low z-scores corresponded directly with those participants (10 laboratories) using ELISA. One participant contacted FAPAS with the observation that, within their laboratory, CAP could be detected by LC-MS/MS but not by ELISA.

Table 1. Stereoisomers of CAP.

Configuration	Stereoisomer	Structure	Common name	Biological activity	Antimicrobial activity
para	*RR-p*-CAP		chloramphenicol or levomycetin	yes	yes
	SS-p-CAP		dextramycin	yes	no
	RS-p-CAP		synthomycin	yes	no
	SR-p-CAP		synthomycin	yes	no
meta	*RR-m*-CAP		none	no	no
	SS-m-CAP		none	no	no
	RS-m-CAP		none	no	no
	SR-m-CAP		none	no	no

Investigation of anomaly

A number of ELISA kit manufacturers were represented in the set of ELISA results, one of which was R-Biopharm. Their investigation centred on the apparent failure of their ELISA kit (RIDASCREEN® Chloramphenicol) in this PT. The investigation involved a number of test samples and CAP reference standards, including the same lot number of standard that had been used to prepare the FAPAS PT 02256 material.

The initial experiment was to analyse two aliquots of the PT sample using the ELISA kit. A third aliquot of the test material was over-spiked (0.1 µg kg^{-1}) with a CAP standard. CAP was not detected in the two non-spiked aliquots but the over-spiked aliquot did have CAP detected in it (0.095 µg kg^{-1}). This result raised the hypothesis that, because ELISA tests are stereo-selective, a different stereoisomer of CAP was used in the preparation of the PT 02256 material. CAP has 8 possible stereoisomers,

of which only one (*RR*-*p*-CAP) is antimicrobially active (Table 1). The R-Biopharm ELISA kit is specific to *RR*-*p*-CAP. (*NB*. CAP has the CAS Number 56-75-7 which does not distinguish stereo-isomeric properties.)

Two different analytical standards of CAP were procured from Sigma-Aldrich (different product codes). The first standard (CAP STD1) was the standard material as used in the RIDASCREEN® Chloramphenicol ELISA kit. The second standard (CAP STD2) was the same make and lot number as that used to prepare the FAPAS PT 02256 material. The ELISA kit buffer was spiked at 0, 0.1, 0.5 µg kg^{-1} with CAP STD1 and at 0.1, 0.5, 1.0 µg kg^{-1} with CAP STD2. The results are shown in Table 2. CAP STD2 was not detected at any spiking level, whereas CAP STD1 was detected at the expected levels.

Characterisation of CAP standards

The two CAP standards were subjected to a number of characterisation studies. The techniques applied to the study were mass spectrometry, NMR, microbial inhibition, chiral liquid chromatography (with MS/MS detection) and immuno-affinity chromatography.

The mass and NMR spectra were essentially identical for the two standards. The microbial inhibition test measured the biological activity of *Escherichia coli* and *Staphylococcus aureus* at 0, 1, 2, 4, 8 and 16 µg mL^{-1} CAP for two lot numbers of CAP STD1 and the one lot number of CAP STD2. Both lots of CAP STD1 increasingly suppressed biological activity of both organisms at increasing concentrations of CAP. CAP STD2 failed to suppress biological activity at any concentration.

The chiral chromatography analysis ran the two CAP standards against references of *RR*-*p*-CAP, *SS*-*p*-CAP, *RS*/*SR*-*p*-isomer, *RR*/*SS*-*m*-isomer and *RS*/*SR*-*m*-isomer. The CAP STD1 was determined to be *RR*-*p*-CAP. The CAP STD2 was determined to be *SS*-*p*-CAP. Neither CAP standard contained any detectable amounts of the other isomers.

The observed bias of the stereoisomers on the ELISA tests was verified by employing a simple immuno-affinity chromatography test. Two samples spiked with the different CAP standards and blank water (control) were applied to EASI-EXTRACT® Chloramphenicol immuno-affinity columns (in triplicate). After washing and elution, each sample was injected onto HPLC for analysis. CAP STD1 was recovered with a mean value of 92%. CAP STD2 had no observable recovery.

Table 2. RIDASCREEN® CAP ELISA test results for spiking experiment with two different CAP standards at multiple levels

Sample	Sample treatment	Result [µg kg^{-1}]
FAPAS Sample 02256	Spiked with CAP STD1 0.1 µg kg^{-1}	0.095
Buffer	Blank	< LOD
Buffer	Spiked with CAP STD2 0.1 µg kg^{-1}	< LOD
Buffer	Spiked with CAP STD2 0.5 µg kg^{-1}	< LOD
Buffer	Spiked with CAP STD2 1.0 µg kg^{-1}	< LOD
Buffer	Spiked with CAP STD1 0.1 µg kg^{-1}	0.084
Buffer	Spiked with CAP STD1 0.5 µg kg^{-1}	0.54

Discussion

There is a demonstrably observable distinction between two reference standards, both pertaining to be CAP, referenced by the same CAS number. Simple experiments have shown that there is a stereo-isomeric dependency on antimicrobial activity and immuno-affinity determination. The stereo-isomeric forms are not distinguishable by conventional LC-MS/MS or NMR. If LC-MS/MS is employed, only a chiral separation will distinguish the isomeric forms. The majority of participants in a proficiency test (and, by extension, in routine analysis) use conventional LC-MS/MS for CAP analysis. These laboratories will be unaware of whether detected CAP residues actually had any anti-microbial activity *in vivo*.

There are obvious commercial implications for the supply of reference standards. It is not the purpose of this paper to discuss these implications but it is worth noting that the CAP STD2 has been withdrawn from supply.

The point of discussion, therefore, is what regulation exists to define stereo-isomeric forms for veterinary medicine application and their appropriate analysis. Commission Decision 2002/657/2002 (EU) does specifically mention the need to demonstrate specificity of closely related substances (including isomers, metabolites, degradation products, endogenous substances, matrix constituents). The current Commission view (personal communication 1), however, is that this is not a requirement for CAP. This is evidenced by the fact that none of the LC-MS/MS laboratories in the FAPAS PT employed separations using a chiral chromatography column. In this regard, the proficiency test was valid in terms of allowing a z-score to be calculated although it unintentionally disadvantaged participants using ELISA kits. The fact that ELISA kits could not detect the analyte verifies the issue.

From a scientific and regulatory perspective, there is a clear need to open up discussion on the differentiation of stereoisomers. This is in terms of both the desired biological activity and the analysis of their residues in the human food chain.

NB. Another ELISA kit manufacturer carried out a similar investigation with complementary results (data not shown or discussed here, personal communication 2).

Conclusion

The comprehensive investigation provides a plausible explanation for the observation in participants' data for FAPAS PT 02256. No other FAPAS PTs for CAP have suffered the same observation and the preparation of CAP test materials now includes an immuno-affinity check. There is a need to open debate to address the potential impact of incompletely specified stereoisomers in veterinary medicine residues analysis.

Acknowledgement

The chiral chromatography analysis was performed by RIKILT Wageningen UR.

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SAMPLE-BASED REPORTING OF OFFICIAL NATIONAL CONTROL OF VETERINARY DRUG RESIDUES

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Abstract
Data collection is an essential prerequisite for assessing compliance of chemical residues in food and for risk assessment. The present system for collecting aggregated data of residues of veterinary medicinal products and other substances in animals and animal products has limitations for risk assessment as well as risk management.
The European Food Safety Authority has been assigned with the task to set up a system for data collection based on individual analytical results. A pilot project has been launched with participants from eleven Member States for parallel reporting of monitoring results from 2015 in aggregated form as well as individual analytical results using a standardised data model. The challenges that face the pilot participants include provisions for categorised sample information, specific method performance data, result evaluation and follow-up actions. Experience gained through the reporting of monitoring data from Denmark will be presented.

Introduction
Presently, all Member States (MSs) of the European Union (EU) are obliged to report their monitoring results for veterinary drug residues and other substances in live animals and animal products according to Council Directive 96/23/EC (EC 1996) through the Sanco Residue web based application for monitoring plans and results (EC 2009). This allows MSs to report on a yearly basis results from samples analysed for the official control according to the directive.

However, samples are grouped in very broad categories according to the directive: *bovine*, pigs, horses, sheep/goats, poultry, aquaculture, milk, eggs, rabbit, farmed game, wild game and honey. Sampling points can be reported as 'farm' or 'slaughterhouse' where relevant and sampling strategy can be reported as 'target' or 'suspect'. The two remaining sampling groups are 'import' and 'others'.

Also residue substances are grouped in broad categories as defined in the directive. These are six groups of substances having anabolic effect and unauthorized substances (stilbenes and stilbene derivatives; antithyroid agents; steroids; resorcylic acid lactones; β-agonists; compounds included in Annex IV to Council Regulation 2377/90), seven groups of veterinary drugs (antibacterial substances; anthelmintics; anticoccidials; carbamates and pyrethroids; sedatives; non-steroidal anti-inflammatory drugs; other pharmacologically active substances) and six groups of contaminants and other substances (organochlorine compounds including PCBs; organophosphorus compounds; chemical elements; mycotoxins; dyes; others). Individual substances are only reported when non-compliant results have been found, and no concentration levels are reported.

The European Food Safety Authority (EFSA) has since the 2008 data collection assisted the European Commission with compiling and publishing a technical report on the occurrence of residues of veterinary drugs and other substances monitored according to the directive (EFSA 2010a).

Due to the limited level of details in reporting positive findings, the reports have been focusing on risk handling aspects of the monitoring, *i.e.* the number of samples in comparison to the National Residue Control Plans that MSs also report to the same web based application, and the frequencies of non-compliant results and/or samples for the different broad sampling groups.

A number of limitations due to the reporting practices have been pointed out in the EFSA reports. The information on sample identification, sample matrix and the corresponding results is not available and thus it is impossible to perform a more elaborate statistical analysis at the matrix level (meat, liver, blood etc.) and to identify the samples non-compliant for more substances (multi-residues samples). Neither is information on the occurrence of veterinary medicinal product residues (VMPR) at levels below MRLs available (EFSA 2010b, EFSA 2015a).

Collection of sample-based data would allow a more elaborate data analysis and would enable the Commission, MSs and EFSA to answer additional questions in relation to VMPR monitoring results.

Pesticides have also been reported to the Commission in aggregated form, but in 2009 EFSA set up a pilot project for sample-based reporting of pesticide residues using a preliminary version of what is now known as the EFSA Standard Sample Description. A revised Guideline was published in late 2009 aimed at the reporting of chemical contaminants and pesticide residues (EFSA 2010c).

In 2013, EFSA published the Guidance on Standard Sample Description version 2.0 (SSD2) (EFSA 2013a) with the purpose of unifying all data collections in a single data reporting model and to a common list of harmonised controlled terminologies. The SSD2 is designed to harmonise the transmission of data from data providers to EFSA covering several data collection domains, *i.e*. food additive and chemical contaminants occurrence data, pesticide residues, and residues of veterinary medicinal products as well as zoonoses, antimicrobial resistance and food-borne outbreak data.

SSD2 is complemented by the Guidance on Data Exchange version 2 (EFSA 2014), which supports SSD2 with harmonised technical specifications about transmission requirements, metadata and general business rules definition.

The National Food Institute at the Technical University of Denmark has been involved in several EFSA pilot projects on data collection using SSD for structuring and transmission of sample-based results from the official national control and monitoring programs under the auspices of the Danish Veterinary and Food Administration (DVFA), which is the national authority for risk management of food. Presently the institute, in cooperation with DVFA, is engaged in the EFSA pilot project on the implementation of SSD2 for reporting residues of veterinary medicinal products for the 2015 monitoring programmes according to Council Directive 96/23/EC (EFSA 2015b).

Materials and Methods

Laboratories

Samples for the official control of residues of veterinary medicinal products in Denmark are almost exclusively analysed at the DVFA control laboratory in Ringsted, Denmark. A few types of analyses are performed at The National Food Institute in Mørkhøj, Denmark, either where the relevant methods have not yet been implemented at the control laboratory or as part of a technical assistance. In addition, a few agreements exist for confirmatory analysis at laboratories outside Denmark.

Analyses for some non-drug substances included in the Directive 96/23 monitoring are performed at the DVFA laboratory in Aarhus, Denmark.

In all cases, the DVFA laboratories are responsible for both sampling and result registration. Consequently, information on sampling and analysis is collected in the same laboratory information system (LIMS) (LabVantage® LIMS 6.0). The LIMS is integrated in the DVFA data warehouse (DW) (Microsoft SQL Server®). The DVFA DW also includes information from other administrative systems such as registers of controlled establishments and inspections databases.

Data extraction

During the previous SSD pilot projects on data transformation and transmission to EFSA, an automated, dedicated extraction procedure has been set up to extract specific data elements from the DVFA DW. Based on a list of programme identifiers these data are transmitted to an Oracle database at the National Food Institute when relevant.

In order to cater for the requirements of the VMPR domain, the previously identified data elements have been expanded with a few elements, including decision limit (CCα) and detection capability (CCβ) (EC 2002).

The extracted data include elements for transformation to the SSD data model as well as supplementary elements for *e.g.* validation of registrations.

Table 1. Example of SSD data definitions and associated transformation columns.

SSD2 Element code	SSD2 Element label	Type	DTU	LIMS	Catalogue	Lookup column
sampCountry	Country of sampling	String(2)	Constant	DK		
sampID	Sample ID	String(100)	Copy	Proeve_ID		
sampArea	Area of sampling	String(5)	[a)]			
sampY	Year of sampling	Integer	Function	year(Dato_udtaget)		
origCountry	Country of origin	String(2)	Lookup	Oprindelse_land	EFSAcountry	countryCode
anMethRefId	Analytical method	String(50)	Copy	Testmetode_ID		
anMethType	Analytical method type	String(5)	Lookup	Testmetode_ID	EFSAmethod	ANLYTYP
anMethCode	Analytical method code	String(5)	Lookup	Testmetode_ID	EFSAmethod	ANLYMD

[a)] *empty*

Transformation

The core functionality has been built around a configurable transformation system based on the Microsoft Excel® version of the SSD2 data definitions (EFSA 2013b). These tables are compiled by a suit of SAS procedures (SAS® Enterprise Guide 6.1; SAS® 9.4) into a program that transforms the extract from the DVFA DW to a SSD2 compatible SAS dataset. The process includes several types of transformation as exemplified in Table 1. The different transformation types are described in Table 2.

The lookup facility is used both for simple translation of national terms (*e.g.* country of origin of the sample) to SSD2 terms/codes but also to facilitate reporting of certain types of information that is not readily available from the data warehouse, such as adding information on method type (screening/confirmation) and method principle (ELISA/LC-MSMS *etc.*).

Some types of information need further processing, *e.g*. result evaluations, follow-up actions and conclusions or handling of analytical substances (parameter codes) for complex residue definitions, like albendazole: "Sum of albendazole sulphoxide, albendazole sulphone, and albendazole 2-amino sulphone, expressed as albendazole".

Table 2. Transformation of data from the data warehouse of the Danish Veterinary and Food Administration to the EFSA Standard Sample Description 2.0.

Transformation type	Explanation
Constant	Value in column LIMS is inserted into the SSD2 Element
Copy	Value of the LIMS element named in column LIMS is inserted into the SSD2 Element
<empty>	Element not included in SSD2 file
Function	Function output of value of the LIMS element named in column LIMS is inserted into the SSD2 Element
Lookup	Value of LIMS element in column LIMS is used as a key for lookup in the translation catalogue named in column Catalogue.

Data validation
Technical validations according to relevant business rules are performed by SAS procedures. Scientific and administrative reviews are performed by specialists at the National Food Institute and/or DVFA laboratories.

XML formatting
The final validated SAS dataset is written as a XML formatted text file (Extensible Markup Language (W3C 2004)) using a dedicated SAS procedure that minimizes the file size by excluding all empty elements.

Transmission
The XML file is manually uploaded to the designated EFSA web page (Data Collection Framework). Following the upload an automatic technical validation will be performed by EFSA before the file is accepted for further processing at EFSA.

Results and Discussion

Challenges in VMPR reporting
The fact that the monitoring activities for official food control in Denmark has been centralised to one administration (the Danish Veterinary and Food Administration) and that the relevant information exists in a centralised data warehouse has simplified the implementation of the system for reporting of VMPR since the major part of the necessary infrastructure was already in place.

Legal limits and evaluations. However, each chemical domain has its special requirements for information; the VMPR domain has several requirements in common with the pesticide domain. In both domains a legal requirement for an annual report from a central European authority exists, and these reports must include information on residues in relation to legally accepted residue levels.

Consequently, there is a need for a detailed reporting of the legal limit that has been the basis for the subsequent evaluation of the result, the administrative actions that have followed for samples evaluated as non-compliant, and also the conclusions drawn from follow-up investigations of non-compliant or suspect samples.

This information is not in all cases directly available in machine readable form from the data warehouse and may need expert judgement from trained personnel. For this reason, all results above the reported limit values and results from suspect samples are extracted to an Excel file and circulated to the responsible person(s) at DVFA who will then correct/supplement information on evaluation, actions taken and conclusions of follow-up investigations. Subsequently this information will be loaded back into the SSD2 structured file before reporting to EFSA.

Multi methods. In many cases the analytical programmes are implemented using multi methods. In most cases only results for the detected substances are reported for these methods or information indicating that the method has been applied without detections. The prescriptions of SSD2 require that an individual result is reported for each substance included in the method scope. This is also the case for screening methods. Consequently, the report lines must be expanded, using information from a method/substance catalogue that is setup in corporation with the laboratory.

For detected substances analytical limits (limits of detection (LOD), quantification (LOQ) and decision (CCα) and detection capability (CCβ)) can be reported in LIMS together the measured result. For substances not reported – or only implicitly reported by the reported method, information on analytical limits must be supplied from the method/substance catalogue. The situation is very much the same in the pesticide domain, and experience and implementations from that domain have been drawn upon for the reporting of VMPRs.

Screening methods. Screening methods are applied and reported for several substance groups. In case of positive screening results a confirmatory quantitative analysis must be performed subsequently and reported in the LIMS. However, double reporting cannot be transmitted in the SSD2 data model, so whenever results from a confirmatory method has been reported for a sample together with a screening result for the same substance, the screening result must be deleted before reporting in the SSD2 data model. Such functionality has already been implemented to cater for the equivalent situation in the additives domain.

Future challenges

The EFSA SSD2 data model for VMPRs has been designed to fulfil the present requirements for reporting according to Directive 96/23. However, the data model will be able to accept more detailed information on the analyses performed, should this be required. Such detailed reporting could be relevant for both risk assessment and risk management, adding value for stakeholders in this field.

Matrix analysed. Today the requirements for detailed reporting of the type of matrix analysed is very limited in relation to result reporting, although the National Residue Control Plans, required by the Directive, often shows more details. The SSD2 data model will allow for a detailed description of the samples taken, both in terms of describing the animal (sex, age *etc.*) as well as the type of matrix analysed (muscle, urine *etc.*). Traditionally, in Denmark, details on these matters have only been reported in a textual file on follow-up measures. A change from reporting '*Bovine*' and details on *e.g.* sex and age in free text (which is not in good keeping with the SSD2 data model) to a categorised form via the LIMS product catalogue would be a limited effort for future reporting, thus making this information available for assessments at EFSA or the EU Reference Laboratories.

Method capabilities. Today's possibilities of using advanced instrumental analyses like liquid-chromatography coupled with mass-spectrometric detection have lead the DVFA laboratories to abandon the traditional microbiological and some of the biochemical screening methods, replacing these with chemical instrumental analyses. These techniques may still be used as screening methods, meaning that analytical efforts are concentrated on samples with residues close to or above the legal limits. Utilising the full potential of these methods to determine residues well below the legal limits would certainly provide more information to assess the impact of residues in products of animal products, but might inflict an increased cost due to a higher rate of necessary quantifications and maybe initially also additional validation.

Conclusions

A system for transformation and transmission of sample based reporting of individual analytical results using a standardised data model has been designed and partially implemented. The existing system for transformation of analytical results from chemical occurrences and pesticide residues is foreseen to be able to cope with the reporting of results from monitoring of residues of veterinary medicinal products with only minor adjustments. Apart from a necessary updating of catalogues to include relevant sample descriptions, methods and substances, a major challenge will be the handling of legal limits and evaluations which might require some manual interaction.

The data model will allow a more detailed description of samples and results than the present reporting of aggregated data, which will improve the usefulness of the data collection for risk management and risk assessment.

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O21
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APPLICATION OF LC-QTOF TECHNOLOGY FOR SCREENING FOR HORMONALLY ACTIVE SUBSTANCES IN MATRICES OF ANIMAL ORIGIN

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Abstract

Hormonally active substances are banned in the European Union (EU) for use in food-producing animals (Directive 96/22/EC) due to health concerns. Substances such as stilbenes, thyreostats, steroids, corticosteroids and resorcylic acid lactones may be misused as growth-promoters. Within the mandatory residue control system LC-MS/MS-based confirmatory methods are routinely used to screen for a predefined analyte spectrum. These targeted approaches, however, are not suited to detect substances not included in the analyte spectrum (*e.g*. unknown newly designed growth promoters). To meet this challenge, we used LC-QTOF technology to record both high resolution full scan MS and MS/MS data with high mass accuracy. A database with accurate masses and retention times of all relevant stilbenes, thyreostats, steroids, corticosteroids and resorcylic acid lactones including relevant metabolites was established. In addition, a PCDL library (personal compound database and library) containing high resolution spectra of three different collision energies (10, 20, 40 eV) of the analytes and relevant metabolites was established. Screening was realised by measuring the samples in the auto MS/MS mode. In combination with the established libraries the QTOF technology allows for the screening of a package of known hormonally active substances. Additionally, it offers the possibility to detect unknown (new) substances or substances which are not in the target analyte spectrum of an analytical method.

Introduction

Based on their growth-promoting effects some hormonally active substances like *e.g*. diethylstilbestrol or anabolic steroids were used extensively in the past in food-producing animals. In the European Union (EU), hormonally active substances were banned for use in food-producing animals already a long time ago by Council Directive 96/22/EC. It was assumed that residues of this substances in edible tissues may cause harmful effects on human health. In contrast, in some countries like *e.g*. the United States and Canada the use of some hormonally active substances such as trenbolone, zeranol, testosterone, estradiol is officially allowed and common practice in cattle fattening.

To control the ban of hormonally active substances in food-producing animals within the EU, residue control is mandatory as stipulated by Regulation (EC) No. 882/2004 and Council Directive 96/23/EC. In the EU, annual national control plans in each member state are established. More specifically, the residue control on stilbenes, thyreostats, steroids (androgens, estrogens, gestagens), corticosteroids and resorcylic acid lactones in the relevant matrices of animal origin is mandatory.

To this end, mostly LC-MS/MS-based confirmatory methods are routinely used to analyse a predefined analyte spectrum in matrices of animal origin, usually in blood, urine, hair, muscle, kidney or liver. These targeted LC-MS/MS-based confirmatory methods allow for the sensitive screening and confirmation of hormonally active substances in matrices of animal origin down to the 1 ng g^{-1} level or even lower, depending on the analyte. On the other hand, these targeted approaches are not suited to detect potentially relevant substances beyond a predefined analyte spectrum of an LC-MS/MS method. Indeed, there are numerous growth-promoting hormonally active substances currently not covered by the targeted approaches of the residue control programs. In this respect, *e.g*. the selective androgen receptor modulators (SARMS) might be relevant due to their selective growth-promoting effects. Furthermore, newly designed steroids developed for improved performance in competitive sports might also have impact in residue control and should therefore also be considered.

Recently, LC-QToF-MS technology was applied successfully in systematic toxicological analysis (Broecker *et al*. 2011). The identification of a variety of chemically different toxic substances in human biological samples was achieved by means of spectra libraries using the accurate mass of precursor and specific fragments in combination with isotope distribution pattern and retention times. The use of spectra libraries allows for the screening of a lot of different substances within one experiment and it therefore holds great promise also for the residue control of veterinary drugs.

In order to overcome the limitations of the targeted LC-MS/MS approaches and to extend the analytic spectrum to further growth-promoting agents, we were prompted to create a spectra library containing the data of more than 80 hormonally active substances. To this end, high-resolution and accurate mass spectrometry as realised by Q-TOF-technology was employed. Accurate mass spectra of more than 80 hormonally active substances were measured at different collision energies. The data were stored in a personal compound database and library (PCDL). In combination with full scan data and data-dependent MS/MS experiments, this PCDL library serves as a powerful tool for the identification of hormonally active substances in matrices of animal origin in an untargeted way.

Materials and Methods

Instrumentation and Software

All measurements were performed using a 6550 accurate-mass Q-TOF LC-MS instrument (Agilent Technologies, Santa Clara, USA). The Agilent 1200 SL series HPLC consisted of a degasser, a thermostated HiP-ALS autosampler, a Bin Pump SL binary pump and a TCC SL column oven. The QTOF was operated with an electrospray ion source ESI Agilent Jet Stream Technology in positive and negative ionization mode, using a quadrupole for isolation of precursor ions in MS/MS mode, a linear hexapole collision cell with nitrogen as collision gas and collision energies from 0 to 40 eV.

Measurement of library spectra

For measurement of the CID mass spectra solutions of the analytes in a concentration of 0.1 µg mL^{-1} were prepared in methanol/water (50/50, v/v). The spectra were recorded in the "targeted MS/MS" mode using flow injection of 1 ng of each substance. The flow rate was 0.1 mL min^{-1} and the composition of the mobile phase is a 1:1 mixture of eluent A (5 mM ammoniumformiate / methanol (90/10, v/v)) and eluent B (0.1% formic acid in methanol). The protonated or deprotonated molecules $[M+H]^+$ or $[M-H]^-$ were selected by the quadrupole with a mass resolution of 1.3 *m/z*. Three MS/MS spectra were generated in a product –ion-scan mode ("targeted MS/MS") at collisions energies of usually 10, 20 and 40 eV. If no significant fragmentation was observed at 40 eV, higher collision energies of 40, 50 and 60 eV were applied. The following QToF conditions were applied: gas temperature 150°C, gas flow 18 L min^{-1}, nebuliser pressure 30 psi, sheat gas temperature 350°C, sheath gas flow 12 L min^{-1}, Vcap voltage 3,500 V, nozzle voltage: 500 V, fragmentor voltage 150 V, mass ranges 100-1100 *m/z* in MS-mode and 50-800 *m/z* in the MS/MS mode. A scan rate of 6 Hz for MS and MS/MS experiments was used. The reference ions for mass calibration were: 121.050873 $[M+H]^+$ and 922.009798 $[M+H]^+$ in the positive ESI mode and 112.985587 $[M+H]^-$ and 1033.988109 $[M-H]^-$ in the negative ESI mode.

HPLC-Method and data-dependent acquisition

A Zorbax Eclipse Plus C18 column (3.5 µm, 2.1 x 150 mm) from Agilent was used applying a flow rate of 0.5 mL min^{-1}. The eluents consisted of A: 5 mM ammoniumformiate / methanol (90/10, v/v) and B: 0.1 % formic acid in methanol. A linear gradient starting from 5 % to 95 % eluent B within 17 min was applied.

Full scan data were acquired in the "auto-MS/MS"- mode using a preferred list and a cycle time of 0.8 s. This cycle time consists of the measurement in the MS mode for 0.2 s and the following three MS/MS experiments, in which a precursor is fragmented. An active exclusion of the precursor after one spectrum for 0.05 min was carried out. The same QTof conditions as for the recording of the spectra was used (see above).

Results and Discussion

Collision-induced accurate mass spectra library

The library of spectra (PCDL-library) is designed for the qualitative analysis of hormonally active substances which are supposed to have a misuse potential in cattle fattening. Spectra were recorded for more than 80 substances, such as steroids (androgens, estrogens, gestagens), steroid esters, corticosteroids, thyreostats, stilbens, resorcylic acid lactones and specific androgen receptor modulators (SARMS). The measurement of the collision-induced- dissociation (CID) spectra was performed by flow injection of 1 ng of each substance in methanol/water (1/1, v/v) at a flow rate of 0.1 mL min^{-1} of a 1/1 mixture of eluent A and B. The pure standard substances were used for the measurement of the spectra. Possible impurities were excluded by the quadrupole by using a mass window of Δ *m/z* = 1.3. Therefore, a chromatographic separation was not necessary. The monoisotopic ions $[M+H]^+$ and $[M+H]^-$ theoretically calculated from the molecular formula were selected by the quadrupole and then fragmented by CID. Nitrogen was used as a collision gas in the collision cell. Collision energies of 0, 10, 20, and 40 eV were applied. For the substance peak between 40 and 50 spectra per collision energy were merged with 1,230 transients per spectrum. For background correction the spectra in front of and after the peak were subtracted. For the PCDL-library mainly the spectra acquired at collision energies of 10, 20 and 40 eV were used and only masses with an intensity above 100 counts were stored. The ions found in each spectrum were identified by their chemical formula. The exact mass was calculated and the measured mass was replaced by the calculated mass while the relative abundance of each ion was maintained. In this way an accurate mass library was established which may serve as a tool for substance identification by routinely comparing fragment ions in real samples with the expected ions.

As an example the three spectra obtained for the compound 17α-nortestosterone are depicted in Figure 1. Whereas the $[M+H]^+$ (*m/z* = 275.20056) is the highest peak at 10 eV, it decreases at 20 eV and is not seen at 40 eV where an increasing number and intensity of fragment ions is visible (Figure 1). The degree of fragmentation at the three different CID energies was depended on the chemical nature of the substance. *E.g*. for 16β-OH-stanozolol the protonated molecule peak still dominated even at 40 eV and a significant fragmentation is only observed at higher collision energies of 50 and 60 eV (data not shown). In these cases, also spectra recorded with higher collision energies were measured and stored in the PCDL library. So

far, the spectra of approximately 80 hormonally active substances and selected metabolites were measured, evaluated and subsequently assigned to the respective substance within the PCDL library. The arrangement of the library and database of hormonally active substances is not finished yet. Steady extension by addition of further compounds is in progress.

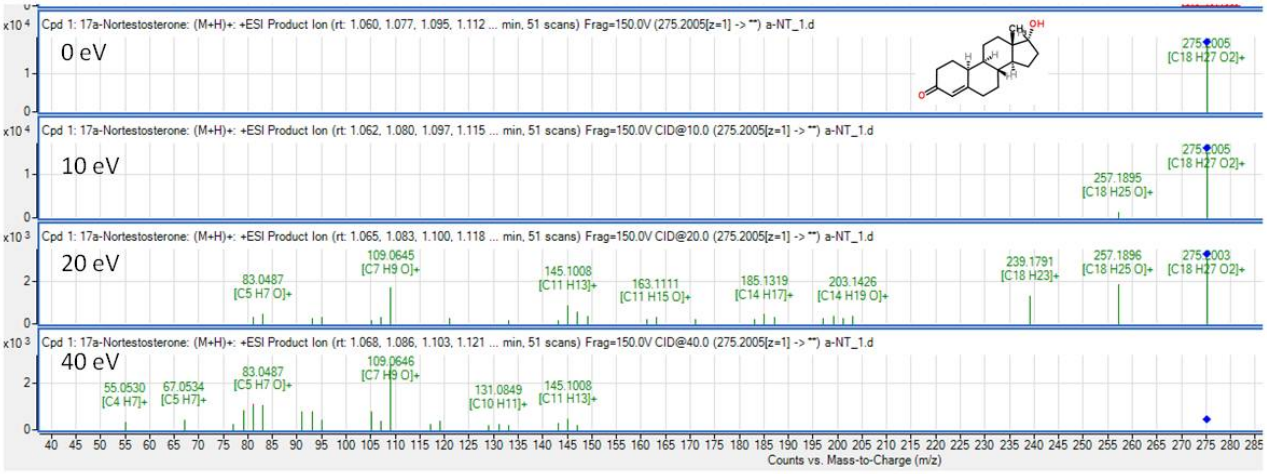

Figure 1. Accurate mass spectra of 17a-Nortestosterone at CID energies of 10, 20 and 40 eV as stored in the PCDL library.

Data-dependent acquisition (auto-MS/MS mode) – method development

To detect as many hormonally active substances within a single HPLC run an HPLC-method was established which allows both the detection of very hydrophilic substances (*e.g*. thyreostats) and very lipophilic substances (*e.g*. steroid esters). Furthermore, the methods showed a sufficient chromatographic separation of the substances belonging to the same substance group (*e.g*. steroid esters). The best results were obtained by using a Zorbax Eclipse Plus C18 column (3.5 µm, 2.1 x 150 mm). The eluents were A: 5 mM ammoniumformiate / methanol (90/10, v/v) and B: 0.1% formic acid in methanol. The flow rate was 0.5 mL min^{-1} and a linear gradient starting from 0% to 95% B within 24 min was applied. The retention time windows of the different hormonally active substances are listed in Table 1.

Table 1. Overview on the substance groups, the number of substances, the individual retention times and the ionisation mode

Substance group	No. of substances	Retention time range (min)	Ionisation mode
Thyreostats (Thiouracil-derivatives, tapazol, mercaptobenzimidol)	7	1.5 – 9.5	ESI (+)
Corticosteroids	16	12.0 – 17.7	ESI (+)
Steroids (Androgens, estrogens, gestagens)	21	15.2 – 19.2	ESI (+)
Steroidesters (Testosteron-, boldenone- ,nortestosterone-, and estradiol-esters)	16	18.9 – 23.8	ESI (+)
Stilbens (Dienestrol, hexestrol, diethylstilbestrol)	3	16.4 – 18.0	ESI (-)
Resorcylic Acid Lactones (Zeranol and metabolites)	6	15.2-16.6	ESI (-)
Selective Androgen Receptor Modulators (Ostarine, andarine, bicalutamide)	3	15.2 -15.9	ESI (-)
Glucuronides and sulfates of selected steroids and corticosteroids	6	1.5- 4	ESI (-)

For the data-dependent acquisition of spectra the instrument was operated in the so-called “auto-MS/MS mode” with steady alternation of MS and MS/MS mode with a defined cylce time. In MS mode, for each 0.2 s the full mass-spectrum was recorded. The precursor ion is selected by the quadrupole using a mass window of $\Delta\ m/z = 4$ and the CID accurate mass spectra were measured during the residual time of the measurement cycle at three different collision energies (usually 10, 20, 40 eV). A preferred list was used which contains the exact masses of the monoisotopic ions $[M+H]^+$ and $[M+H]^-$ theoretically calculated from the molecular formula in combination with the relative retention times.

By data-dependent acquisition in the “auto-MS/MS” mode” so-called “compounds” are identified by the software either by the command “find by auto-MS/MS” or by the “find by formula” command. The MS/MS spectra of the resulting “compounds” can be compared with MS/MS spectra stored in the PCDL-library. To this end a library search using the PCDL library was carried out. As search criteria both the agreement of accurate fragment masses and the fragment abundance ratio are used for identification of the spectra. A weighted score of these criteria is used. The data-dependent acquisition method as described above was tested using mixtures of the hormonally active substances in solution. Both in the positive and in the negative ESI mode all substances could be identified by a library search.

Conclusions

High-resolution and accurate mass spectrometry as realised by Q-TOF-technology was used to screen for hormonally active substances. First, a database containing the exact masses of the $[M+H]^+$ and $[M-H]^-$ ions and the respective retention times was created. Second, a personal compound database and library (PCDL) was established which contains additionally substance-specific spectra recorded at defined collision energies. To this end, accurate mass-spectra of more than 80 hormonally active substances were recorded at three different collision energies using flow injection methodology. The PCDL library allows for the screening of hormonally active substances in samples of animal origin after data-dependent MS/MS experiments. Substances can be identified by their accurate mass, by their isotope distribution pattern and by their spectra (accurate mass and abundance ratio of the fragments). The arrangement of the library and database of hormonally active substances is not finished yet but is steadily being extended by addition of further compounds.

Furthermore, full scan data acquisition by QTof-technology allows both for the screening of hormonally active substances based on the library and it principally enables the detection of “new” or “unknown” substances. Thus, this untargeted approach represents a significant advantage to the conventional targeted LC-MS/MS methods currently used in residue control for hormonally active substances.

Acknowledgements

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O22
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TARGETED PROTEOMICS FOR THE INDIRECT DETECTION OF DEXAMETHASONE TREATMENT IN *BOVINES*

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Abstract
The illegal use of pharmacologically active compounds for growth-promotion in food-producing animals is still matter of concern in European Union due to the risks for consumer health and animal welfare. Surveillance of such practices is necessary, however it is an analytically challenging. Methods currently adopted for the quantification of drug residues are based on HPLC-MS/MS, but their efficacy can be negatively affected due to the undetectable residual concentrations in biological matrices. Consequently, techniques focusing on the indirect biological effects of the administration of exogenous compounds have been proposed as more effective and sensitive surveillance methods. Targeted protein markers profiling has been suggested as a fast screening approach for detection of illegal treatments in meat production. The purpose of the present study is to develop an analytical method based on selected reaction monitoring for the absolute quantification of protein markers in skeletal muscle for the indirect detection of illicit dexamethasone treatments. A set of protein markers was previously identified by 2D-DIGE proteomics approach and a group of them was analysed using the selected reaction monitoring method developed in the present study. The gathered data enabled to define a promising predictive model based on logistic regression to detect dexamethasone treated animals.

Introduction
The use of pharmacologically active compounds aimed at improving animal growth in food producing animals is banned in the European Union (Council Directive 96/22/EC). To enforce the ban on anabolic compound abuse, effective monitoring, detection, identification and confirmation methods have been developed. However, the benefits of increased beef production yields are clear, and in spite of the current legislative regulation and intense inspection, the illicit use of these substances continues on European farms (Courtheyn *et al*., 2002). In this regard, the synthetic corticosteroid dexamethasone (DXM) has been reported to be illicitly employed for growth-promotion in veal calves and beef cattle at low dosage and primarily through oral administration, either alone or in combination with other anabolic agents (Gottardo *et al*., 2008). Indeed, DXM is available on the market, though approved only for therapeutic application with defined withdrawal periods between treatment and slaughtering to prevent risks for consumer's health. Yet, long term and low dosage administration of DXM can yield enhanced meat quality traits and increased beef production (Tarantola *et al*., 2004). The development of efficient analytical methods is crucial for detecting illegal practices. In spite of the progress in analytical techniques, the direct detection of administered drugs or related metabolites, may be unsuccessful whenever a compound is subjected to extensive metabolism or when different compounds are administered at low dosage, alone or in combination (Courtheyn *et al*., 2002).

To improve monitoring and detection of illegal anabolic practices, screening methods based on the detection of changes in biological components have been proposed to develop more effective tests (Mooney *et al*., 2009; Pinel *et al*., 2010). Protein-marker profiling has been suggested as screening approach because proteins remain altered even after the complete excretion of anabolic compounds, extending the detection time window. The potential of proteomics to identify the effects of anabolic agents and to be used as a screening tool has been investigated in previous studies that proposed a number of potential protein markers of treatment (Della Donna *et al*., 2009; Draisci *et al*., 2007; Guglielmetti *et al*., 2014; McGrath *et al*., 2013; Stella *et al*., 2014). The present study describes a targeted proteomics approach based on selected reaction monitoring (SRM) to develop an analytical method for the absolute quantification of previously identified candidate protein markers of illicit DXM treatments in bovines.

Materials and Methods

Animal treatment and sampling
Three sets of animals were used for the proteomic profiling study described here. The first set of animals was composed of Clinically healthy Charolaise bulls (n = 24) at 19-21 months of age were divided into three groups of eight animals each. The first group (n = 8) was used as control, the second one (n = 8) was treated with DXM administered via feed at 0.75 mg animal^{-1} day^{-1} for 42 days. The third group (n = 8) was treated with an increasing dose of clenbuterol via feed: 2 mg animal^{-1} day^{-1} during the first week, 4 mg animal^{-1} day^{-1} during the second week, and 6 mg animal^{-1} day^{-1} during the third and the

fourth week (28 days in total) in combination with DXM at 0.66 mg animal^{-1} day^{-1} for 21 days, starting from the second week of treatment.

The second animal set was composed of 16 clinically healthy 18-20 months old mixed-bred (Charolaise x Limousine) bulls that were divided in two groups of 8 animals each. The first group was used as control. The second group was treated with DXM administered via feed at 0.75 mg animal^{-1} day^{-1} for 43 days.

In both cases animals were housed in ventilated stables and the experiment began after three weeks of acclimatization. The animals were managed in agreement with the regulations regarding the protection of animals used for experimental or other scientific purposes, subsequently enforced by the Directive 2010/63/EU. During the experimental trials, hay and water were available to the animals *ad libitum*. The selected pharmacological treatments were chosen to mimic putative growth promoting schedules.

The third set of animals consisted of 12 cross-bred Friesian male veal calves aged between 5.5 and 6.5 months that were randomly and blindly selected from different abattoirs located in Northern Italy during a large monitoring plan conducted between 2006 and 2009. They were healthy when examined *ante-mortem* at the slaughterhouse and passed the *post mortem* inspection. These animals were divided in suspected of being treated with DXM, and untreated on the basis of the histological analysis conducted on the thymus sampled at slaughterhouse following the procedure described in a previous study (Vascellari *et al*., 2008).

Small biopsies of the skeletal muscle *biceps brachii* were collected from each animal at the slaughterhouse, flash-frozen in liquid nitrogen, and stored at -80 °C prior to analysis. Finally, muscle samples were divided in two distinct classes: untreated animals that were assigned to Control class, comprising 20 animals, and treated or suspected animals that were assigned to DXM-treated class, consisting of 32 animals.

Sample preparation

Samples were prepared starting from 150 mg of *bovine biceps brachii* skeletal muscle. Proteins were extracted with 1.5 mL of lysis buffer containing 8 M urea, 4% CHAPS, 5 mM MgAc, 30 mM Tris-HCl at pH 8.5, and protease inhibitor cocktail. The protein concentration was determined using a Micro-Lowry assay kit, and samples were diluted using lysis buffer to a final concentration of 10 µg µL^{-1}. A known amount of chemically synthesized isotopically labelled ($^{13}C_6$,$^{15}N_4$-Arg or $^{13}C_6$,$^{15}N_2$-Lys) synthetic peptides (i.e. 25 pmol) were added to 100 µg of protein extract before reduction (25mM DTT, 1h, 55°C), alkylation (55mM iodoacetamide 45 min, room temperature, in the dark), trypsin digestion (37 °C, 16 h, 1/100 enzyme/protein ratio) and C_{18} purification steps to account for losses during sample preparation. A calibration curve of six points was prepared by spiking purified synthetic peptides in 100 mM NH_4HCO_3 to obtain final concentrations of: 50, 75, 100, 250, 500, 750 fmol µL^{-1}. A fixed amount of synthetic isotopically labelled peptides (*i.e*. 25 pmol) was also added to the calibration curve. Target protein concentrations in skeletal muscle samples from Control and DXM-treated groups were determined by extrapolation of corresponding calibration curves.

SRM analysis

A Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced to an API 4000 triple quadrupole mass spectrometer with a turbo ion spray source (AB Sciex, Framingham, MA, USA) was used for SRM analyses. Sample extracts were analysed in positive polarity electrospray. Source temperature was set to 450°C with an ion spray voltage of 5000. MS/MS experiments were performed using nitrogen as collision gas. Ion acquisition was performed in SRM using the transitions from the molecular ion to the three most abundant fragments (2 peptides per protein).

The chromatographic separation of tryptic digests was achieved using a Brownlee SPP peptide ES C_{18} column (100 x 2.1 mm, 2.7 µm, Perkin Elmer). Elution solvents were 0.1% formic acid (A) and ACN/0.1% formic acid (v/v) (B), using a 20 min linear gradient from 10:90 to 25:75 (A:B) at a flow rate of 0.3 mL min^{-1}, injecting 10 µL of sample, corresponding to 5 µg of digested proteins. Peptide quantification was achieved using Skyline software (version 2.1.0.4936 (MacCoss lab software) exporting peptide ratio to standard values as a Microsoft Excel spreadsheet. A linear regression curve was calculated for each peptide including zero as first calibration point.

Statistics and data handling

Statistical analysis was performed using R. Potential differences of protein concentration values among different animal groups were explored performing one-way analysis of variance (ANOVA). Quantitative data were also explored to detect possible pattern in the dataset applying unsupervised principal component analysis (PCA). For comparisons of single variables among treated and control groups, the Mann-Whitney test was used. To select variables to be included or excluded the Akaike information criterion (AIC) value was calculated for the different models tested.

Results and Discussion

Global strategy

For the absolute quantification of a protein, the selection of peptides has to be performed carefully because in targeted proteomics approaches the proteins are not quantified directly, but one or more signature peptides are quantitatively measured. Therefore, the peptides were selected in order to be unique to the protein of interest within the considered proteome. A scheduled SRM method was developed using Skyline software (MacCoss Lab) to monitor 24 peptides deriving from 12 considered protein markers of DXM treatment: fructose-bisphosphate aldolase (ALDO), subunit beta of mitochondrial ATP synthase (ATPB), carbonic anhydrase 3 (CAH3), desmin (DESM), β-enolase (ENOB), glyceraldehyde-3-phosphate dehydrogenase (G3P), creatine kinase M-type (KCRM), myosin regulatory light chain 2 (MLRS), pyruvate kinase (PKM), troponin T, slow skeletal muscle type (TNNT1), troponin T, fast skeletal muscle type (TNNT3), and triosephosphate isomerase (TPIS).

In the present study, the absolute quantification of peptides (AQUA) method was used to achieve accurate quantitative measurements by exploiting the principle of stable isotope dilution MS (Kettenbach *et al*., 2011). The concentration values of protein markers measured in the skeletal muscle of DXM-treated and untreated animals were then used to select the most reliable markers for the indirect detection of DXM abuse, and develop a predictive model based on logistic regression for animal classification (Figure 1).

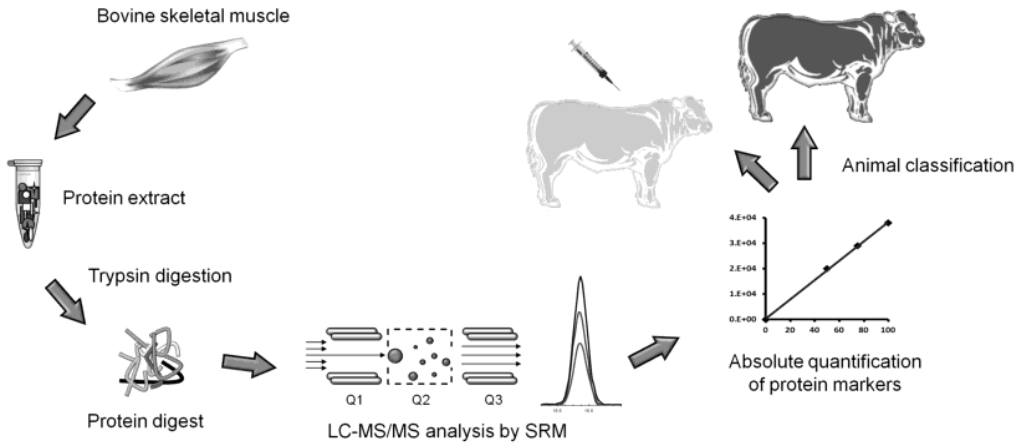

Figure 1. Schematic representation of the analytical workflow used in the present study.

Method performance

The linearity of the response was studied using a calibration curve for each peptide within the corresponding concentration range of interest. Linearity regression was confirmed by correlation coefficients (r) that was always better than 0.97. Intra-laboratory variability was evaluated by comparing concentration values acquired analysing 20 independently prepared protein digests from one representative muscle extract, in three distinct analytical sessions. The average CV% for the 24 monitored peptides was found to be 7.7%. The divergence of concentration values measured for the two peptides coming from the same protein marker was also estimated and found to be, on average, lower than 15%. This result confirms once more that reliable quantification of proteins is achievable even in absence of instrumental platforms such as nano-LC systems.

Data interpretation

Quantitative data of 12 proteins from the 52 animals of this study were analysed using PCA to obtain a general overview of distribution and grouping of samples. The unsupervised PCA underline a significant difference between the three animal breeds, which is able to mask in part the effect of DXM treatment on protein abundance (Figure 2).

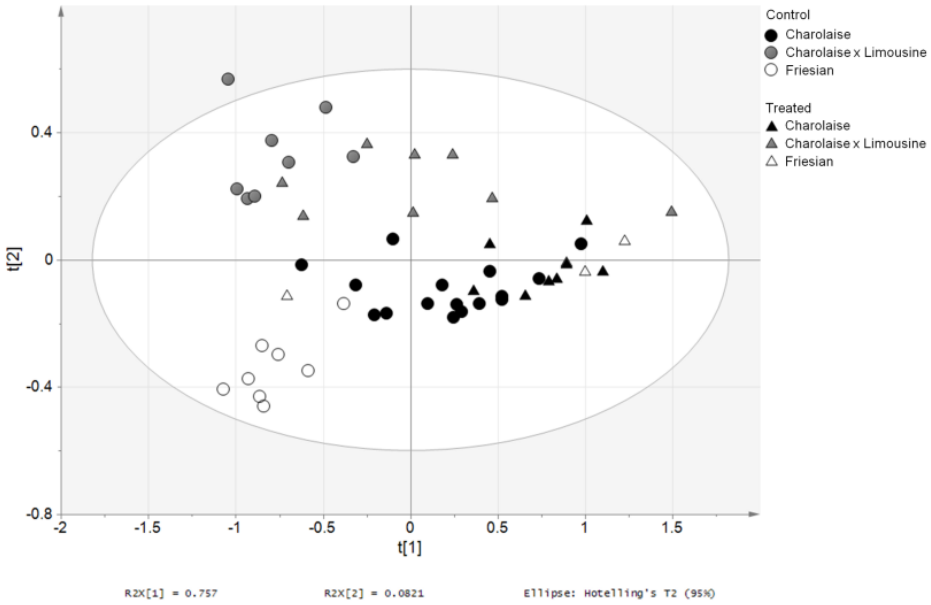

Figure 2. PCA score plot based on the 12 selected candidate markers. Protein concentration values were transformed into principal components, obtained by linear combination of the original variables and maximizing the explained variance within the original dataset. On x-axis the first principal component is reported, and on y-axis the second principal component is reported.

Then, ANOVA was applied on the entire dataset to test if the interaction effect between animal breed and DXM treatment was significant. Results from ANOVA indicate that the overall effect of DXM treatment does not depend on breed, as also attested by the conserved trend in the fold change of protein content induced by the treatment (Figure 3). Indeed, the effect of breed consists mainly in different amount of protein markers present in the skeletal muscle of control animals, which changed in a conserved proportion among the three animal groups upon DXM treatment. To face such different protein content due to breed influence, concentration values measured for the different proteins were normalized, allowing for the direct comparison of corresponding normalized values.

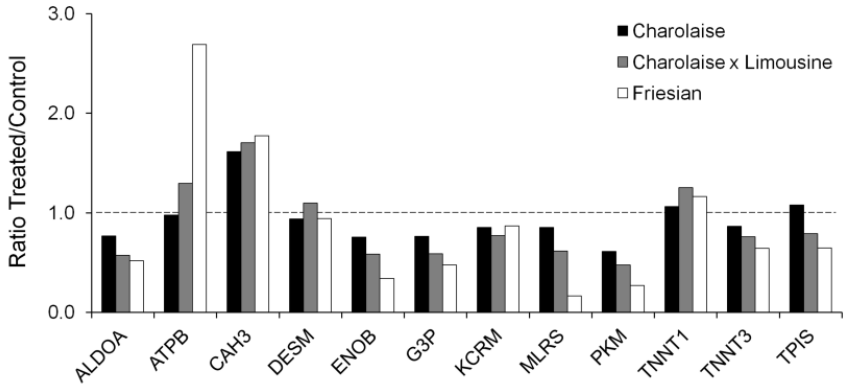

*Figure 3. Ratio values (treated/control) for the different candidate protein markers of treatment. In the bar graph the average treated/control ratio values for the three animal groups analysed are reported (*i.e. *Charolaise bulls, n=24; Charolaise x Limousine bulls, n=16; Friesian veal calves, n=12).*

Development of the linear regression model

For the development of the linear regression model, the animal set comprising the higher number of animals (*i.e*. Charolaise bulls, n=24) was used as training set in order to take in account the widest possible animal to animal variability. In this animal set, quantitative data of 6 among the 12 analysed candidate markers of treatment (*i.e*. ALDO, ENOB, G3P, KCRM, PKM, TNNT3) were confirmed to be significantly altered after DXM treatment (p value < 0.05, Mann-Whitney test). Correlated variables were excluded to avoid multi-colinearity in the predictive linear regression model. The variables to be included or excluded from the model were further selected on the basis of AIC values, thus a final model with a unique biomarker (PKM) as an explicative variable was obtained.

The fitted model presented a global error rate of 8.3% in predicting the observed data of the training set, correctly detecting all the 16 treated animals (100%) and accounting for 2 false positive animals in the control group (25%). The performance of the model was subsequently assessed using two external datasets. Using the animal set composed by 16 Charolaise x Limousine bulls the global error rate was 12.5%, with two false positive animals (25%), and all the treated animals correctly detected (100%). Applying the model to the animal set consisting of 12 cross-bred Friesian male veal calves the global error rate was 8.3%, with only one false positive animal (25%), and all the treated animals correctly detected (100%).

Conclusions

Targeted SRM assays have been used successfully for quantifying peptides. Protein quantification results demonstrated that protein abundances are strongly influenced by animal breed and therefore the selection of a common cut-off concentration for sample classification is probably not viable. However, using appropriate statistical tools and taking into account the breed, it was possible to define one promising biomarker among the ones tested (*i.e*. PKM) to screen for DXM treatment. Clearly, biomarkers of growth promoters may be affected by several factors such as age, breed, feed consumption and growth. Therefore, it is proposed to perform additional studies on a larger population of DXM-treated and untreated animals to further validate the proposed method. Although the official controls rely on conventional detection method that are required by current regulation to prove the illegal veterinary practice, the developed method fulfils the minimum requirements needed for its applicability (95% detection rate) and may serve to complement the existing screening techniques.

Acknowledgements

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THE HISTOPATHOLOGICAL APPROACH FOR THE MONITORING OF THE ILLEGAL ADMINISTRATION OF GROWTH-PROMOTERS IN FOOD-PRODUCING ANIMALS

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Abstract
It is well known that, despite the EU ban and the high frequency of official controls, growth-promoters are used illegally in livestock. Among them, steroid hormones represent a high-danger category of anabolic substances. Besides the positive effects exerted on the muscles and tissues of the treated animals, they can also accumulate as residues with severe long-term effects on consumers, representing a potential risk as carcinogens. The current monitoring strategy within the EU for sexual hormones, is a screening phase followed by confirmatory analysis of non-compliant samples. The number of non-compliant samples from the execution of the National Residues Control Plans are negligible (less than 0.01%), but the true extent of the illegal administration may be underestimated. The use of low dose drug cocktails or of natural compounds (*i.e*. 17β-estradiol) or the natural presence of synthetic hormones (*i.e*. nandrolone - 17β,19-nortestosterone in many species), combined with the different illicit treatments (the so-called weekend-treatment, single administration of rapidly excreted compounds which residues disappear from urine within 24-48 h), makes the control of the abuse of these substances difficult. The European Food Safety Authority has recently underlined the weaknesses of the screening methods for residue monitoring and has stated the need for new detection strategies, shifting the target from the identification of the molecule/metabolite towards the highlighting of the biological effects. All this considered, this paper is to illustrate the results and perspectives of the histopathological approach and of biomarkers related to growth promoters in calves for the detection of illegal administration within the framework of the Italian Residue Control Plan.

Introduction
The use of anabolic substances like steroid hormones as growth-promoters, in food producing animals is strictly forbidden by the Council Directive 96/22/EC. The use of hormones in food-producing animals poses a serious risk to consumers, due to a great variety of long-term adverse effects. In fact, most estrogens and estrogen-like compounds are known to cause cancer in humans, whereas androgens and progestagens are associated to the development of neoplasms in experimental animals.

Despite the ban, sex steroids are still being used in farm animals, as reported by numerous seizures of illegal unidentified preparations (usually containing several anabolic substances, *e.g*. both estrogens and androgens at low level) at farm level. Since the late 80s, the European Union has focused its efforts in fighting the illegal use of sexual hormones for growth-promoting purposes by implementing Communitarian Residues Control Plans to monitor the presence of residues of hormones and veterinary drugs in food and feedstuffs (Directive 96/23/EC). In spite of the intense official control activity (over 400,000 targeted samples reported to the European Community by the 28 Member States – EFSA report on MVPs), in the group of steroids (A3) only 0.04% (35 of the 42,846) samples were found non-compliant in *bovines*.

Therefore, the European Agency for Food Safety underlines the inefficiency of the screening strategy implemented in the Official Monitoring Plans, suggesting and encouraging the development and use of biologically-based methods, in order to improve the effectiveness of the official screening step of Residues Control (EFSA opinion, 2013). In this context, the histopathological approach is known to have temping characteristics: it is used to describe the lesions induced by sexual hormones, glucocorticoids and thyrostats in *bovines* through the microscopic examination of the target organs (*i.e*. sexual accessory glands, Thymus and thyroid). These lesions persist far over the already apparently false compliance status of the official matrices and they are specific of illicit hormones administration. Sex hormones are known to cause squamous metaplasia in target organs of treated animals, as demonstrated by several animal experiments (Pezzolato *et al*., 2011).

In 2008, the Italian Ministry of Health introduced the histological method as complementary monitoring test in the framework of the Italian Residues Control Plan. It consists of a sampling step at the slaughterhouse, when target organs (prostate and bulbo-urethral glands for sex steroids in male cattle) are sampled, and a histological evaluation step, when lesions are analysed giving a suspect or negative result. Through a pilot monitoring plan, the goal was to assess the spread of illicit treatments in healthy slaughtered cattle to enable an estimate of the prevalence of the phenomenon at national level.

The aim of the present work is to increase the reliability of simple, reliable and robust biomarkers of illicit sex steroid administration by evaluating the specificity and the sensitivity of the previously described histological markers. The final goal is the proposal of a new Histological Surveillance Plan to investigate more closely the illegal administration of sex steroids in calves. This Plan will coordinate and address the official control within the Italian Residues Monitoring Plan.

Materials and Methods

Animal and experimental design

Several animal experimental studies have been conducted to provide matrices of interest. A total of 135 male Friesian veal calves, aging between 15 and 35 days and bought from the same local breeder, were randomly divided into 4 groups. They were treated as follows: group A (63 calves) 17β-estradiol (5 mg/head; 4 weekly injections), group B (20 calves) nandrolone (50mg/head; 4 weekly injections) and group C (20 calves) the association of the two steroids (5mg estradiol + 50 mg nandrolone/ head; 4 weekly injections), while group K (52 calves) was kept as control. Each group of animals was raised in separate multiple pens, in order to avoid any kind of cross-contamination, under controlled conditions over a 7-months' period. Experiment was executed according to the animal welfare legislation (Directive 86/609/EEC). Each pen had its own crib, multiple drinking troughs, and a dedicated milk feeder automated system. All animals were vaccinated against rhinotracheitis (IBR), parainfluenza 3 (PI3), respiratory syncytial (BRS) and viral diarrhea (BVD) viruses (Cattlemaster 4, Pfizer Animal Health, New York, USA) and were clinically evaluated by a veterinarian with daily observations and, if needed, complete physical examination.

The animals were fed with a standard milk replacer, available on the market until the 4th month of age, then 0.2 to 0.5 kg of corn was added to the diet twice a day until slaughtering, and had free access to fresh water.

At the age of 6 months, the animals belonging to group A, B, C were treated according to the scheme presented above and blood and urine were periodically sampled for further studies not included in this paper. Fifteen days after the last injection, all animals were slaughtered in a EU-certified slaughterhouse.

Samples preparation

For the present study, tissue samples of sexual accessory glands (prostate and bulbo-urethral glands) of each animal sampled at the slaughterhouse were fixed in neutral buffered formalin, trimmed, routinely processed, embedded in paraffin wax and serially sectioned at 3 ± 2 µm for histological evaluation. The tissue slides were stained with haematoxylin and eosin according to our laboratory standard operating procedures.

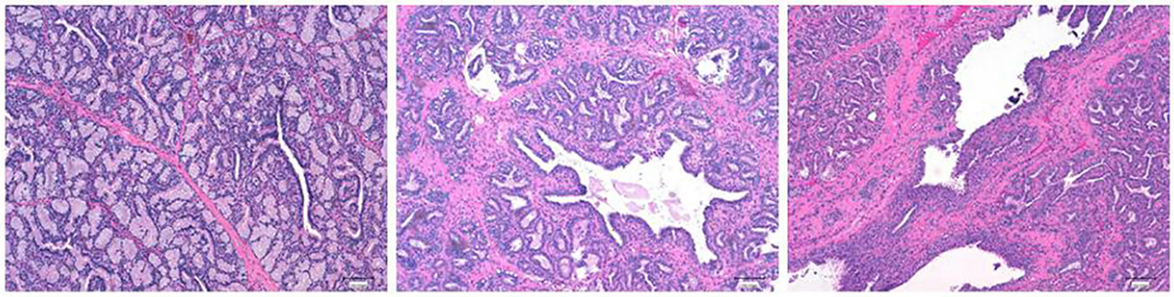

Figure 1. Minor lesions: bulbo-urethral hyperplasia (left), prostate (pars glandularis) hyperplasia (middle) and bulbourethral gland duct hyperplasia (right) from treated calves. HE

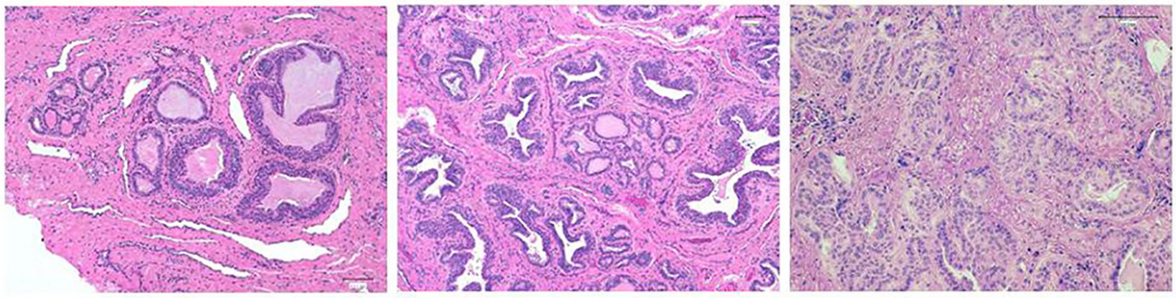

Figure 2. Major lesions: bulbo-urethral hypersecretion and metaplasia (left), prostate (pars glandularis) moderate metaplasia (middle) and prostate squamous metaplasia (right) from treated calves. HE

Histopahology

Histopathological examination was performed blindly by three veterinarians. The slides were examined and major and minor lesions were described, taking into account the different anatomical regions within the target organs: urethra and *pars glandularis* for prostate, glandular alveoli and ducts for bulbo-urethral glands. The lesions were classified as follows: urethral hyperplasia, prostate and bulbo-urethral glands hyperplasia and cysts as minor lesions (Figure 1); prostate and bulbo-urethral

glands metaplasia and hypersecretion as major lesions (Figure 2). The data on the presence of these lesions for each experimental animal was recorded into an electronic data set.

Statistical analysis

The results of the microscopic examination were entered into an *ad hoc* database and analysed using Stata 13 (Stata-Corp, College Station, TX, USA). For each lesion, sensitivity and specificity were calculated. The combination of different histological lesions was evaluated using parallel test interpretation in order to maximize the validity parameters of the histological test.

Application of the surveillance programme

The validity parameters calculated and the results of the pilot monitoring plan conducted in 2008 have been finally used to increase the performance of the surveillance system proposed in order to investigate in a more efficient and effective way the illicit treatment phenomenon at national level

According to the results of the pilot monitoring plan and based on the validity results of this study a new sample size for the surveillance plan was calculated.

Results

In our evaluation, urethral hyperplasia was found to be not statistically associated with illicit administration of sex-hormones in veal calves, while urethral metaplasia was not constantly present in treated calves. Moreover, prostate and bulbo-urethral hyperplasia were found both in treated and in control animals. Thus prostate and bulbo-urethral glands cysts were not constantly found in treated calves and appeared to be present in untreated calves as well. Instead, prostate and bulbo-urethral glands metaplasia was found to be significantly associated with treatment. Namely, found in nearly all estrogen-treated calves, in 14 out of the 20 androgen-treated calves and in all animals treated with the combination of 17β-estradiol and nandrolone. Hypersecretion was found to be specific of the cocktail treatment group, while it was not consistent in the estradiol-treated group. The results of the histopathological evaluations, combined together with the statistical analysis are summarised in Table 1. For each lesion, the sensitivity and specificity were calculated.

Table 1. Sensitivity (Se) and specificity (Sp) of lesion analysis.

Organ	Lesion	Se	IC Se 95%	Sp	IC Sp 95%
Prostate	Hyperplasia	18%	11%-26.9%	82.7%	69.7%-91.8%
	Metaplasia	83%	73.9%-89.7%	100%	93.2%-100%
	Hypersecretion	86%	77.6%-92.1%	67.3%	52.9%-79.7%
	Cysts	41%	31.3%-51.3%	76.9%	63.2%-87.5%
Bulbo-urethral glands	Hyperplasia	19%	12.3%-28.4%	76%	61.8%-86.9%
	Metaplasia	82%	73.8%-89.3%	96%	86.3%-99.5%
	Hypersecretion	60%	50.1%-69.7%	62%	47.2%-75.3%
	Cysts	21%	13.2%-29.7%	70%	55.4%-82.1%
Bulbo urethral gland - ducts	Hyperplasia	30%	21.5%-39.9%	42%	28.2%-56.8%
	Metaplasia	95%	89%-98.4%	92%	80.8%-97.8%

As the table shows, hypersecretion presents low sensitivity and specificity values, even if it is recognized to be a good marker of illicit administration of estrogens and androgens cocktails, while metaplasia (both in the prostate and the bulbo-urethral glands) is the major feature associated with illicit administration of sex hormones, and is the histological marker of the illegal use of these molecules (Table 2).

Table 2. Histological biomarker sensitivity and specificity.

Lesion	Se	IC SE 95%	Sp	IC SP 95%
Metaplasia	98.1%	93.2%-99.8%	90.4%	79%-96.8%

In order to define an effective surveillance system able to detect illicit treatments, a representative multi-stage survey was defined. In particular, a two-stage survey was designed considering the uneven distribution of the phenomenon investigated. In the first stage (animal-level) the sample size was calculated assuming 80% and 95% test specificity and sensitivity, respectively, and an animal-level prevalence of 80%. The number of animals to be sampled within the same batch of animals sent for slaughter is shown in Table 3.

In the second stage (herd-level), the number of slaughtered batches to be sampled (indirect indicator of herds) was calculated assuming a design prevalence of 10% and a 95% survey sensitivity and specificity (Table 4).

Table 3. Number of animals per batch.

Number of animals slaughtered	Number of animals to be sampled
< 12	5
> 12	6

Table 4. Number of batches to be sampled per region.

Region	Number of batches
Piemonte	20
Lombardia	20
Veneto	20
Trentino Alto Adige- Sud Tirol	5
Emilia Romagna	3
Liguria	3
Marche	3
Toscana	3
Valle d'Aosta	3
Abruzzo	1
Basilicata	1
Calabria	1
Campania	1
Friuli Venezia Giulia	1
Lazio	1
Molise	1
Puglia	1
Sardegna	1
Sicilia	1
Umbria	1

After the histopathological evaluation of target organs, according with the sensitivity and specificity of the surveillance system, the sampling batch is considered suspect of illicit treatment when only one animal is found to develop prostate or bulbo-urethral glands metaplasia.

Discussion and Conclusions

The administrations of sex hormones in food-producing animals for growth-promoting purposes is strictly forbidden by law in the EU. However, several seizures of unknown illicit cocktails and feed containing a mixture of hormones at farm level, as well as a wide black market of illicit drugs, demonstrate that the phenomenon of illicit treatments is overwhelming. The presence of residues of sex hormones in food-producing animals is a serious health-threat for consumers. In fact, estrogens are known to cause cancer in humans, while androgens and progestagens are related to the development of different neoplasms in animals. Steroid hormones are known to determine squamous metaplasia in target organs (accessory sex glands) of veal calves. The first aim of this study was to test the validity and to standardize the histological method at a national level by describing simple and robust histological markers of illicit treatment. The second aim was to integrate the method in a specific surveillance plan, specifically designed with the performance features of the test and the required detection level in consideration. Its purpose is to improve the monitoring of illicit use of anabolic treatments. In our study, metaplasia was found to be the best candidate biomarker of illicit administration of estrogens and androgens, each alone or as a combination treatment.

Based on the validity of the test through the analysis of the results obtained from over 100 calves were treated experimentally with (a combination of) estrogens and androgens, and on the results of the pilot monitoring histological plan, we were

able to define the features of the surveillance plan. The design prevalence was fixed at 10%, the sensitivity and specificity of the test were fixed at 80% and 95%, respectively, to reach a high specificity of the surveillance system. For the same reason, the judgment of suspect of illicit treatment was defined when over 80% of animals are found to develop the specific lesions (prostate or bulbo-urethral glands metaplasia).

All things considered, the histological method has showed tempting characteristics in the evaluation of the illicit administration of sexual hormones in calves: it is a cost-effective, robust and consolidated test that allows to record the administration of certain substances. Since its application, the evaluation of the lesions induced by growth-promoters has constantly developed and the diagnostic scheme within the Italian Residues Control Plan has been standardized. By implementing a new histological monitoring plan, the attempt is to strengthen its role as complementary analytical tool, with high values of sensitivity and specificity.

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SITE-ENCODED DNA STRATEGIES FOR RESIDUE ANALYSIS

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Abstract
Microarray technologies can be excellent screening tools to process simultaneously many samples for many analytes. Unfortunately, manufacturing protein microarrays is not as straightforward as DNA microarray chips. An alternative to circumvent these limitations is the use of DNA-Directed Immobilization (DDI) strategies. Using DDI we have been able to convert a DNA chip into a hapten-microarray based on fluorescent or surface plasmon resonance (SPR) sensing principles. As proof-of concept, this bioanalytical approach has been used for developing multiplexed platforms for anabolic androgenic steroid (AAS) analysis. The fluorescent microchip developed in this way is able to detect several illegal substances with sufficient detectability to be used as screening method according to the World Antidoping Agency (WADA) and the European Commission (EC) regulations in the sport and food safety fields, respectively. Moreover, the same principle has been used to create biofunctional plasmonic nanostructures suitable for multiplexed localized plasmon resonance (LSPR) biosensing AAS.

Introduction
The use of microarrays permits simultaneous detection of a large number of substances being ideal for high-throughput analysis. Technological advances in micro (nano)biotechnology have provided the possibility to develop multiplexing bioassays through two strategies: a) achieving spatial multiplexing (planar microarrays) or b) using multiple quantitation tags (non-planar microarrays). The first case, in which the identity of the target analyte is encoded by its location, is the most widely used approach. Today, DNA microarrays are considered standard tools for analysing complex genetic information and the food field for detecting food-borne pathogens (1,2) or genetically modified organisms (3).

Besides genomics, microarrays for peptide, protein or small molecule analysis represent a big challenge in clinical, food safety and environmental fields. For example, chemiluminiscent microarrays have been very useful to detect bacterial toxins and mycotoxins (4,5) in addition to some other interesting targets for these fields (6). However, protein microarray technology is not as straightforward as DNA technology due to the molecular variability and complex nature of proteins (different hydrophobicities, acidic or basic characters, functionality, etc.). Unlike nucleic acids, which are relatively homogeneous in terms of structural and electrostatic properties, proteins can be extremely diverse regarding chemical structure and biological properties. Preventing protein denaturation and maintaining structural conformations are key issues in microarray technology (see Ref. 7 for a recent review on immobilization strategies). This is the reason why DNA microarrays are much more standardized.

An alternative to circumvent these limitations consists through the use of oligonucleotide probes with well-known sequences and their subsequent hybridization with corresponding complementary oligonucleotides already immobilized on the surface. This strategy, known as DNA-Directed Immobilization (DDI), has been used to spatially assemble mixtures of molecular components, such as nanoparticles, proteins and polypeptides (8-14). It not only provides greater immobilization efficiency than conventional adsorption techniques (9), but also allows reversible immobilization of biomolecules allowing development of reusable microarrays and biosensor chips. In combination with antibodies, DDI may also provide a useful strategy to construct microarrays based on different immunochemical analytical approaches, taking advantage of the wide variety of selectivities provided by the antibodies and their exceptional features as natural bioreceptors (15). In this paper we will present different technological approaches in which DDI has been used to create multiplexed platforms for the detection of AAS based on different principles to detect the specific biomolecular interactions.

Materials and Methods

Reagents and Immunoreagents.
The immunoreagents for stanozolol (St), boldenone (B) and tetrahydrogestrinone (THG) used in this study have been described before (16-18). Other immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The "As cocktail" is a combination of the three antisera (St: As147, diluted 4,000 times; THG: As170 diluted 2,000 times; B: As138 diluted 16,000 times, all in PBST). Tetrahydrogestrinone was synthesized in our laboratory (17) and other analytes were purchased from Sequoia Research Products, Ltd. (Oxford, UK) and Sigma-Aldrich®. The analytes used are norstanozolol (NorSt), 16β-OH-stanozolol (16bOH-St), 3'-OH-stanozolol (3'OH-St), α-boldenone (aB), methylboldenone (MB), gestrinone (G), norethandrolone (NEth), methyltestosterone (MT), testosterone (T), nandrolone (NT), ethynilestradiol (EES), progesterone (P), trenbolone (Tr), estrone (E1), estradiol (E2), androstenedione (A1), androstadienedione (A2) and dihydrotestosterone

(DHT). Stocks solutions were prepared 10 mM concentration in DMSO. The synthesis of the oligonucleotides (*N1down, N2down and N3down)* and of the hapten-oligonucleotide conjugates (St: 8-*N1up,* THG: hG-*N2up* and B: 13-*N3up*) has been performed as desribed (19). The "H-OLG cocktail" is a mixture of the three conjugates at 0.01 µg mL^{-1}. The anti-Rabbit IgG-TRITC is purchased from Sigma-Aldrich®. Other chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The O-(methyl)-O'-(2-mercaptoethyl)-hexaethylene glycol (m-PEG-SH) was supplied by Polypure. Chloroauric acid ($HAuCl_4 \cdot 3H_2O$), trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and *N*-hydroxylamine from Sigma-Aldrich® were employed. Glycerol and tris-base were employed for the final suspension of the synthesized AuNP-DNA conjugates. NaN_3 was used as preservative of the final conjugates. DL-dithiothreitol (DTT) was purchased from Sigma-Aldrich®.

Buffers
PBS was 0.01 M phosphate buffer in a 0.8% saline solution (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl) at pH 7.5. PBST was PBS with 0.05% Tween-20. Printing buffer consisted of 150 mM sodium phosphate (pH 8.5) with 0.01% sodium dodecyl sulphate (SDS). Hybridization buffer was 10 mM TRIS, 1 mM EDTA, 1 M NaCl (pH 7.2). The final washing buffer was saline-sodium citrate buffer (SSC) (15 mM NaCl + 1.5 mM sodium citrate), 0.05% SDS (pH 7.5). Disulfide cleavage buffer was 170 mM phosphate buffer (pH 8.0). Final reconstitution buffer of the AuNP-DNA conjugates was 20% glycerol, 20 mM Tris base, 0.05% NaN_3. For LSPR measurements the slides were washed with 0.3 M ammonium acetate, pH 7.0.

AuNP-DNA conjugates.
The preparation of gold nanoparticles (AuNPs) was accomplished using described methods (20, 21). Coupling of the oligonucleotides to the AuNPs was carried out following the procedures described by Taton (22) and Hill (23).

Preparation of the Hapten-Microarray
Oligonucleotide chains (*N_{1-3} down*, 200 µg mL^{-1} in printing buffer) were spotted onto poly-L-lysine substrates using a BioOdissey Calligrapher MiniArrayer (Bio-Rad Laboratories, USA) provided a high humidity chamber, maintained for 30 min at room temperature and finally dried in an oven for 30 min at 60°C. Each glass slide contains 24 wells. A 5x3 spot matrix was printed on each well with five spots replicates for each oligonucleotide Nxdown. The slides were placed on a microplate microarray ArrayIt® hardware system allowing 96-well formatted experimentation with up to four glass substrate slides (Telechem International). The system consisted of a silicon gasket that demarcates 24 wells for slide. Before starting the assay, the slides were blocked (100 µL $well^{-1}$ blocking solution) for 30 min and washed four times with PBST. Once ready, the H-OLG cocktail was added (0.01 µg mL^{-1} in the hybridization buffer, 100 µL $well^{-1}$ in all cases) and after 30 min of incubation at RT, the slide was washed and dried. In this way, the chip was ready for use.

Multiplexed Hapten SPR microarray
For this experiment a spotReady™ substrate with 16 gold spots per chip, with a spot diameter of 1 mm, was used and the solutions of *Nxdown-SH* oligonucleotides were deposited per triplicate for 1 h at room temperature (RT). Then, with the chip inside the SPR set up, a solution containing a mixture of the three steroid-oligonucleotide conjugates (*8-N1up/ hG-N2up/ 13-N3up*) was flowed over the cell for about 20 min, until a steady-state situation. Following, the chip was washed (10 min) and the blocking solution was passed (10 min).

Preparation of Hapten Microarray on Nobel Metal Nanostructured Surfaces.
The capture oligonucleotide chains were spotted onto the epoxy-activated slides in a controlled humidity chamber at 60% and maintained for 3 h at RT. Afterwards, the biohybrid AuNP-DNA were added onto the microarray at different concentrations, incubated for 30 min at RT, washed and dried. Under these conditions the slides could be stored at 4°C on a dry chamber for more than 1 month, although in most of the experiments the slides were immediately used or the day after for running the assays. Prior the assay, a solution of the corresponding hapten-oligonucleotide bioconjugate was added to the corresponding wells of the, incubated for 30 min at RT and the slide was rinsed with PBST.

Multianalyte Immunoassay on the Microchips
Standard (0.1 nM - 1 µM (St), 0.1 nM - 1 µM (THG) and 0.5 nM - 5 µM (B), all in PBST) or the samples were added to the wells (50 µL $well^{-1}$ in PBST) followed by the "AS Cocktail" (50 µL $well^{-1}$, PBST) and incubated for 30 min at RT. The slides were washed and the anti-IgG-TRITC solution (1/250 in PBST, 100 µL $well^{-1}$) was added. After incubation step of 30 min at RT, the slide was washed with the final washing buffer, dried with N_2 and read with the scanner.

Results
We have applied the DDI strategy to create a hapten-microarrays based on fluorescent, SPR and LSPR through the use of well-defined hapten-oligonucleotide conjugates. This type of microarrays could allow determination of small organic molecules under competitive conditions, using the well-know indirect immunoassay format, using DNA universal multiplexed platforms due to the reversible nature of the DNA hybridization approach.

Fluorescent Hapten Microarray.
The microarray chip was prepared printing *$N_{1\text{-}3}$down-NH2* oligonucleotide sequences. The slides were used in this manner for the preparation of the AAS hapten-microarray by hybridization of the oligonucleotide chains with their complementary sequences (*$N_{1\text{-}3}$up).* For this purpose, a cocktail of hapten-oligonucleotide conjugates (H-OLG cocktail) with the complementary oligonucleotide sequences linked to the St (stanozolol), THG (tetrahydrogestrinone) and B (boldenone) haptens was used. Hybridization on the surface of the DNA-chip gave a hapten-microarrray (see Figure 1) in a very selective maner. The complementary oligonucleotide sequence directed hapten immobilization to the appropriate prositions of the microarray.

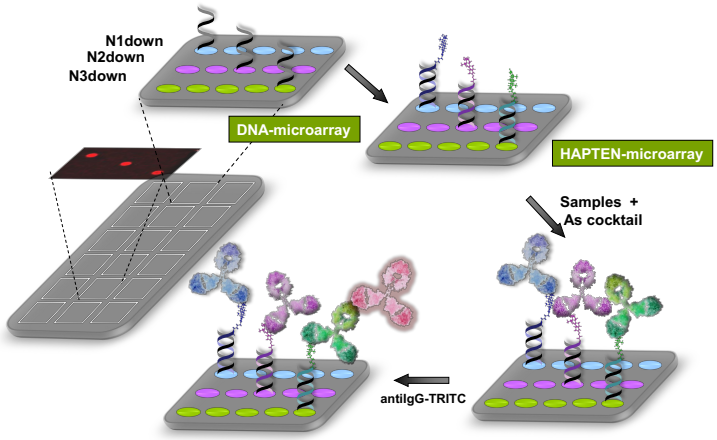

Figure 1. Scheme of the preparation and use of the hapten-microarray

Analysis of S, THG and B was performed by exposing the hapten-microarray to solutions containing these analytes mixed with the As cocktail and with no analyte. The detectability accomplished is in compliance with the WADA and EC requirements regarding the MPRL proposed by those agencies (see microchip features in the Table). Thus, the experiments performed demonstrate that St, THG and B can be detected in buffer with a limits of detection of 0.32, 0.14 and 0.48 µg L^{-1}, respectively, when the MRPL values proposed by WADA are 10 µg L^{-1}.

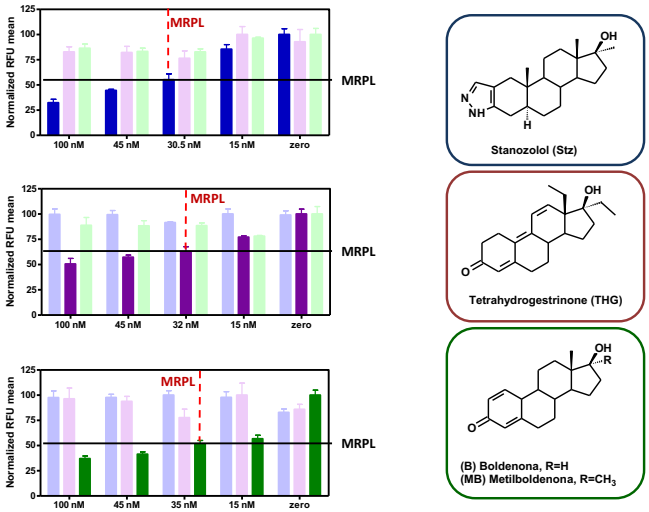

Figure 2. Results from the measurement of blind samples with the hapten microarray. Steroids were tested at different concentrations and the RFU value compared with the response at the samples at the MRPL and zero concentrations. Data shows that the hapten microarray provides sufficient detectability to work in compliance with the regulations. Samples with concentrations of steroids over the MPRL still provide an inhibition of the fluorescent signal. Bars show the average and standard deviation of signals recorded from 15 spots and 3 chips.

Assessment of the microarray precision as screning tool demonstrated that the %CV at the MPRL (minimun required performance level, according to WADA) of these residues was in most of the cases below 20%. The AAS hapten-microarray developed can clearly detect samples that contain AAS even below the MRPL.

The assay could be run in about 2 h and provided specific response on a different microarray section depending on the target analyte. This platform was able to detect and identify these hormones in blind samples, and allowed the discrimination between compliant, non-compliant or blank samples (see Figure 2).

SPR Hapten Chip

The chips were biofunctionalized with *N_{1-3}down-SH* oligonucleotide sequences and subsenquetly used to immobilized the haptens by flowing solutions of the *N_{1-3}up-hapten* bioconjugates. Specific hybridization could be monitored by the change produced in the refractive index (Figure 3) to obtain hapten-biofunctionalized SPR chip. The SPR-chip allowed monitoring of specific antibody binding and the experiments performed demonstrated that the signal recorded was specfic and that the chip could be easily regenerated recovering again the same signal on subsequent measurement cycles.

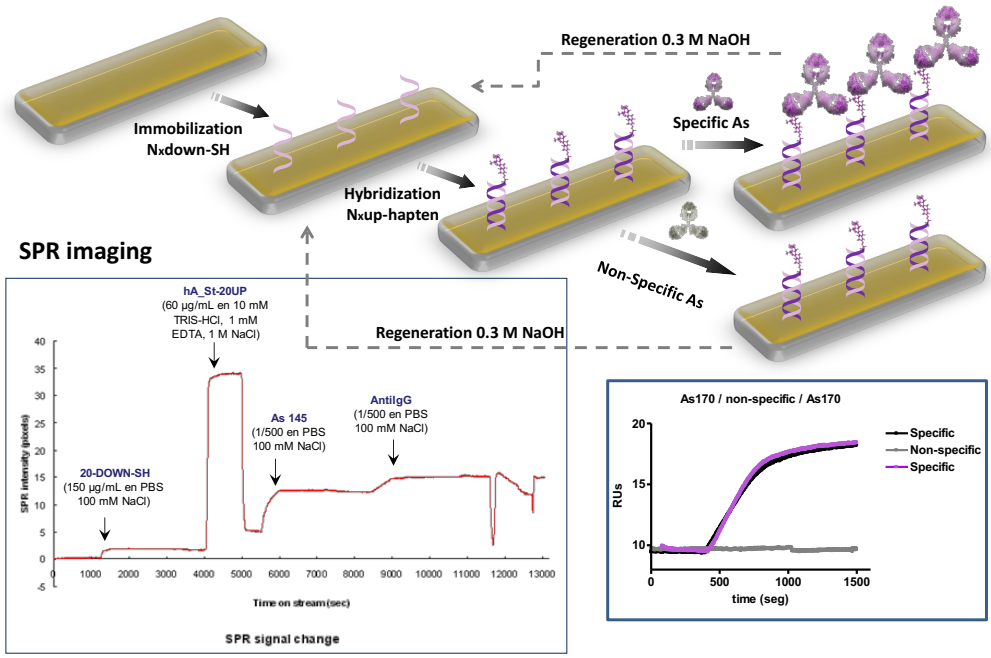

Figure 3. Scheme showing preparation of the hapten-biofunctionalized SPR chip exposed to specific (purple) and nonspecific (grey) antibodies. The chips could be regenerated recovering again the DNA-biofunctionalized SPR chip which could be re-used again for hybridization with any other molecular probe containing the appropriate complementary DNA sequences. Bottom-Left: representative SPR sensogram for ST showing all steps, including anti-IgG biding. Bottom-Right: Representative sensogram for THG showing the lack of signal when exposed to nonspecific antibodies and the recovery of the signal on the second cycle after regeneration.

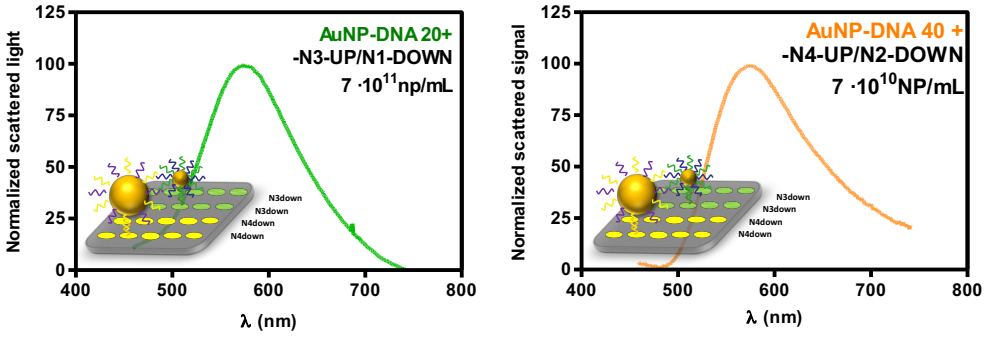

Figure 4. LSPR sensograms of chips biofunctionalized with 20 and 40 nm AuNP using DDI.

DDI for LSPR hapten chips: LSPR chips have been produced chemically by DNA-directed immobilization of multifunctional AuNP particles biofunctionalized with oligonucleotide sequences that are complementary to the DNA chip and oligonucleotide sequences which are complementary to hapten-oligonucleotide probes. Scanning electron microscopy allowed to

demonstrate that specific immobilization of noble metal nanoparticles, with distinct optical properties, on different spots of the microarray is possible using DDI (see Figure 4). Moreover, biofunctionality and plasmonic properties of the chip could be demonstrated by fluorescence and LSPR measurements.

Conclusions and Discussion

Immunochemical methods show great potential for diagnostics and screening purposes in many fields. Combining with the microarray technology the analytical capabilities of those methods can be extraordinarily increased. Major concerns are related to the immobilization of the proteins to the surfaces which may compromise their functionality. To circumvent this limitations DNA-directed immobilization methods have open the possibility to develop universal platforms for the analysis of a great variety of substances (proteins, peptides, cells) using microarray technology. Thus, we show here results related to fluorescent, SPR and LSPR microarrays in which DNA has been used to immobilized haptens and plasmonic nanoparticles on a selective manner. For this purpose, hapten-oligonucleotide bioconjugates have been synthesized with single stranded DNA sequence that is complementary to the oligonucleotide immobilized on the chip. SPR results have demonstrated that the chip can be regenerated easily. This opens the door to use DNA chips as universal platforms for the analysis of a variety of substances as long as the corresponding complementary oligonucleotide probes are available. As a proof-of concept, the hapten biofunctionalized chips have been used to detect and quantify AAS using a site-encoded configuration where the identity of the target analyte is encoded by its location in the detection platform. The strategy presented here is universal and it can be used to detect other analytes of interest in biomedical, food and environmental fields.

Acknowledgements

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LIPIDOMICS: AN ALTERNATIVE AND COMPLEMENTARY TOOL TO HIGHLIGHT BIOMARKERS OF GROWTH-PROMOTING PRACTICES

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Abstract
As leaner meat is expected upon the use of growth-promoting agents in livestock, it can be hypothesised that lipid profile may be disrupted as a consequence of such practice. Although the link between steroids and lipid metabolism has been highlighted decades ago in farm animals, analytical strategies to investigate thoroughly these fine changes did not follow. Technological advances in liquid-chromatography, mass-spectrometry technologies, ^{1}H-NMR and omics strategies are now well suited to address this issue; the question stills need to be addressed in the anabolic research field. The current research presents an analytical strategy for characterizing lipids profile disruption in serum of anabolic treated animals (*bovine* and *porcine* species). Lipidomics data have been assessed as complementary to those obtained by metabolomics from urine and serum investigations and allow deepening our understanding of metabolic pathways disruption upon anabolic practices in *bovine*. All these emerging strategies are efficient ways to highlight candidate biomarkers of such practices in livestock and are considered by the community as promising tools for ensuring safer food to the consumer.

Introduction
For more than twenty years, the use of growth promoting agents in livestock has been banned in Europe (Council Directive 88/146/EEC). To identify potential abuses, various analytical strategies have been implemented, mostly based on the targeted analysis of incriminated compounds or their direct metabolites (Courtheyn *et al.*, 2002; Le Bizec *et al.*, 2009). However, such approaches still hold a few drawbacks and can be blind to the administration of low-dose cocktails or new compounds arising from the black market. Consequently, this led to the development of innovative untargeted approaches, consisting in the investigation of the physiological effects induced by illegal practices. Such so-called omics strategies mainly refer to transcriptomics (study of changes in mRNA-expression), proteomics (study of changes in the protein profile) and metabolomics (the large-scale measurement of small molecules). Applied to the highlighting of anabolic practices in rearing animals, these strategies already proved their relevance using either transcriptomics (Riedmaier *et al.*, 2009; Reiter *et al.*, 2007; Pinel *et al.*, 2010) or proteomics (Mooney *et al.*, 2009; Pinel *et al.*, 2010; Nebbia *et al.*, 2011).

Metabolomics gathered even more interest from the community and appeared to be an efficient approach to investigate biomarkers related to growth-promoting practices (Courant *et al.*, 2009; Dervilly-Pinel *et al.*, 2011; Dervilly-Pinel *et al.*, 2015). Compared to other omics strategies, biomarkers evidenced upon metabolomics studies are considered as easier to subsequently monitor since involving targeted analytical strategies similar to those already available in control laboratories. The metabolome however is very broad which can make the biomarker biological interpretation quite a difficult challenge. Therefore, an alternative trend is to focus on the discovery of biomarkers in reduced parts of the metabolome, such as the steroidome (Kaabia *et al.*, 2013; Blokland *et al.*, 2012), or more recently the lipidome. The lipidome can be considered as a promising field of investigation as past studies hinted at a link between the use of growth-promoting agents and the expression of lipids (Dunshea *et al.*, 1998; Dunshea, 1993).

Using an innovative analytical strategy combining both RPLC-HRMS and HILIC-HRMS, a particular class of lipids was recently highlighted as potential biomarkers for trenbolone acetate and estradiol abuse on bovines (Kouassi Nzoughet *et al.*, 2015a). Although such research work illustrated the relevance of lipid profiling, this study also showed that precise biomarker identification remains a bottleneck and that there is a need for a deeper characterisation of the lipidome. To resolve those difficulties, we propose an original combined lipidomics strategy involving both NMR and mass-spectrometry, for the identification of biomarkers related to the use of trenbolone acetate in *bovine* and ractopamine in *porcine*.

Materials and Methods

Animal experiments
Two separate studies were performed, regarding different growth-promoting agents and animal species as follows:

Ractopamine in porcine. Ten animals were involved and respectively divided into treated and control groups (n = 5 pigs each). Feed containing 10 ppm ractopamine hydrochloride was delivered daily to treated animals, equivalent to a dose of 0.45 mg / kg bw/ day. Blood samples were regularly collected for both groups during the 30-day experiment. Serum was then obtained by centrifugation at room temperature and stored at -80°C.

Trenbolone acetate and estradiol in bovine. Thirty-two 10-14-month-old steers were randomly divided into control and treatment group (n = 16 animals each). Treated animals received a subcutaneous implant of Revalor-XS® containing trenbolone acetate (200 mg, 20 mg/pellet) and estradiol (40 mg, 4 mg/pellet). Regularly, blood samples for both groups were collected during the ten-weeks experiment. Serum was obtained by centrifugation, frozen in liquid N_2 and stored at -80°C.

Sample preparation

Prior to LC-HRMS or ^{1}H-NMR analysis, serum lipid extracts were prepared according to a protocol already described (Kouassi Nzoughet *et al*., 2015), which derived from the Bligh and Dyer protocol (Bligh *et al.*, 1959) further modified by Bird *et al.* (Bird *et al.*, 2011). For each sample destined for LC-HRMS analysis, 30 µL of serum were used to which were successively added 190 µL of methanol, 380 µL of chloroform (containing a 0.5mg L^{-1} internal standard mixture) and 120 µL of water. The mixture was vortex-mixed for 20 s after each solvent addition. After room temperature equilibration, the samples were centrifuged at 8,000 *g* at 4°C for 10 min. A portion of 190 µL of the lower chloroform phase was then taken and evaporated to dryness at 25°C under nitrogen stream. Each extract was then reconstituted in 200 µL of an acetonitrile:isopropanol:water 65:30:5 mixture prior to LC-MS analysis. For the preparation of samples destined for ^{1}H-NMR analysis, a similar protocol was performed up to the evaporation, but using 300 µL of serum, ten-fold solvent volumes (*e.g*. 1,900 µL MeOH, 3,800 µL $CHCl_3$, 1,200 µL of H_2O) and collecting 300 µL of $CHCl_3$ phase. The samples were then reconstituted in 700 µL of deuterated chloroform ($CDCl_3$) and transferred into NMR tubes.

LC-HRMS analysis

Bovine and *porcine* serum extracts were both analysed by liquid-chromatography coupled to high resolution mass-spectrometry. An Agilent 1200 infinity series HPLC system coupled to a Thermo Exactive Orbitrap mass spectrometer equipped with an electrospray (H-ESI II) source was used. Two complementary phases were used for chromatographic separation. Lipid species separation was performed on a reversed-phase Waters CSH C_{18} (100 x 2.1 mm i.d., 1.7 µm particle size) column using ACN:H_2O (60:40) and IPA:ACN:H_2O (88:10:2) as solvent A and B, respectively; both containing 10 mM ammonium acetate + 0.1 % acetic acid. The system was programmed to perform gradient elution as follows: 40–50% B in 2 min, 50–70 %B over a 10 min period, 70–99 %B in 5 min, maintained at 99 %B during 1.5 min, returned to initial conditions over 1.5 min and then hold of these conditions for a further 15 min. The flow-rate was 300 µL min^{-1}, the injection volume was 5 µL, and the column oven was maintained at 55°C. On the other hand, lipid classes were separated using a BEH HILIC (100 x 2.1 mm i.d., 1.7 µm particle size) column from Waters using a buffer solution of 10 mM ammonium acetate/acetic acid, pH 6.8 and ACN as solvent A and B, respectively. The chromatographic separation was performed in gradient elution as follows: 5–30% A in 1.5 min, 30–40% A over a 15 min period, returned to initial conditions over 5 min and then hold on these conditions for a further 13 min. The flow rate was 300 µL min^{-1}, the injection volume was 5 µL, and the column oven was maintained at 35°C. For RPLC experiments, the mass spectrometer was used with "all ion fragmentation" (AIF) MS/MS mode on a 100-2000 *m/z* mass range (Resolution 50,000 FWHM). In-source CID fragmentation was applied (50 eV). For HILIC separation, a full-scan mode was used on a 100-2,000 *m/z* mass range (resolution 100,000 FWHM). Electrospray ionisation mode (ESI) applying polarity switching (positive/negative) was used for all HRMS experiments.

NMR analysis

All the ^{1}H-NMR spectra were recorded at 303 K on a Bruker Avance III 700 NMR spectrometer operating at 700.13 Hz equipped with a ^{1}H/^{13}C/^{15}N/^{2}H cryogenically cooled probe. Various mono- and multi-dimensional pulse sequences were tested in order to assess the most appropriate for lipid profiling of serum extracts. A standard pulse-acquire sequence was used for ^{1}H 1D profiling. For conventional 2D experiments, COSY (^{1}H), TOCSY (^{1}H), and HSQC (^{1}H /^{13}C) were performed. Non-uniform-sampling TOCSY (^{1}H) experiments were also tested with different NUS percentages (*e.g.* number of FID points actually acquired in the F1 dimension / total number of FID points in the F1 dimension) : 100% (=conventional 2D acquisition), 50%, 25%, 12%, 5%. Ultrafast COSY (^{1}H) experiments were also performed.

Data analysis

LC-HRMS data were pre-processed using Xcalibur 2.2, extracted and processed with XCMS, after a conversion with MSConvert (Kessner *et al.*, 2008). Data analysis was then performed with Excel® and SIMCA-P+®. Metabolites of interest were identified using LIPID Metabolites And Pathways Strategy (LipidMaps, www.lipidmaps.org). Pathways were constructed and analysed for disciminant metabolites using metabolomic pathway analysis (MetPa) (Xia *et al.*, 2010) and MetExplore (Cottret *et al.*, 2010). NMR data were processed with Bruker TopSpin 3.2.

Results and discussion

Combining RPLC- and HILIC- HRMS for characterizing lipids profile disruption in serum of anabolic implanted animals.
Two different stationary phases have been used for chromatographic separation of serum lipids. Reversed-phase liquid chromatography (RPLC), using a C18 phase, allowed separating the lipids according to their alkyl chains (Figure 1A), while using a complementary HILIC separation enabled investigating lipid classes according to their hydrophobic head (Figure 1B).

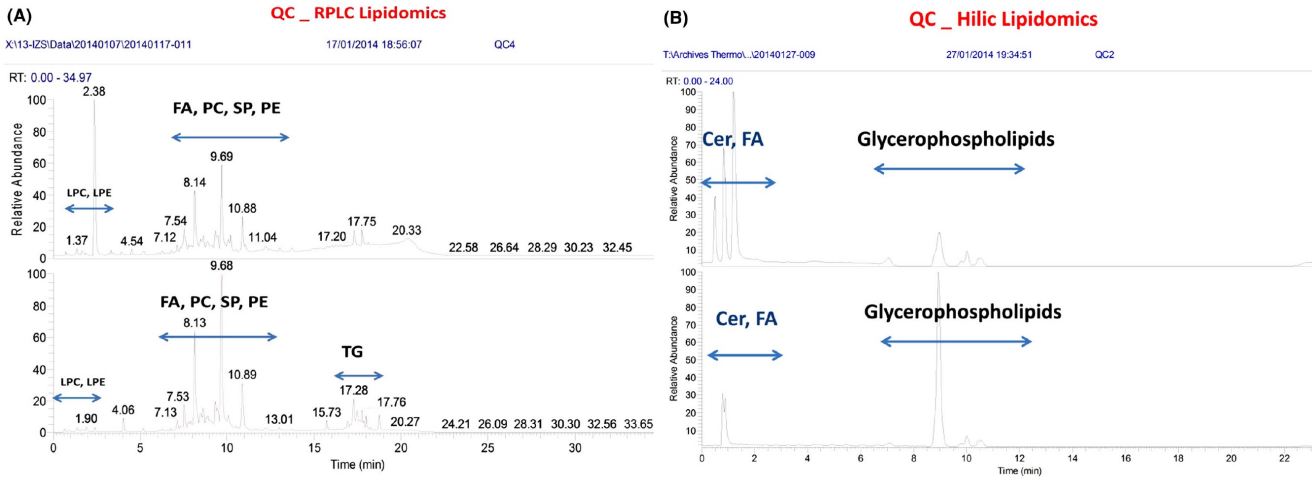
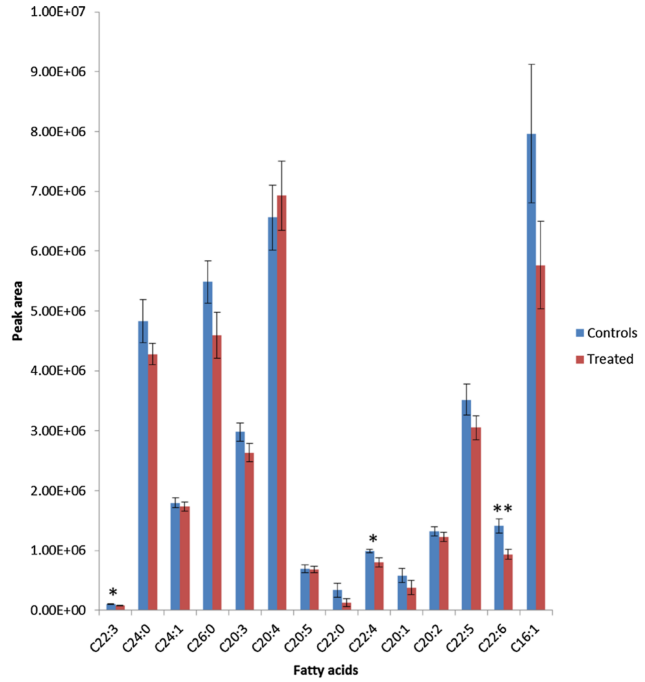


Figure 1. LC-HRMS lipid profile acquired from serum sample in HESI$^+$ (lower graph)/HESI$^-$ (upper graph). A) Total ion chromatograms in RPLC and B) Total ion chromatograms in HILIC. PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, Lyso phosphatidylcholine; LPE, Lyso phosphatidylethanolamine; TG, triglycerides; SP, sphingolipids; Cer, ceramide; FA, fatty acids.

The combination of these two chromatographic separations was used to characterise lipid disruption induced by trenbolone acetate/estradiol in *bovine* serum. The different levels of fatty acids were thus investigated upon HILIC separation and involved a combination of univariate and multivariate statistics to highlight relevant information. Based on HILIC-HRMS data, C22 fatty acids were found to be discriminant between the control and treated groups three weeks after implant administration (Figure 2). Differences in fatty acids levels were significant for C22:3 ($p < 0.05$, Fold Change: 1.29), C22:4 ($p < 0.05$, FC: 1.24) and C22:6 ($p < 0.05$, FC: 1.51), with decreased concentrations in serum from treated animals. Such findings were found in accordance with previous observations reported upon testosterone administration where the levels of unsaturated fatty acids were also observed as decreased (Cinci *et al.*, 1993). A depletion of glycerophospholipids with C22 FA chain three weeks post-implantation could also be measured upon RPLC separation in the present study. Hence, a good matching between RPLC and HILIC observations was obtained. Using these two complementary methods, complex lipids with the same alkyl chain have been putatively identified. Such findings highlight the impact of anabolic steroids on the metabolism of glycerophospholipids such as phosphatidylglycerols, phosphatidylethanolamine, phosphatidylcholine and phosphatidic acid (Kouassi Nzoughet *et al.*, 2015a). Metabolic pathways involved were further visualised using MetExplore (Figure 3) and MetPA softwares, by entering putative lipid class names. Applying both tools to our lipidomics data confirmed glycerophospholipid metabolism as strongly affected.

Development of an NMR lipidomics analytical strategy
To provide deeper and complementary characterisation of the lipidome disruption upon anabolic practice, NMR analysis of serum lipid extracts has been investigated in parallel to MS characterisation. In literature, 1D proton NMR has already been reported for lipid profiling (Jayalakshmi *et al.*, 2010; Annarao *et al.*, 2008; Ekman *et al.*, 2009) whereas 2D NMR has been shown as an appropriate tool for metabolomics, providing a better separation of metabolite signals (Le Guennec *et al.*, 2014). 2D NMR applied to lipid profiling therefore appears as a promising perspective and various sequences from the most advanced multidimensional techniques have been tested in order to assess the most appropriate.
Conventional ^{1}H COSY and TOCSY experiments have first been carried out and compared based on pig serum samples. For the same acquisition time, TOCSY appeared as the most appropriate for the profiling of serum lipid extracts, as more peaks were observed with a better sensitivity. On the contrary, ^{1}H/^{13}C HSQC was not sensitive enough due to the low natural abundance of ^{13}C and this sequence has been discarded.



** p-value < 0.01

* p-value < 0.05

Figure 2. Fatty acid levels in control and treated animals at 3 weeks post-implant (HILIC HESI⁻); significant changes in C22 FA.

Figure 3. Metabolite pathway obtained using MetExplore. The dark blue circles show the metabolites used as input, small light blue circles represent metabolites resulting from the network construction and small squares indicate connecting enzymes (names not shown).

Non-Uniform-Sampling (NUS) acquisition has also been performed. NUS is an acquisition mode in NMR that allows to only acquire a portion of the time increments in the indirect dimension, resulting in the reduction of the acquisition time (for the same number of points in the F1 dimension) or in the improvement of spectral resolution (for the same experiment time) (Le Guennec *et al.*, 2015). TOCSY acquisitions were carried out with different percentages of NUS and compared. A 25% NUS ac-

quisition appeared as the most relevant compromise, yielding an optimal separation of signals in the indirect dimension without reconstruction artefacts as observed with lower NUS percentages such as 5%. The resulting spectrum can be seen in Figure 4a.

The use of hybrid methods based on ultrafast 2D NMR has also been investigated. This approach, relying on a spatio-temporal encoding of the spin interactions, allows the acquisition of 2D NMR spectra with a reduced acquisition time (Frydman *et al.*, 2002). During the last few years, ultrafast NMR has become a powerful analytical tool experiencing an expanded scope of applications (Giraudeau *et al.*, 2014). Its potential for high-throughput quantitative metabolomics was recently demonstrated (Jézéquel *et al.*, 2015). Here, the COSY pulse sequence has been selected, as it is the most robust and sensitive ultrafast pulse sequence. The resulting serum lipid spectrum has been obtained in 26 min (48 scans) and can be seen in Figure 4b. These spectra allowed performing a preliminary peak attribution, although an identification strategy is still to be developed.

While preliminary, these results open promising perspectives regarding the application of multi-dimensional NMR in the field of lipidomics. We expect the multi-dimensional NMR approaches to provide a much better discrimination between different groups of a lipidomics study, as it has been demonstrated for metabolomics (Le Guennec *et al.*, 2014).

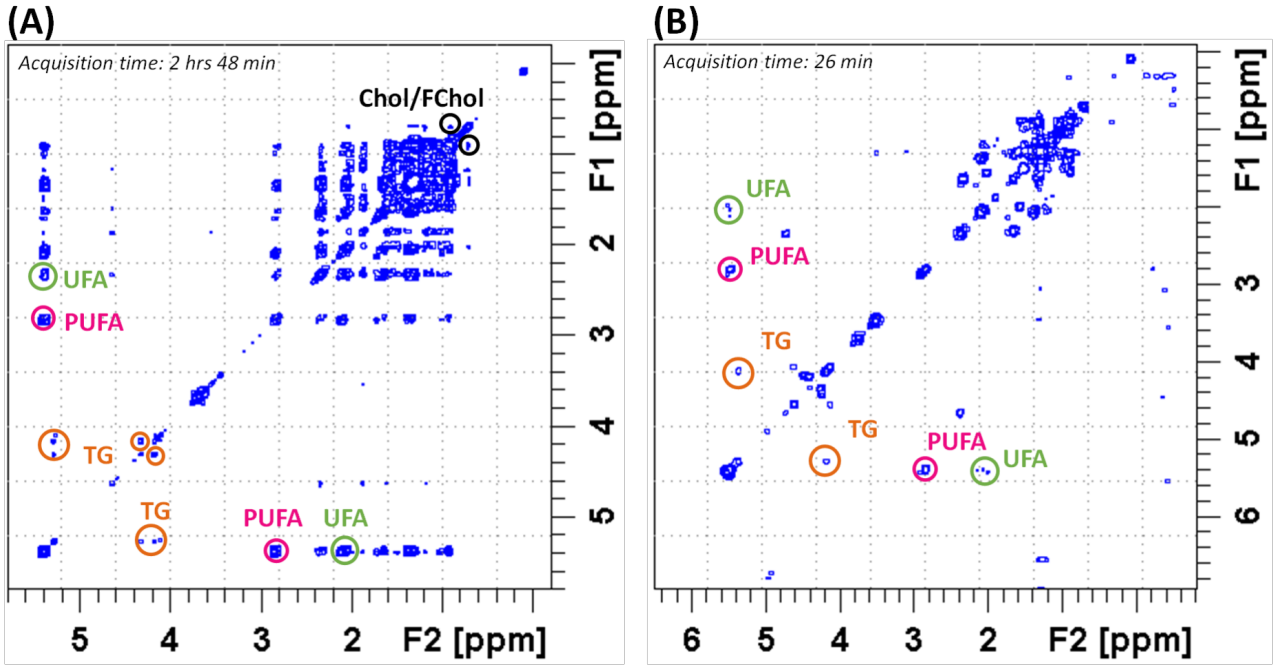


Figure 4. 2D ^{1}H-NMR spectra of a pig serum lipid extract in $CDCl_3$. A: Symmetrised TOCSY acquired in NUS mode (25%). B: Symmetrised ultrafast COSY (48 scans). UFA Unsaturated fatty acids, PUFA Polyunsaturated fatty acids, TG Triglycerides, Chol Cholesterol, FChol Free Cholesterol.

Conclusions

Profiling lipids in serum therefore appears as relevant to highlight the administration of growth promoters in rearing animals. The present study also demonstrated that lipidic biomarkers reveal their interest in more distant dates of administration compared to biomarkers that may have been discovered investigating either the global metabolome (Nzoughet *et al.*, 2015b) or an alternative biological matrix such as urine (Dervilly-Pinel *et al.*, 2011; Jacob *et al.*, 2015). Therefore, both metabolomics and lipidomics derived biomarkers may be considered as complementary allowing detecting growth-promoting practices over large detection time windows.

In order to deepen the interest of lipid profiling, a strategy combining LC-HRMS and NMR is proposed and its preliminary work is presented, applied to the misuse of ractopamine in *porcine*. NMR and in particular NUS TOCSY acquisition and ultrafast COSY appear as very promising for lipid profiling. This strategy still needs to be optimised and completed with proper sample preparation but will hopefully allow us to perform a thorough characterisation of the lipidome and reveal relevant biomarkers.

Acknowledgements

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O26
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THE DUTCH APPROACH FOR THE DETECTION OF (SYNTHETIC) NATURAL STEROIDS IN THE NETHERLANDS: A RETROSPECTIVE OVERVIEW

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Abstract

For years, laboratories are struggling in the residue field with the detection of illegally administrated synthetic natural hormones in cattle. From seized preparations we know that these synthetic natural hormones are still used to fatten cattle. In the last years, a new state of the art analytical techniques was developed to detect abuse of synthetic natural steroids. In The Netherlands, we have implemented a unique control strategy of first screening analysis for a large number of steroids (steroid profiling) followed by confirmation analysis based on GC-C-IRMS measurements. This approach has been applied in Dutch National Residue Control Plan for the last two years. From more than 600 samples, individual steroid profiles were determined. Each individual steroid profile was then projected into reference populations. In case the sample was projected in the population of treated animals, it was reanalysed with GC-C-IRMS. Our current GC-C-IRMS confirmatory analysis enables confirmation of synthetic testosterone and estradiol in urine. Next to a working control strategy, we now have insight in steroid profiles and distributions of individual steroid concentrations of the Dutch *bovine* population. In this presentation a retrospective overview of the results of the steroid-profiling analysis and GC-C-IRMS confirmatory analysis is presented.

Introduction

The analytical problem of the presence of (synthetic) natural hormones in biological samples has been an issue of concern and debate for decades. Synthetic natural hormones used to fatten animals are in general testosterone and estradiol. The compound testosterone is the major androgenic steroid in all food-producing animals and is an effective growth-promotor. The use of estradiol as growth-promoter is highly debated in the past decades. The hormone dispute for the World Trade Organization (WTO) in fact completely focussed on this compound and its possible harmful properties. Testing for these synthetic natural sex hormones should be performed in accordance with Council Directive 96/23/EC (live *bovine* animals at the farm or at the slaughterhouse). A level of estradiol or testosterone exceeding those given in Table 1, in blood plasma of at least one *bovine* animal, was an indication that possible treatment with synthetic natural steroids occurred.

Table 1. Recommended values for routine monitor for synthetic natural hormone abuse.

	Age (months)	Maximum plasma concentrations	
		Male *bovine* (µg L^{-1})	Non-pregnant female *bovine* (µg L^{-1})
Free 17β-estradiol	≤ 6		0.04
	≤ 18	0.04	
Free 17β-testosterone	≤6	10	
	6 – 18	30	
	≤ 18		0.5

These values remained the "officially recommended values" for decades, only slightly modified in 2007 when the CRL Guidance Paper was published. The importance of confirmatory analyses made it necessary to increase the level of 0.04 µg L^{-1} to 0.1 µg L^{-1}, the lowest limit for confirmatory analyses practically achievable at that time. Confirmation in this case meant confirmation of the identity of the compound, not whether it is synthetic or natural.

Detection of natural hormone abuse can roughly be divided in screening and confirmation analysis. Examples of screening analysis are: the threshold approach as described above, indirect measurement of a unique marker after treatment, changes in steroid profiles after treatment. All of these approaches are indirect. None of the methods provides an indication of the presence of a synthetic natural hormone of which the mass-concentration and identity can be subsequently confirmed during confirmatory analyses. Examples of such confirmation analysis are: detection of steroid-esters in hair, isotope ratio mass-spectrometry. Confirmatory methods for natural hormones or their markers must fulfil two separate conditions under EU regulations. As for all banned substances, their identity must be confirmed on the basis of criteria laid down in Commission Decision 2002/657. However, the compound(s) determined must be sufficiently diagnostic to "prove" abuse with a synthetic natural hormone. Some examples of screening and confirmation analysis are provided in the next paragraphs.

Screening analysis

To determine concentrations of natural hormones, immunoassays are popular, which are frequently based on methods originally developed for diagnostic purposes in human medicine. Also methods based on analytical techniques such as GC-MS and LC-MS/MS are used frequently to determine concentrations. These concentrations can be used for monitoring using the threshold approach (Table 1).

Biomarkers are substances which can be used to identify specific animals, *e.g*. animals treated differently from corresponding control animals (1) published a metabolomic study in which the influence of a treatment with estradiol or progesterone on the high-resolution mass-spectrometric profile was determined. This untargeted approach resulted in sets of potential biomarkers for both treatments. The structure of the most important biomarkers was elucidated and the discriminating power of this biomarkers was evaluated (2). Another novel approach is based on transcriptomics RNA-sequencing. In a study (3) in heifers, of a set of 40 selected mRNA candidates, a set of 20 was significantly regulated. With principal component analyses it was possible to discriminate animals treated with a combination of trenbolone-acetate and 17β-estradiol from a control population.

A relatively new approach for screening is the use of steroid profiling. Individual steroids are part of a complex physiological system balancing between the different active steroids, their precursors and metabolites. The hypothesis for screening methods based on steroid profiling, which implies the quantitative analyses of a large number of individual compounds, is that the combined effect on all the individual compounds provides a diagnostic tool for detecting abuse. Anizan *et al*. (4) published a study in which the excretion of 25 known conjugated compounds (phase II metabolites) before and after administration of androstenedione was followed. Blokland *et al*. (5) published a study on the effect of treatment with several natural hormones on the steroid profiles for 17 steroids, aglycons, glucuronides and sulphates. Multivariate statistical analyses showed that the model could be used to classify animals into a treated or untreated group. Both studies show the potential of steroid profiling as a promising strategy to determine whether *bovine* animals have been treated with (natural) hormones or not.

Confirmation analysis

The detection and confirmation of steroid-esters in biological samples is one of the oldest analytical approaches for proving abuse of (natural) hormones. However, for many years its applicability was limited to the analyses of alleged injection sites. In recent years the analysis of intact hormone esters gained renewed interest based on new knowledge concerning the incorporation of steroid-esters in hair and the increased analytical possibilities to detect and confirm very low concentrations of steroid-esters in serum. However, the use of hair for the direct analysis of steroid esters has been limited. Part of the reluctance to use hair as an analytical matrix is due to the risk of external contamination of the animal. A study (6) showed that, at least for clenbuterol, the chances of such (accidental) external contamination are very limited.

In a study of Kaabia *et al*. (7), a new UPLC–MS/MS method was described allowing the detection of steroid-esters in serum of breeding (*bovine*) and racing (*equine*) animals. The time during which the steroid-esters can be detected in the blood, after intramuscular injection, depends on two factors: the time needed for the steroid ester to reach the blood stream and the efficiency of the esterase activity present in the blood for the specific steroid ester. The next critical parameter is the sensitivity of the analytical method. In order to allow the simultaneous detection of estrogenic and androgenic esters, it was necessary to derivative the hydroxyl-group of estradiol and related structures through dansylation. This derivatisation step has a positive influence on the detection limit, which was 0.02 µg L^{-1} for most estradiol esters, with the exception of estradiol decanoate (0.1 µg L^{-1}). For the androgens testosterone and nandrolone the limit of detection (LOD) ranged from 0.02 to 0.05 µg L^{-1}. After intramuscular injection of 17β-estradiol-benzoate the maximum concentration in serum was reached after nine days. The release of the esters is more rapid for shorter chain than longer or aromatic ester chains, explaining their shorter detection time window.

Isotope Ratio Mass Spectrometry (IRMS) is a versatile application to determine the isotopic composition – usually expressed as a ratio – of a wide range of materials and compounds. IRMS is suitable to determine isotope ratios of the lighter elements, which include bio-elements such as carbon (δ13C), nitrogen (δ15N), hydrogen (δ2H), oxygen (δ18O) and sulphur (δ34S). Because isotope ratios provide crucial information about fundamental processes and the patterns that emerge from these processes, IRMS is used in many research fields. By use of IRMS it is possible to distinguish natural (endogenous) and synthetic (exogenous) hormones in sportsmen and farm animals, for which compound-specific isotope analysis is required (8-10).

For the Dutch Routine monitoring a control strategy was developed to detect natural hormone abuse, which consist of a screening based on steroid profiling and confirmation based on IRMS measurement.

Materials and Methods

Over the last years, more than 600 samples of urine from male and female animals were collected. Most of the samples were from young animals aged less than nine months. Samples were collected at different periods over the year. First the samples were screened by steroid profiling analysis. After statistical analysis, samples which had a profile different from that of the reference population, were selected for confirmatory analysis. Confirmation analysis was performed by GC-IRMS-MS (11) by which the occurrence of synthetic natural hormone testosterone and estradiol can be confirmed. For this purpose the $^{13}C/^{12}C$

isotope ratio of the main metabolites 17α-estradiol and 17α-testosterone were compared with dehydroepiandrosterone (DHEA) as an endogenous reference compound (ERC) to prove the exogenous or endogenous origin in cattle urine. The use of ERCs is necessary to compensate for variability of the $\delta^{13}C$ values mostly caused by differences in animal diet. If a significant difference of $\delta^{13}C$ values between ERC and the target metabolite is observed, this is considered proof of administration of synthetic natural hormone administration.

Results and Discussion

The new Dutch control strategy for detection of synthetic natural hormone abuse was introduced in 2014 in routine control programs of the Netherlands and replaced the till then applied threshold approach. The screening analysis (steroid profiling) consist of the analysis of a large number of pro-hormones, steroids and metabolites, a change in their mutual relation can be caused by external administration of synthetic natural hormones but also by other external influences. Steroid profiles from non-treated populations and of animals treated with DHEA, pregnenolone, estradiol and testosterone were measured. The profiles of the treated animals are compared by multi variate statistical analysis with the non-treated groups. See Figure 1 for example of statistical analysis of the different groups.

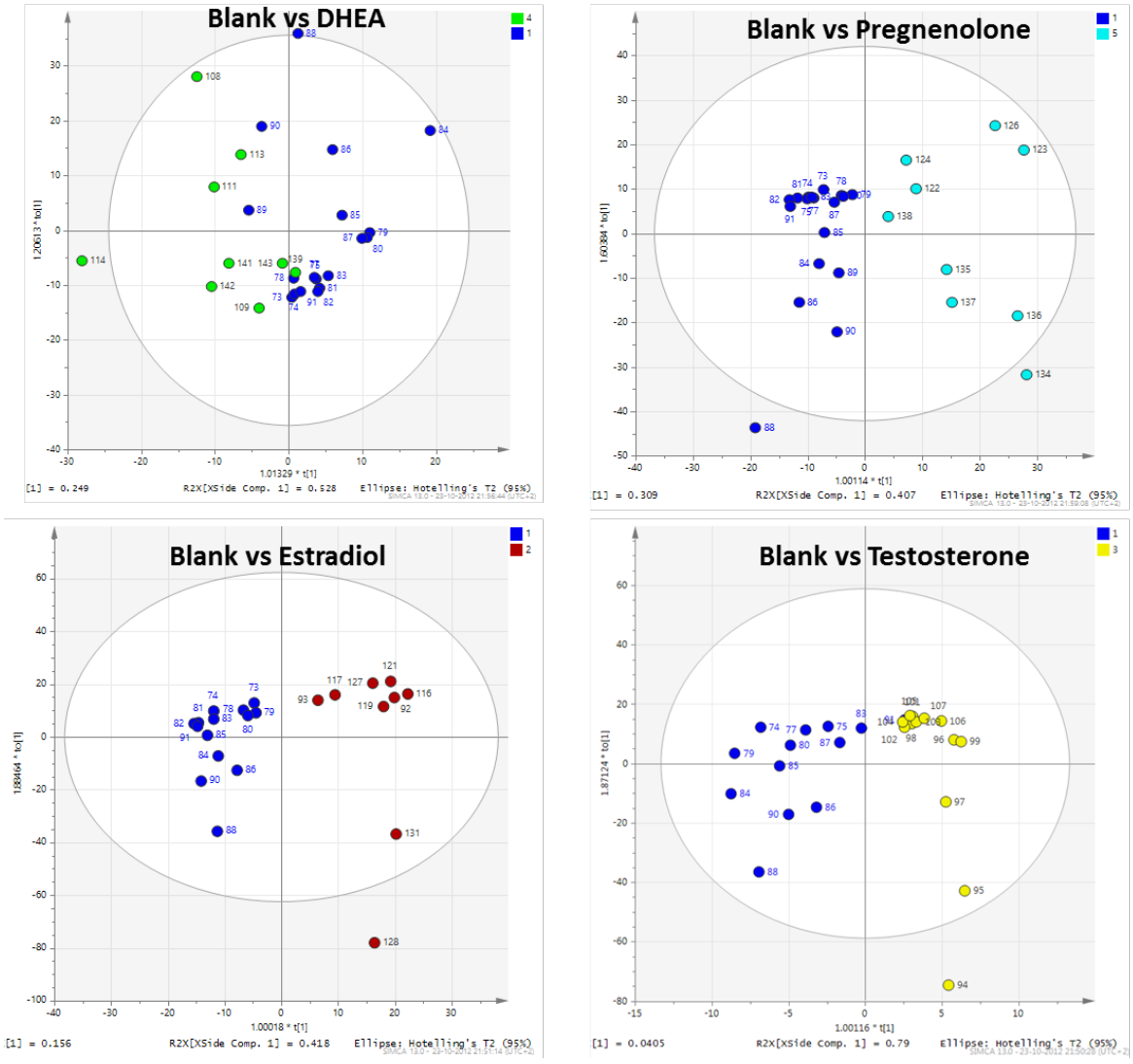

Figure 1. OPLS-DA analysis of different treatments with blank population.

In 2014 and 2015, samples taken at farms for routine monitoring are analysed with steroid profiling. The measured steroid profiles are projected in the different models as depicted in Figure 1. After projection, samples are classified as normal or different from the normal population. More than 600 samples were screened with steroid profiling, most samples were considered as normal but approximately 10 percent was considered as different. These samples were further analysed with isotope ratio mass spectrometry.

Confirmation analysis is performed by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). In Figure 2 examples are given of GC-C-IRMS analyses of animals that were treated with testosterone and estradiol.

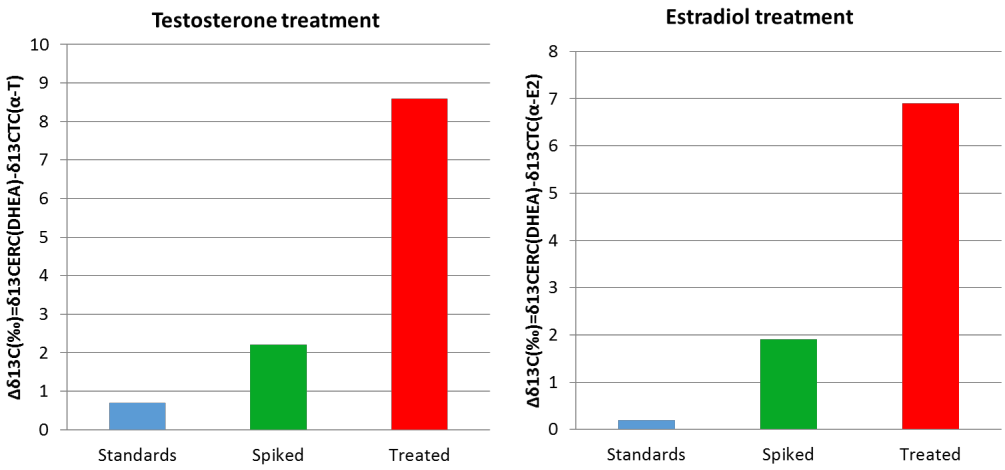

Figure 2. Comparison of the Δδ13C (‰) values of standards, spiked samples of urine and treated animals for testosterone and estradiol treatment.

By comparing urine samples from treated and untreated animals for testosterone and estradiol the resulting $|\Delta\delta^{13}C|$ values were 6.9‰ for 17α-estradiol and 8.6‰ for 17α-testosterone. In our laboratory, a threshold value of four is used to separate non-treated from treated animals. In this example the samples of the treated animals are above this value, clearly showing the capability of the method to detect positive samples.

Most samples selected from the steroid profiling had a delta value lower than 4 for estradiol and testosterone, indicating that these compounds are natural steroids. In one sample a delta value for testosterone that was higher than 4 was found, indicating that the origin was from a synthetic natural hormone.

At this stage it is clear that the majority of "different" profiles is not caused by the presence of synthetic natural hormones, several explanations are possible and remain to be studied further.

1. The differences in steroid profiles are not caused by natural hormones, but by other (synthetic) hormones not tested for at this stage;
2. The differences in steroid profiles are caused by synthetic natural hormones, but the levels in the sample are too low to be detected as such (the effect remains present longer than the residues);
3. The differences are not only caused by synthetic hormones, but also by other mechanisms, *e.g*. stress.

Conclusions

The last two years, a unique control strategy to detect natural hormone abuse was applied in routine control monitoring in The Netherlands. It consists of screening analysis for a large number of steroids (steroid profiling), followed by a confirmation analysis based on GC-C-IRMS measurements. For more than 600 samples, individual steroid profiles were determined. From the screened samples approximately 10 percent was selected for confirmatory analysis with GC-C-IRMS. From this analysis, one sample had an isotope pattern that indicates it was treated with a synthetic natural hormone.

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ARE BIOMARKERS UNIVERSAL AND TRANSFERABLE?

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Abstract
Within the particular context of controlling residues in food from animal origin, an alternative to targeted approaches has emerged; it consists in the characterization of physiological perturbations induced upon exposure of the animal to a given chemical substance to highlight suitable biomarkers addressing safety and/or regulatory issues. Metabolomics in particular has been investigated in the hope of identifying such biomarkers, and some studies have from that time forward demonstrated the efficiency of the strategy. Steps toward official or commercial implementation of corresponding (screening) tools are however still to be taken. Besides the need to harmonise and establish internationally accepted protocols to be applied to such methods, scientists are still facing the question of biomarkers universality and transferability.
Biomarker's universality is closely related to its specificity and its ability to be considered as generic toward, for instance, animal species, route of exposure, chemical analogues, chemical mixtures, while biomarkers' transferability refers to the capacity of, first, highlighting them and then monitoring them on different instruments, different technologies and in different laboratories. The associated issues will be discussed during this presentation.

Introduction
Issues encountered in controlling the use of growth promoters in livestock's led over the last past 10 years to the development of innovative untargeted approaches, consisting in the investigation of the physiological effects induced as a consequence of illegal practices. The objective is to reveal biomarkers of exposure that may subsequently be monitored for screening purposes. Such so-called omics strategies mainly referring to the study of changes in mRNA-expression, protein or small molecules profiles, have already proven their relevance using respectively either transcriptomics (Reiter et al. 2007; Riedmaier et al. 2009b, 2009a), proteomics (Cacciatore et al. 2009; Mooney et al. 2009a; Mooney et al. 2009b; Nebbia et al. 2011), or metabolomics (Courant et al. 2009; Pinel et al. 2010; G. Dervilly-Pinel et al. 2012 and 2015).

Compared to other omics strategies, metabolomics gathered more interest from the residue-control world since it involves analytical platforms similar to those already available in research laboratories in this area. Furthermore, biomarkers evidenced upon metabolomics studies are considered as easier to subsequently monitor since involving targeted analytical strategies close to those already available in corresponding laboratories in charge of the control.

Although metabolomics research work performed up to now is a mandatory step in initial assessment of the strategy, steps toward official or commercial implementation of corresponding screening tools are still to be taken (Esslinger *et al.* 2014; Riedl *et al.* 2015). As these methods do not target specific chemical species *per se* there is in particular a need to harmonise and establish internationally accepted validation and standardisation protocols to be applied to such methods (Creek *et al.* 2014; Gika *et al.* 2014; Martin *et al.* 2015). These have to be thoroughly developed and shared with actors implicated in the field (researchers, control laboratories, risk assessors, policy makers) in order to enable universal acceptance.

Although many guidelines for validating targeted methods are available and provide actors with performance indicators (*e.g.* pesticides (SANCO 12495/2011), pharmacological drugs (Dec 2002/657/EC), such equivalent guidelines for untargeted method validation are not available yet, which certainly explains their limited application for routine or official control purposes. The main difference between non-targeted and targeted approaches is related to the multivariate nature of fingerprints in the former case, compared to single or multi-chemical species analysis in the latter one. In targeted strategies, results and method performance are evaluated compound-by-compound using univariate statistics, while multivariate analysis (which are generally referred to chemometric methods) are required to evaluate relevance of patterns arising from fingerprinting approaches (Godzien *et al.* 2015; Alonso *et al.* 2015). Guidelines for biomarkers/models discovery and validation are therefore urgently required by the scientific community (Naz *et al.* 2014; Goodacre *et al.* 2007).

Besides the need to harmonise and establish internationally accepted protocols to be applied to such methods, scientists are still facing the question of biomarkers universality and transferability. Biomarker's universality is closely related to its specificity and its ability to be considered as generic toward for instance animal characteristics, route of exposure, chemical analogues, chemical mixtures, while biomarkers' transferability refers to the capacity of, first, highlighting them and then monitoring them on different instruments, different technologies and eventually in different laboratories.

This study aims to illustrate these different aspects by taking various examples of metabolomics and steroidomics studies conducted over the past years and consisting in the research of biomarkers, in urine or blood, signing in animals the administration of growth promoters, mainly steroids and β-agonists.

Materials and Methods

Biological samples

Urine and blood samples have been collected in the frame of several animal experiments as described in Table 1. In addition, a large number of control samples have been including in all studies to ensure significant variability to be considered within the developed models.

Table 1. Details of animal experiments protocols used to assess biomarkers robustness

Exp.	Animals	Country	Growth promoters		Treatment	Matrix
A	18 Calves (12 C / 6 T)	FR	β–agonists	Clenbuterol	Oral, 8 µg kg^{-1} bw, 21 days	Urine
B	17 Calves (12 C / 5 T)	FR	β–agonists	Clenbuterol	Oral, 6 µg kg^{-1} bw, 6 days	Urine
C	1 Calf	FR	β–agonists	Clenbuterol	Oral, 6 µg kg^{-1} bw, 6 days	Urine
D	3 Calves	FR	β–agonists	Clenbuterol, ractopamine, cimaterol, zilpaterol, mabuterol	Oral, 1 µg kg^{-1} bw each, 5 days	Urine
E	3 Heifers	FR	β–agonists	Clenbuterol, ractopamine	Oral, C: 10 µg kg^{-1} bw or R:200 µg kg^{-1} bw or C+R 1 µg kg^{-1} bw and 20 µg kg^{-1} bw, once	Urine
F	16 Calves (8 C / 8 T)	IT	β–agonists	Clenbuterol	Oral, 8 µg kg^{-1} bw, 21 days	Urine
G	12 Calves (6C / 6T)	FR	Steroids	Boldenone undecylenate	IM, 2 mg kg^{-1} bw	Urine
H	32 Steers (16C / 16T)	IT	Steroids	Trenbolone acetate + estradiol benzoate	Implant, TA: 200 mg, 20 mg/pellet, E : 40 mg, 4 mg/pellet	Urine / Blood
I	24 Calves (12C / 12T)	NL	Steroids	Estradiol benzoate + Nortestosterone laureate	IM, EBz: 25 mg, NT: 150 mg	Urine
J	1 Stallion	FR	Steroids	Nortestosterone laureate	IM, 600 mg	Urine / Blood
K	1 Gelding	FR	Steroids	Testosterone esters (propionate, phenylpropionate, isocaproate, decanoate)	IM, 500 mg	Urine
L	1 Gelding	FR	Steroids	Testosterone esters	IM, 500 mg	Urine
M	1 Mare	FR	Steroids	Boldenone undecylenate	IM, 400 mg	Urine

Steroidomics general workflow

A large panel of steroids of interest (n>20) are extracted from *equine* urine and plasma samples using a C18 solid-phase cartridge. The analytes are submitted to methanolysis in order to release the free forms. Subsequently, liquid-liquid and solid-phase extractions purifications are performed before derivatisation and analysis on GC-MS/MS for quantification. Data are further analysed with SIMCA-P+ software to set up descriptive and predictive models and highlight candidate steroid biomarkers.

Metabolomics general workflow

Urine and serum samples are filtered over 10 kDa membranes under centrifugation at 10,000 *g* at 5°C for 20 min. Urine samples are further normalised. Chromatographic separation is performed with a high performance liquid chromatography (HPLC) system (1200 Infinity Series from Agilent Technologies, Santa Clara, California, USA) on a Hypersil Gold C18 column (2.1 mm x 100 mm, 1.9 µm particle size, Thermo Fisher Scientific) or a BEH HILIC (100 9 2.1 mm2 i.d., 1.7 lm particle size). Mobile phase consists in water containing 0.1% acetic acid (A) and acetonitrile containing 0.1% acetic acid (B). Liquid Chromatography - High Resolution mass spectrometry (LC-HRMS) fingerprints are acquired either on an LTQ-Orbitrap/ Exactive or Q-exactive mass-spectrometer (Thermo Fisher Scientific, Bremen, Germany) in switching (+/-) heated electrospray ionization mode. Full scan mass-spectra are acquired from *m/z* 80 to *m/z* 1000 using a mass resolution of 50,000 FWHM at 400 *m/z* in centroid mode. Data are processed by the open-source XCMS software and analysed with SIMCA-P+ software to set up descriptive and predictive models and highlight candidate biomarkers.

Results and Discussion

Biomarkers specificity

Toward experimental conditions and animals characteristics (breeding conditions, location, age, gender,...). Once robust models have been established and biomarkers highlighted as the result of untargeted approaches, next step is to check the validity of both the models and the markers toward a range of parameters that may induce high variability and could interfere with the markers response, leading thus to decreased performances of the models and possible erroneous status prediction. Such specificity assessment therefore constitutes a pre-requisite for models validation. While parameters such as breeding conditions, feeding, geographical place etc. are expected not to disturb the biomarkers' response, parameters related to animals age, breed or sex may affect biomarkers response in a stronger way. Evaluating these different parameters contributes at establishing the biomarkers, and corresponding prediction model's, scope of application.

A metabolomics model dedicated to β-agonists screening in calves (Holstein-Friesian) has been established based on exp. A (Table 1). The specificity of the established model has been tested, using the same drug and dose, with i) different calves of the same breed, reared at the same place (France) 2 years later (exp. B), ii) 1 calve of a different breed (Montbéliard) (exp. C), iii) different calves (Friesian) reared at a different place (Italy) 5 years later different (exp. F) (Figure 1), and finally iv) adult female animals (exp. E). Associated prediction results allowed extending the application of the tool to a range of calves's breeds and also to bovines other than male calves, whatever the breeding place and conditions, ensuring thus a potential for applicability of the newly developped screening tool (Dervilly-Pinel *et al.* 2015).

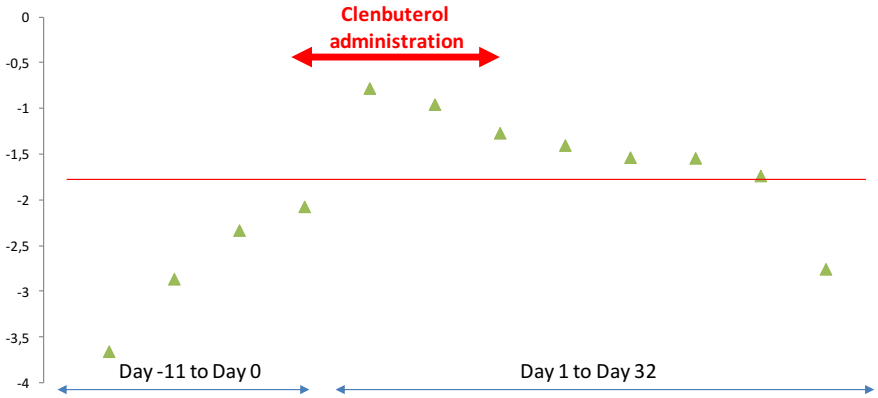

Figure 1. Prediction of urine samples (exp. F) status in the model created based on exp. A. Red line = suspicious threshold.

Toward different drugs and/or doses. Experiments D and E further enabled assessing performances of the metabolomics model with regards to compounds, doses and coktails. Besides confirming relevance of the model to predict different β-agonists treatments than the clenbuterol-based original one, the model was proven efficient in screening also lower treatment doses, as well as mixtures. The results therefore suggest an extended scope of application to the screening of any β-agonists related practice in *bovine* animals (Dervilly-Pinel *et al.* 2015).

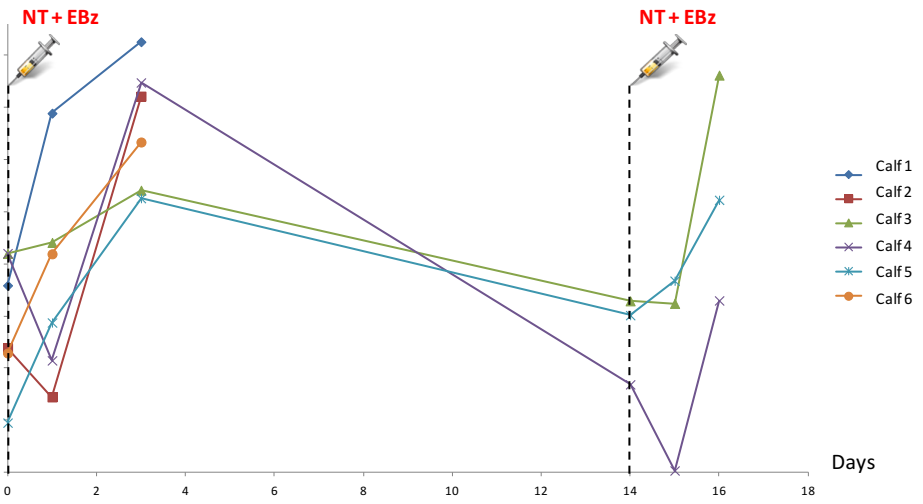

Figure 2. Prediction of urine samples collected on steroid treated calves (exp. I) status, using the validated β-agonist model (exp. A to F).

Toward different growth promoters classes. Biomarkers with proven relevance in highlighting β-agonists abuse in *bovine* have further been tested for their potential ability in signing steroids administration (exp. H and I). As shown in Figure 1 for exp. I, the biomakers' profiles in urine of treated animals appears as modified upon steroids administration which means such biomarkers are not only specific of β-agonists treatment but are also involved in steroids related effect. These biomarkers can therefore be considered as rather generic, allowing extended screening of growth promoting practices in bovines, although suspicious thresholds should be adapted in that context.

Regarding *equine* species, a metabolomics model was established to screen for testosterone abuse using exp. K and validated with samples from exp. L as challenge test. Specificity of the model regarding other anabolic steroids was further assessed using samples collected within exp. J and M. As illustrated in Figure 3, the model which involves four biomarkers is efficient in highlighting modified profiles, therefore enabling broad screening related to androgens administration in horses.

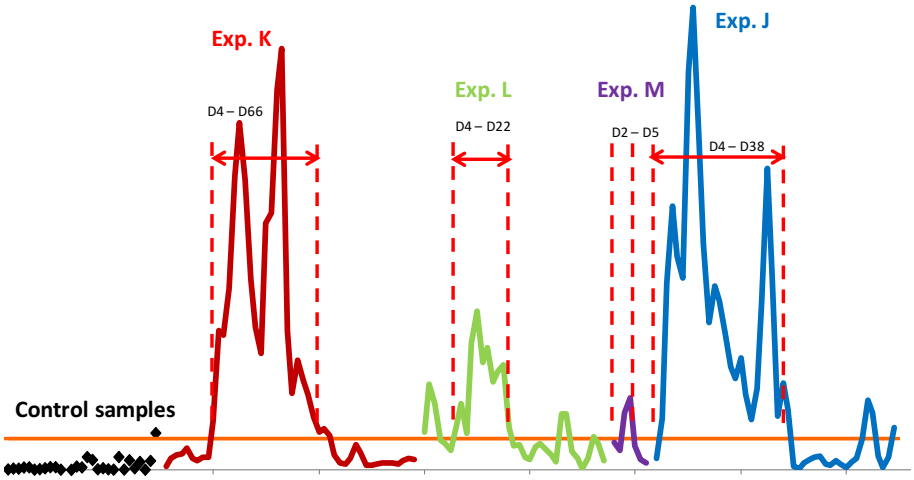

Figure 3. Prediction of urine samples (exp. L, J and M) status using a model validated to screen for testosterone abuse in horses (exp. K).

Depending on the biomarkers origin. While some biomarkers highlighted as the result of global untargeted metabolomics studies may be in some particular cases, as illustrated above, considered for broader applications than the one for which they were initially discovered, the question of such generalisation may be asked when considering a reduced part of the metabolome to investigate biomarkers in, such as the steroidome one.

The steroidome constitutes indeed a particular interesting sub-metabolome fraction to look for biomarkers in, as recently demonstrated using urine and plasma samples collected in the frame of exp. J (Kaabia *et al.* 2014). In particular, a model based on the combined contributions of eight specific urinary biomarkers was proposed for efficient long term screening of nandrolone abuse in the horse (exp. J). Applying a similar steroidomics workflow, urine samples collected during exp. K enabled highlighting 9 steroid biomarkers to screen for testosterone esters administration in horses. The comparison of these two models showed that although some biomarkers were found in common (*e.g.* DHEA, estradiol and testosterone), their respective weights in the models where different, but overall, additional and different steroid markers were required in both models to ensure efficient long term screening of the considered practice, even though both cases involved drugs belonging to the androgens class (Figure 4).

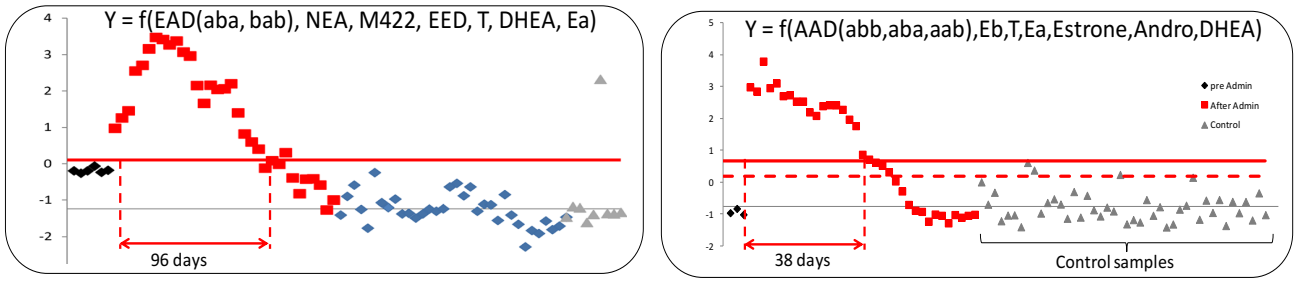

Figure 4. Statistical models built on the basis of the combined contribution of selected steroids in urine and prediction of samples collected within exp. J (left) and K (right), together with control samples.

All these results serve to highlight the comparative advantages of both approaches: biomarkers discovered by untargeted metabolomics strategy generally allow for a more comprehensive screening of anabolic practices while steroid biomarkers

highlighted using semi-targeted profiling provide a more restrictive but specific screening, over longer periods of time than those generally proposed by metabolomics based biomarkers.

Biomarkers transferability

While biomarkers specificity as described above is related to the biology of the animals and the physiological effects induced upon growth-promoters administration, biomarkers' transferability refers to technological aspects, such as the capacity of detecting and monitoring them on different instruments, using different technologies and in different laboratories.

Discovering relevant biomarkers using different analytical workflows. Although main steps of metabolomics studies are well described and generally involve sample preparation / sample fingerprinting / data processing / data analysis and structural elucidation steps, the parameters selected by the researchers at these different steps of the workflow may differ from a study to another one, from a research group to another, which poses the question of the possibility to highlight the same biomarkers when different analytical conditions are used. To answer such question, the investigation of urine samples collected in exp. H using either an Exactive or a Q-Tof sytem for the fingerprinting step was performed. While global fingerprints were slightly different, in terms of number of detected features, similar relevant ions (n=23) could however be highlighted in both studies, enabling establishing similar robust descriptive and predictive models (Figure 5), confirming the (expected) possibility to reveal same biomarkers using different analytical platforms.

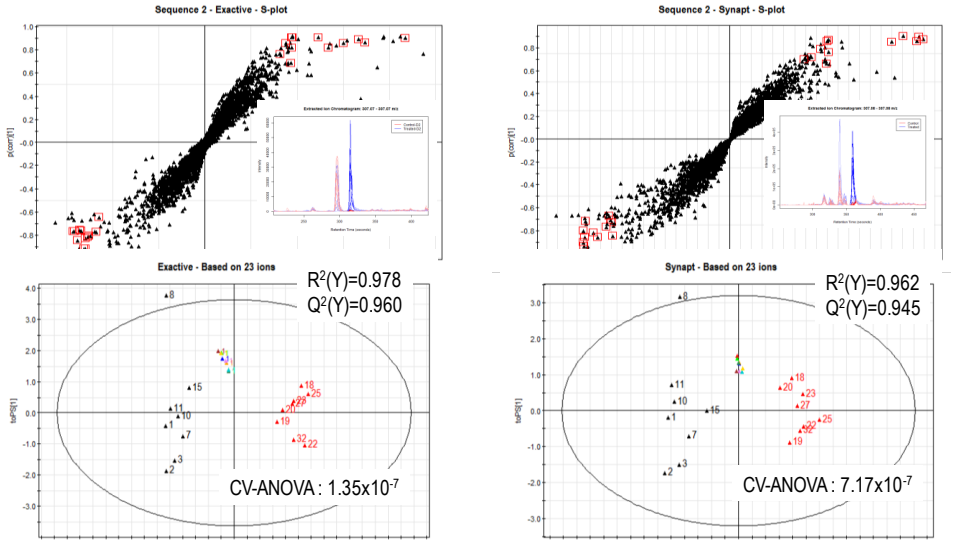

Figure 5. Statistical models built on the basis of the contributions of 23 urinary biomarkers (exp. H) highlighted using two different high resolution mass spectrometric technologies, an orbital trap (left) and a TOF (right).

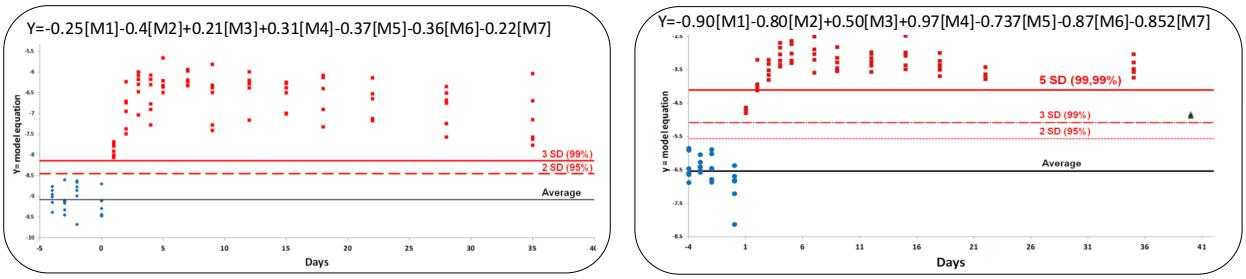

Figure 6. Statistical models built on the basis of the contributions of 7 urinary biomarkers (exp. G) and involving (left) untargeted detection using full HRMS-Q-Exactive and (right) targeted detection using SRM-QqQ with refined model's equation.

Transferring biomarkers monitoring on routine instrument. Once biomarkers have been discovered and validated typically on a research dedicated instrument (*i.e.* involving High-resolution MS), next expected step consists in transferring their monitoring to more routine dedicated instruments (*e.g.* triple quadrupole MS). A number of key challenges however has to be overcome to deliver such objective: i) the identification of specific fragmentation patterns to establish SRM transitions for targeted monitoring and ii) the adaptation of chromatographic conditions to both allow detection of the signal of interest in a known Rt range and to reduce the chromatographic separation time to fulfil high sample throughput requirements. Once efficient transfer of biomarkers detection from an untargeted to a targeted approach is effective, the weighted contribution of each biomarker involved in the model needs being recalculated to establish new models with up-dated equations, and

finally setting revised suspicious threshold. Such strategy was tested using samples collected within exp. G. The performances of the refined model, ready for routine targeted LC-MS/MS analysis, were in accordance with previous untargeted models obtained from HRMS instruments, resulting in a sensitive, reliable and high-throughput method based on targeted quantification of specific metabolites (Figure 6).

Conclusions

While technological issues associated to biomarkers transferability are still challenging tasks, they can be overcome and should not be considered as bottleneck for the routine implementation of biomarkers-based screening tools. The real challenge lies in the biological validation of the discovered biomarkers, using crash tests, and the definition of the scope they allow covering (*i.e*. their specificity), with associated performances in terms of false negative rate.

Acknowledgements

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DISCOVERY OF A URINARY BIOMARKER TO DISCRIMINATE BETWEEN SEMI-ENDOGENOUS AND EXOGENOUS THIOURACIL IN CATTLE

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Abstract
In the European Union, the use of thyreostats for animal fattening purposes has been banned since 1981. The main reason for this relates to their teratogenic and carcinogenic properties, which implicates that any thyreostat residue may connote a risk for human health. However, monitoring potential abuse is strongly hampered by the semi-endogenous prevalence of some thyreostats, including thiouracil (TU). Although a recommended urinary concentration of 10 μg L^{-1} has been proposed to discriminate between endogenous and exogenous TU, recent epidemiological work has shown that this threshold is not conclusive. Therefore, this study aimed to reveal a urinary biomarker to unambiguously describe the true origin of detected TU. For this purpose, a comprehensive *in vivo* study was carried out in which *bovines* (n = 34) were subjected to a synthetic TU administration (1 g per day), a rapeseed-enriched (30%) diet (known to induce TU excretion) or a control treatment. In order to point out relevant metabolome differences between treatments, a metabolomics strategy was applied. Urinary fingerprints (up to 40,000 metabolites) were thereby acquired through UHPLC coupled to high-resolution hybrid Orbitrap™ MS and processed by multivariate data-analysis (OPLS-DA). As such, two promising candidate biomarkers were revealed for both cows and calves. The use of such biomarkers in practice would constitute a valuable complement to the residue control plan, permitting a justified decision on potential illegal use of TU in livestock.

Introduction
In livestock, the administration of thyreostats has been associated with a considerable live weight gain, which is primarily due to increased water retention in the edible tissues and filling of the gastrointestinal tract. As such, these growth-promoting agents exert a negative effect on the meat quality (Batjoens *et al*., 1996). In addition, xenobiotic thyreostats are assigned teratogenic and carcinogenic properties, which implicates that any residues in the edible tissues connote a possible risk for human health (International Agency for Research on Cancer, 2010). Therefore, based on the listed arguments, the use of thyreostats for animal fattening purposes has been banned in the European Union since 1981 (European Community, 1981). This ban implies a strict zero-tolerance concerning the use of thyreostatic drugs, whereby their non-presence in animal derived matrices such as muscle and organ tissue is requisite. To this end, a constant monitoring is effectuated by the individual member states to discourage thyreostat abuse, being at risk of severe penalties.

In recent years, however, a semi-endogenous origin of TU has been reported for urine of livestock upon ingestion of glucosinolate-rich crops, belonging to the Brassicaceae family (Kiebooms *et al.*, 2012; Pinel *et al*., 2006; Vanden Bussche *et al*., 2011;). The discovery of this semi-endogenous origin was a startling finding as the rigid link between TU detection in *bovine* urine and its illegal use was no longer binding. In addition, this could explain the presence of low urinary TU levels in livestock, for which there was no direct evidence for illegal administration (EFSA Report, 2013). Although further research is designated to elucidate the exact formation mechanisms and define other (dietary) triggers, the possibility of a semi-endogenous origin strongly hampers the decision-making process concerning a potential illegal use of TU upon detection. In this regard, the community of EU reference laboratories (EURLs) introduced urinary TU concentration levels for which a semi-endogenous origin of detected TU is presumed; *i.e.* below 10 μg L^{-1} (CRL, 2007). Unfortunately, a systematic occurrence of urine samples exceeding the 10 μg L^{-1} threshold was observed, pointing towards an inadequate threshold value. As a result, national and international epidemiologic surveys have been conducted (Le Bizec *et al.*, 2011; Wauters *et al.*, 2015; Wozniak *et al.*, 2012) to set a threshold value that is more accurate in differentiating between semi-endogenous and exogenous TU. These survey results were taken into consideration by the latest reflection paper of the EURL, in which a new threshold value of 30 μg L^{-1} was suggested (EURL, 2014). However, using this threshold-based approach, incorrect decision-making is still possible. An alternative strategy for discriminating between endogenous and exogenous TU is thus highly needed. In this regard, untargeted profiling approaches may enclose significant potential as these may reveal valuable marker molecules, being descriptive towards the origin of detected TU. Analysis of descriptive biomarkers, as part of the national control plans, would indeed lead to a justified decision on the origin of urinary TU.

In this study, it was aimed to reveal a urinary marker that is able to define the true origin of detected TU. To this end, a comprehensive *in vivo* study was carried out in which bovines were subjected to various treatments, including synthetic TU administration. In order to point out relevant metabolome differences and marker metabolites between treatments, a metabolomics workflow was applied, thereby using hybrid high-resolution Orbitrap™ MS.

Materials and Methods

Experimental set-up of the in vivo trial

The *in vivo* trial concerned a parallel-like design with various treatment groups (Figure 1). Each of the treatments was preceded by a 2-week acclimation phase during which all test animals were fed a commercial diet of concentrate (27% crude protein content) with *ad libitum* access to water and hay. Whereas this dietary regimen was maintained for the control and TU treated group, one fraction of the test animals received a diet in which 30% of the concentrate was replaced by rapeseed cake (37% crude protein content). With respect to the TU treated group, animals were orally administered a daily dose of 0.2 g TU per 100 kg body weight (TU analytical powder, Sigma Aldrich, St. Louis, MO, USA) by means of a filled capsule. Following this TU treatment, a short washout period was appended to assess the excretion profiles of potential markers in urine. This study was approved by the Ethical Committee of the Centre d'Economie Rurale (CE/Sante/ET004).

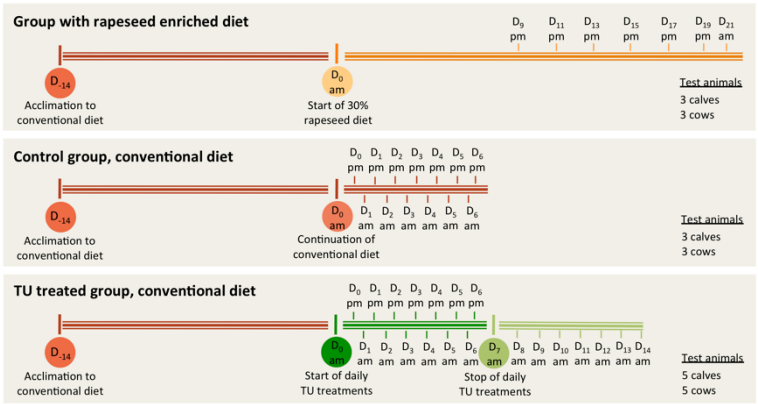

Figure 1. Schematic representation of the in vivo *trial. Within a first treatment group, test animals received a diet that was enriched with rapeseed cake, which intends to promote the semi-endogenous formation of TU. A second group of animals was fed a commercial diet during the entire course of the trial and thus served as control group. Finally, a group of animals was treated with TU on a daily basis for one week, which was followed by a washout period. Acclimation periods were incorporated for adaptation to the imposed dietary regimen. The small ticks represent moments of urine sampling, being relatively expressed towards treatment starting points (*i.e. *days (D)).*

Chemicals and materials

The analytical standard 2-thiouracil (TU) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the deuterated internal standard 6-propyl-2-thiouracil-d_5 (PTU-d_5) from Toronto Research Chemicals (Toronto, Canada). Stock (1 mg mL^{-1}) and working solutions (1 and 0.1 ng μL^{-1}) were prepared in methanol and stored in dark glass bottles at -20°C. Reagents were of analytical grade when used for extraction purposes and of LC-MS grade for UHPLC-MS applications. They were purchased from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK), respectively. Phosphate buffer was adjusted to pH 7 and saturated with 1% of DL-dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA). Ultrapure water (0.055 $\mu S\ cm^{-1}$) was obtained by means of a purified-water system (VWR International, Merck, Darmstadt, Germany).

Sample collection, preservation and extraction

Urine was collected in several 5-mL aliquots, whereby samples were treated with 0.1 M hydrogen chloride (final pH 1) and EDTA (final concentration of 0.1 M). These pre-treatment conditions have been demonstrated to strongly inhibit thyreostat degradation during urine preservation (Vanden Bussche *et al.*, 2012). Urine samples were stored at -20°C and thawed prior to extraction. For extraction, the methodology of Vanden Bussche *et al.* (2010) was adopted with some minor modifications.

Liquid chromatography and mass spectrometry

The UHPLC system consisted of a Dionex Ultimate 3000 XRS pump, coupled to a Dionex Ultimate 3000 RS column compartment and autosampler (Dionex, Amsterdam, The Netherlands). Chromatographic separation was thereby achieved on an Acquity HSS T3 column (1.8 μm, 2.1 x 100 mm) (Waters, Zellik, Belgium), whereby a gradient program using 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) was applied. A constant flow rate of 300 $\mu L\ min^{-1}$ and a column oven temperature of 25°C were set.

Mass-spectrometric analysis was carried out using a high-resolution hybrid quadrupole Q-Exactive™ Orbitrap mass spectrometer (HRMS/MS) (Thermo Fisher Scientific, San Jose, USA), which was equipped with a heated electrospray ionisation source (HESI), operating in polarity switching mode. In addition to these full-scan MS events, separate MS/MS experiments were afterwards performed for identification of revealed biomarkers. Instrumental control and data processing were carried out with Chromeleon Express and Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, USA).

Metabolic fingerprinting of urine samples

As a key strategy towards biomarker discovery, it was opted to compare the urinary metabolic fingerprints of the control group with these of the TU treated group (excl. washout). To this end, urine samples from both treatment groups were randomly extracted and analysed, although batches for cows and calves were separately handled. A same randomization was applied for the samples coming from the rapeseed-enriched diet and washout group. The metabolic fingerprints from these latter groups were used to evaluate the performance of discovered markers to discriminate between exogenous and semi-endogenous TU.

During mass spectrometric analysis of all urine extracts, quality control measures were taken by using external and internal quality control (QC) samples. External QC samples were used for instrument stabilization, whereas internal QC samples were included to monitor and correct for any instrumental drift.

Acquired full-scan MS data of study samples were imported into the Sieve^{TM} 2.2 software package (Thermo Fisher Scientific, San Jose, USA) to compose metabolic fingerprints. Hereby, metabolite features from total ion current chromatograms were extracted, applying peak alignment and integration. Each of the detected features was characterized through its retention time (t_R), *m/z*-value, and peak intensity. Normalization was conducted based on the associated internal QC samples.

Multivariate data analysis

The normalized multivariate data matrix was analysed by SIMCA 13.0 (Umetrics, Malmö, Sweden), whereby it was aimed to discover metabolome discrepancies between the TU treated and control group. Hereby, data was log-transformed and pareto-scaled to induce normality and standardize the peak intensity ranges, respectively. In first instance, unsupervised segregation was checked by principal components analysis (PCA), allowing to evaluate clustering of QC samples and identify potential outliers. Subsequently, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to model variation and establish separation between investigated treatment groups. Based on the validated OPLS-DA models, metabolites that are responsible for differentiation between TU treated and control samples could be revealed.

Results

Metabolic fingerprinting of urine samples

Urinary fingerprints were separately established for cows and calves, thereby distinguishing between positively and negatively charged ions. Fingerprints inclosed 29,128 and 39,961 monoisotopic ions for calves and cows, respectively. The majority of these ions was obtained in positive ionization mode (*i.e.* 69.8% and 63.1%). The associated data matrix of peak intensities was normalized on the basis of iQC samples and subsequently analysed by multivariate statistics to extract useful information and reveal candidate biomarkers.

Multivariate data analysis

First PCA-X modelling was carried out to evaluate the instrument's stability during analysis, reveal the natural patterning of samples, and detect potential outliers. Using this unsupervised modelling strategy; class membership of urine samples was not taken into account. PCA-X score plots (Figure 2, presented for cows only) pointed at acceptable instrument stability, separations of samples that are quite well in line with treatments, and the presence of some deviating samples. Although the presence of these outliers could be confirmed by the Hotelling's T^2 test (99% critical level), any removal of samples was only executed during the subsequent OPLS-DA modelling. It was noted that samples were most scattered in the case of calves, which may reflect the metabolic changes that are associated with growth and maturing (Egli and Blum, 1998).

Subsequent to unsupervised PCA-X, metabolic fingerprints were subjected to OPLS-DA modelling, which aimed to differentiate between the TU treated and control sample population (Figure 3). In that case, information about sample class membership was used. Valid models could be generated for cows and calves, and this for both ionization modes.

Based on these OPLS-DA models, selection of discriminating metabolites was performed, thereby using the S-plot (Figure 3, presented for cows and positive ions only), VIP-score and Jack-knifed confidence intervals (Cho *et al*., 2008; Wager *et al.*, 2014; Wicklund *et al.*, 2008). Other selection criteria included peak shape and diagnostic selectivity/specificity. As such, 31 metabolites were retained for the cows and 18 for the calves.

Assessment of candidate marker's potential to describe the origin of detected TU

The diagnostic potential of the candidate markers was further evaluated by considering their presence in secondary samples. These included urine samples, obtained from the group with a rapeseed-enriched diet and during the washout period. After all, a low prevalence or absence of the markers in the urine samples from the rapeseed-enriched diet group (where endogenous TU formation can be expected) is requisite. In addition, candidate markers that can be detected for several days after ending the TU treatment are of course most promising, which was covered by the washout period. Taking these additional elements into account, for both the cows and calves, two candidate biomarkers were retained.

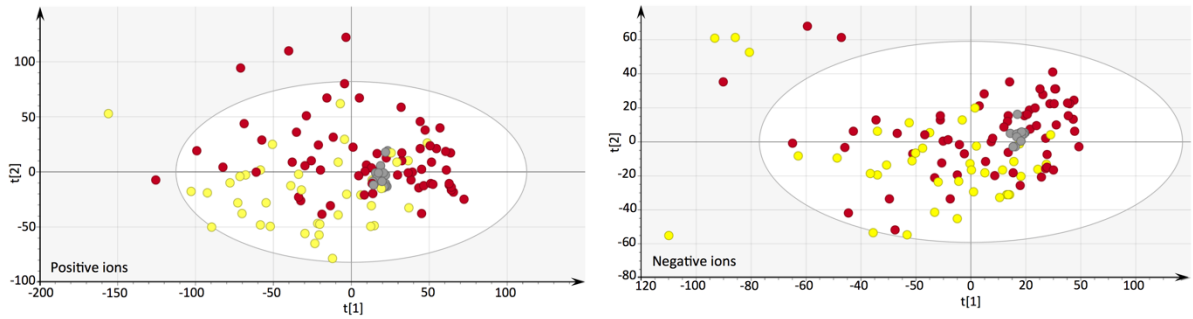

Figure 2. PCA-X score plots for urine samples that were derived from the cow's TU treated group (n = 65) or untreated control group (n = 39). Figure panels present the positively (left) and the negatively (right) charged metabolites.

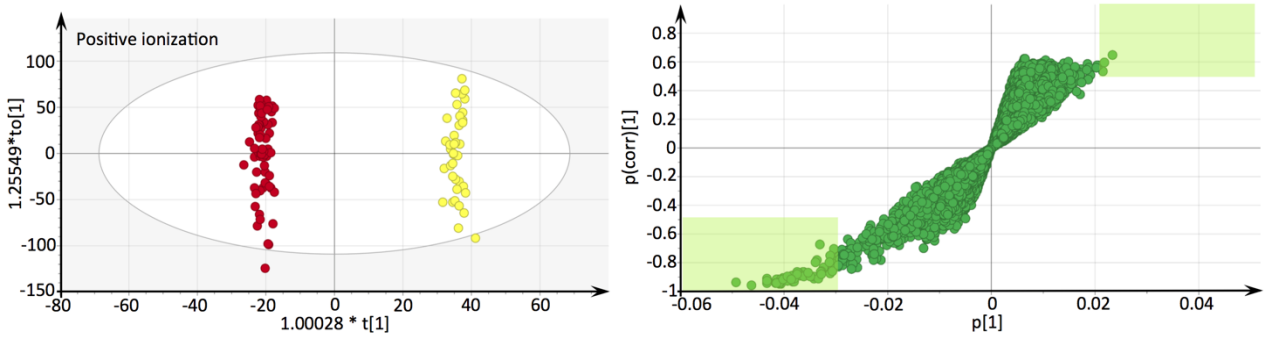

Figure 3. OPLS-DA score plot (left) for urine samples that were derived from the cow's TU treated group (n = 65) or untreated control group (n = 39). Based on the associated OPLS-DA model, discriminating ions were selected based on various selection strategies, including the S-plot (right). Coloured areas represent relevant discriminating ions.

Conclusions and Discussion

The applied metabolomics workflow was able to reveal some valuable candidate markers, which may have potential to unambiguously differentiate between endogenous and exogenous TU in urine. More specifically, two markers were found for both cows and calves (4 in total), being discriminative towards the origin of TU and up to two days detectable in urine after ending the TU treatment. It can thus be stated that the first steps towards an alternative strategy, supportive towards the decision-making on potential TU abuse, have been made. However, additional efforts are needed to ensure the suitability and accuracy of these markers in practice. A comprehensive clinical qualification and validation are hereby requisite. In this context, identifying the most promising biomarkers and connecting their intensities to biological pathways will be most challenging. Nevertheless, successful completion of all validation and qualification phases is essential in order that these biomarkers find their way in practice and are implemented as part of current monitoring strategies.

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POSTER PRESENTATIONS

CONTRIBUTIONS

P1. Simultaneous 5-in-1-sample preparation and multiplexed automated analysis by ChemWell® bioanalyzer of chloramphenicol and nitrofuran metabolites AOZ, AMOZ, AHD and SEM in shrimp.
(194) Czymai T, R-Biopharm AG, Germany

P3. A unified approach for the detection of unknowns in complex matrices: a case study a β-agonist in urine.
(198) Gerssen A, RIKILT - Wageningen UR, The Netherlands

P4. Development and validation of a chiral HPLC-MS/MS method for the identification of chloramphenicol isomers and quantification of (*R,R*)-para-CAP.
(204) Domisse SM, Ducares, The Netherlands

P6. Validation of a Method for the Determination of Residues of ß-Lactam Antibiotics in Milk by LC-MS/MS.
(210) Bohm DA, Federal Office of Consumer Protection and Food Safety (BVL), Germany

P7. Validation of a Method for the Determination of Residues of Triphenylmethane Dyes in Aquaculture Products by LC-MS/MS.
(215) Eich J, Federal Office of Consumer Protection and Food Safety (BVL), Germany

P8. Development and validation of a confirmatory method for the determination of amphenicols in muscle and kidney of several animal species.
(220) Moragues F, Public Health Laboratory of Valencia-Fisabio, Spain

P9. Amphenicols analysis in meat by ultra-performance liquid chromatography coupled to tandem mass spectrometry.
(226) Staub Spörri A, Official food control authority and veterinary affairs of Geneva, Switzerland

P10. Development of a VICH guidance for conduct of residue depletion studies in aquatic species.
(230) Smal M, Elanco, Australia

P11. Determination of nitroimidazole residues in poultry feathers using SupelMIP SPE and HPLC-MS/MS.
(233) Církva A, Institute for State Control of Veterinary Biologicals and Medicines, Czech Republic

P12. Rapid detection of the resistance to β-lactam antibiotics in gram-negative bacteria by instrumental analysis.
(239) Stolker AAM, RIKILT, The Netherlands

P13. Feather segmentation analysis in order to monitor off-label use of antibiotics in the poultry sector.
(243) Jansen LJM, RIKILT, The Netherlands

P14. Strategies to distinguish synthetic from naturally produced chloramphenicol.
(246) Gerritsen H, RIKILT, The Netherlands

P15. Deposition and depletion of maduramicin and monensin residues in eggs resulting from misuse of feed for target species.
(250) Varenina I, Croatian Veterinary Institute, Croatia

P17. Production of secondary antibody for the development of a screening method for the determination of tetracyclines residues in milk.
(257) Nesterenko ES, FGBU VGNKI, Russian Federation

P18. Veterinary drug analysis in animal origin food and feed and their relevant products: a modern multi-class, multi-residue method using UHPLC-MS/MS.
(261) Zhao H, Covance Food Solutions, USA

P19. Development and validation of a confirmatory method for the determination of corticosteroids in meat, milk and liver.
(266) Yunin MA, VGNKI, Russian Federation

P20. Investigation of corticosteroids profiles in *bovine* urine: Part A. Development of a method for cortisol, prednisolone and their metabolites determination.
(269) Gili M, Istituto Zooprofilattico Sperimentale PLV, Italy

P21. Results of proficiency testing for the analysis of (fluoro)quinolone residues in trout fish (*Oncorhynchus mykiss* species).
(274) Fuselier R, Anses, France

P23. Antibiotic residues in milk during the cheese making process. Part I. Effect of the pasteurization step.
(279) Pellicciotti S, Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Italy

P24. Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) as a Tool to Distinguish between Endogenous and Exogenous Boldenone and Nortestosterone in Urine.
(283) Gebbink WA, RIKILT, The Netherlands

P25. Antibiotic residues in milk during the cheese making process. Part II. Effect of the skimming step.
(287) Pellicciotti S, Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Italy

P26. Validation of a screening and confirmatory method for tiamulin in liver by LC-MS/MS.
(291) Palmqvist V, Danish Veterinary and Food Administration, Denmark

P27. Towards responsible use of antibiotics without loss of a good udder health.
(294) Supré K, MCC-Vlaanderen, Belgium

P29. *In vitro* growth inhibition of *bovine* intramammary streptococci against betalactams.
(299) Supré K, MCC-Vlaanderen, Belgium

P30. Screening for veterinary drug residues and steroids in meat using HRMS and a data-independent acquisition mode.
(303) Van Poucke C, ILVO, Belgium

P31. Development and validation of a multi-residues confirmatory method for determination of macrolides, lincosamides and pleuromutilines by HPLC-MS/MS.
(306) Batov IV, The All-Russian State Center for Quality and Standardization of Veterinary Drug, Russian Federation

P32. Target and post-target analysis of antibiotics in animal tissue by UHPLC-HRMS.
(310) Igualada C, Public Health Laboratory of Valencia, Spain

P33. Excretion profile of 17ß,19-nortestosterone and its main metabolite in *bovine* urine after intramuscular administration.
(316) Ferranti C, Istituto Superiore della Sanità, Italy

P34. Non-steroidal anti-inflammatory drug analysis in milk by QuEChERS and LC-MS: low and high resolution detection and confirmation approaches.
(322) Rúbies A, Laboratori de L'Agencia de salut Publica de Barcelona, Spain

P35. Pharmacokinetics of abamectin in combination with monepantel is not impacted by cytochrome P450 induction.
(326) Smal M, Elanco, Australia

P36. Research of tylosin metabolites in manure to detect tylosin treatment and to environmental exposure.
(331) Bor G, RIKILT, The Netherlands

P38. A comprehensive method for the determination of 20 coccidiostats residues in various food matrices.
(335) Pietruk K, National Veterinary Research Institute, Poland

P39. The occurrence of chloramphenicol residues in tissues of chickens exposed to low dietary concentrations of the chloramphenicol.
(340) Rejtharová M, USKVBL, Czech Republic

P40. The determination of testosterone esters and estradiol esters in *bovine* and *porcine* blood serum.
(345) Rejtharová M, USKVBL, Czech Republic

P41. Screening method for the identification of banned compounds in urine.
(348) Zuidema T, RIKILT, The Netherlands

P42. Effect of diet rich in cruciferous plants to the presence of endogenous thiouracil in urine and milk of cattle.
(353) Wozniak B, National Veterinary Research Institute, Poland

P43. True non-targeted analysis of antimicrobial active compounds using bio-assay directed screening followed by LC-HRMS.
(358) Zuidema T, RIKILT, The Netherlands

P44. Residues of veterinary medicinal products in foods of animal origin in the EU RASFF (1979-2014).
(362) Papapanagiotou E, Aristotle University of Thessaloniki, Greece

P46. Determination of fenbendazole residues in fermented dairy products by ion-pair liquid chromatography.
(367) Vousdouka V, Aristotle University of Thessaloniki, Greece

P48. Analysis of isomeric pyrrolizidine alkaloids by online multiple heart-cutting 2D-LC QToF-MS.
(372) Van de Schans MGM, RIKILT, The Netherlands

P49. QTRAP®LC-MS/MS method for determination of estradiol in *bovine* serum with 4-(dimethylamino) benzoyl chloride derivatization.
(376) Matraszek-Zuchowska I, National Veterinary Research Institute, Poland

P51. Determination of antibiotic residues in honey.
(381) Schoeman MG, RIKILT, The Netherlands

P52. Determination of the metabolites of nitrofuran antibiotics in animal tissues and associated products by liquid chromatography-tandem quadrupole mass spectrometry.
(386) Hird S, Waters, United Kingdom

P53. Liquid Chromatography-Tandem Mass Spectrometry for the Determination of Nortestosterone Glucuronide in Rainbow Trout Muscle.
(390) Chu P-S, FDA, USA

P54. A simplified multi-class method for simultaneous determination of growth-promoters by LC-MS/MS.
(395) Kaplan M, Tubitak Marmara Research Centre, Turkey

P57. Drugs of abuse and other influences on steroid profile using new validated methods.
(397) Popova R, Bulgarian Anti-doping Centre, Bulgaria

P59. Fast quantitative multi method for analysis of prohibited substances using LC/MSMS.
(402) Petrova A, Bulgarian Anti-doping Center, Bulgaria

P60. UHPLC-MS/MS analysis of thyreostats in *bovine* faeces.
(410) Witek S, National Veterinary Research Institute, Poland

P61. Determination of polypeptide antibiotic residues in muscle and milk samples by liquid chromatography-tandem mass spectrometry.
(414) Bladek T, National Veterinary Research Institute, Poland

P62. Transfer of nitroimidazoles from contaminated beeswax to honey.
(419) Mitrowska K, National Veterinary Research Institute, Poland

P63. Tissue distribution and residue depletion of metronidazole in rainbow trout (*Oncorhynchus mykiss*).
(424) Mitrowska K, National Veterinary Research Institute, Poland

P64. Confirmatory analysis of antibacterial residues in food of animal origin in Poland.
(431) Gajda A, National Veterinary Research Institute, Poland

P65. Combining standard addition with blank addition.
(437) Steliopoulos P, CVUA Karlsruhe, Germany

P66. Determination of flubenzurons in seafood BY LC-MS-QQQ.
(439) Hannisdal R, NIFES, Norway

P68. Confirmatory method for the determination of acidic and basic NSAIDs in milk by LC-MS/MS.
(442) Kittler K, Federal Office of Consumer Protection and Food Safety, Germany

P69. LC-MS/MS determination of corticosteroids and non-steroidal anti-inflammatory drug residues in food, development and comparison with LC-HRMS.
(448) Giannetti L, IZS-Lazio e Toscana, Italy

P70. Comparing the performances of MS/MS and HRMS analysers in the fast analysis of multi-class antibiotic residues in milk.
(451) Biancotto G, Istituto Zooprofilattico Sperimentale delle Venezie, Italy

P71. Development and validation of a multi-residue LC-MS/MS analysis for the detection of aminoglycosides in milk.
(455) Daeseleire E, ILVO, Belgium

P72. Analysis of anthelmintic residues in liver by multiplex screening approach: comparison of a biochip array versus LC-MSMS approach.
(458) Benetti C, Istituto Zooprofilattico Sperimentale delle Venezie, Italy

P74. Multimethod for antibiotic analysis using 2-D liquid chromatography mass spectrometry.
(464) Zuidema T, RIKILT, The Netherlands

P75. Multi-residue and Multi-class determination of Antibiotics and Anthelmintics in Feed by High Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry.
(468) Robert C, CER Groupe, Belgium

P77. Rapid and specific extraction of anabolic steroids (A1, A3, A4) and corticosteroids in urine before detection and identification by UPLC-MS.
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P81. Passive samplers, as surrogates for biological monitoring, to measure emerging (micro)pollutants in the marine environment.
(481) Huysman S, Ghent University, Belgium

P82. Production of antisera to phenylbutazone and oxyphenylbutazone for use in immunochemical detection assays.
(486) Traynor I, Agri-Food and Biosciences Institute, United Kingdom

P84. Abuse of anabolic agents in beef cattle: bile as a possible alternative matrix for official control.
(491) Galarini R, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Italy

P85. Which analytical techniques can reduce matrix effects in LC-MS analysis.
(497) Kaklamanos G, Veterinary Laboratory of Serres, Greece

P86. Implementation of an analytical methodology for detection of Sulfachlorpyridazine (SCP) residues in broiler chickens feathers by liquid chromatography tandem mass spectrometry.
(502) Cornejo J, University of Chile, Chile

P87. Depletion study of oxytetracycline (OTC) and 4-epi-oxytetracycline (4-epi-OTC) residues in feathers of broiler chickens by liquid chromatography tandem mass spectrometry.
(507) Cornejo J, University of Chile, Chile

P89. Validation of an analytical methodology for the detection of florfenicol (FF) and florfenicol amine (FFA) residues in feathers by Liquid chromatography-tandem mass spectrometry (LC-MS/MS).
(512) Cornejo J, University of Chile, Chile

P92. Enrofloxacin and ciprofloxacin residues in broiler chicken feathers after oral administration.
(519) Mestorino N, Veterinary Faculty, Argentina

P93. Quechers method for simultaneous determination of veterinary drugs and pesticides analysis in milk by LC-MS/MS
(524) Öktem Olgun E, Tubitak, Turkey

P95. Dye residues in aquaculture products: implementation of targeted and non-targeted approaches.
(526) Dubreil E, ANSES, France

P96. Multiplex bead-based assay for the simultaneous on-site detection of forty-two antimicrobials in drinking water and feathers.
(532) Bienenmann-Ploum M, RIKILT, Wageningen, The Netherlands

P97. Screening and verification of steroid esters in *porcine* hair using LC-QTOF-MS.
(538) Frandsen HL, National Food Institute, Technical University of Denmark, Denmark

P98. Proficiency test for resorcylic acid lactones in *bovine* urine.
(542) Elbers IJW, RIKILT, The Netherlands

P99. Effect of storage, thermal processing and pH on the stability of penicillins G and V in milk.
(547) Koukouranos A, Aristotle University of Thessaloniki, Greece

P100. Long term detectability of recombinant *bovine* somatotropin in serum and milk.
(553) Smits NGE, RIKILT, The Netherlands

P101. Treatment of laying hens with nitroimidazoles - detectability of residues in eggs, feathers and preen oil.
(556) Polzer J, BVL, Germany

P102. Gel-permeation chromatography clean-up for the determination of growth promoters in kidney fat by liquid chromatography-tandem mass spectrometry.
(560) Kaklamanos G, Veterinary Laboratory of Serres, Greece

P103. Proficiency Testing in Food control - Added value by offering traceability to SI-units?
(564) Polzer J, BVL, Germany

P104. Investigation of matrix effects on selected veterinary drugs in LC-MS.
(569) Polzer J, BVL, Germany

P105. Intestinal and plasma concentrations of florfenicol in pigs after (non-)conventional oral and intramuscular treatment, within the context of resistance selection.
(573) De Smet J, Ghent University, Belgium

P108. Determination of ampicillin residues below their EU-regulatory limits in muscle, liver and plasma of chicken by LC-MS/MS.
(577) Mompelat S, ANSES - Fougères Laboratory, France

P110. In-house validation of an analytical methodology for detection of oxytetracycline (OTC), chlortetracycline (CTC) residues and their metabolites in feathers by liquid chromatography tandem-mass spectrometry (LC-MS/MS).
(581) Pokrant E, University of Chile, Chile

P111. Rapid analysis of sedatives, basic and acidic NSAIDs in kidney and muscle by LC-MS/MS.
(587) Söderlund ST, National Food Agency, Sweden

P112. The depletion of doxycycline residues in poultry tissues.
(592) Mestorino N, Veterinary Faculty, Argentina

P113. Use of Cephalosporins in Veterinary medicine Results of the German National Antibiotic Resistance Monitoring (GE*RM*-Vet).
(597) Kaspar H, Federal office of consumer protection and food safety, Germany

P114. Doxycycline residues in edible tissues of pigs.
(599) Mestorino N, Veterinary Faculty, Argentina

P115. Resolution of a disputed albendazole result in the UK Official Control System - time for more guidance?
(604) Firpo L, LGC Group, United Kingdom

P116. Analysis of 17ß-nortestosterone metabolites to distinguish between abuse and natural occurrence.
(609) Meijer T, RIKILT, The Netherlands

P117. Chloramphenicol residues to tissues, plasma and urine after oral intake low doses by pigs.
(614) Törnkvist A, National Food Agency, Sweden

P118. Validation of the Betastar S Combo for the rapid screening of milk for residues of ß-lactams and tetracyclines.
(617) Reybroeck W, ILVO, Belgium

P119 Development, validation and applicability of a multi-residue LC-MS/MS method for the detection of anthelmintics in milk.
(621) Daeseleire E, ILVO, Belgium

P120. B ZERO CAP HS: a new master-curve calibrated immunoassay for the detection of chloramphenicol in milk.
(627) Gon F, Tecna s.r.l., Italy

P121. Residue depletion of ivermectin in chickens.
(632) Mestorino N, Veterinary Faculty, Argentina

P124. Novel design and synthesis of chloramphenicol immunoreagents for the analysis of biological samples.
(636) Hernández-Albors A, CIBER-BBN/IQAC-CSIC, Spain

P126. Wide-range screening of banned substances in *bovine* urine.
(642) Moretti S, Istituto Zooprofilattico Sperimentale Dell'Umbria E Delle Marche, Italy

P127. New approach based on immunochemical techniques to monitor selective estrogen receptor modulators (SERMs) in clinical samples.
(648) Salvador JP, CIBER-BBN/IQAC-CSIC, Spain

P129. A new sorbent for clean-up of seafood extracts prior to multi-residue veterinary drug LC-MS analysis.
(654) Young MS, Waters Corporation, United Kingdom

P132. A novel multispot ELISA for antibiotic screening.
(657) McNamee SE, Queen's University Belfast, United Kingdom

P135. Validation of 4SENSOR® Milk KIT060 for the testing of raw milk on the presence of ß-lactams, tetracyclines, (dihydro)streptomycin and chloramphenicol.
(661) Reybroeck W, ILVO, Belgium

P136. The impact of enzymatic hydrolysis in the proper quantification of ractopamine as a drug residue in swine muscle matrix.
(665) Caldeira LR, Ministério da Agricultura, Pecuária e Abastecimento, Brazil

P137. Pilot Project for a Chilean National Reference Laboratory.
(670) San Martín B, Universidad de Chile, Chile

P140. Development and validation of a multi-class method for the determination of antibiotic residues in honey using liquid chromatography-tandem mass spectrometry.
(673) El Hawari K, CNRS-CLEA, Lebanon

P141. Determination of growth hormone-releasing hexapeptide in *bovine* urine by LC-MS/MS.
(689) Viilup G, RIKILT, The Netherlands

P142. Deconjugation of steroids in urine: Is there an ultimate method for complete deconjugation of steroids in urine?
(694) Van den Brink J, RIKILT, The Netherlands

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SIMULTANEOUS 5-IN-1 SAMPLE PREPARATION AND MULTIPLEXED AUTOMATED ANALYSIS BY CHEMWELL®BIOANALYZER OF CHLORAMPHENICOL AND NITROFURAN METABOLITES AOZ, AMOZ, AHD AND SEM IN SHRIMP

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Abstract

Chloramphenicol and the nitrofuran antibiotics furazolidone, furaltadone, nitrofurantoin and nitrofurazone are banned for the application to animals used for food production due to their mutagenic and carcinogenic characteristics. Nevertheless, they are frequently used, *e.g.*in aquaculture of shrimp. Chloramphenicol and nitrofuran metabolites bear a health risk for consumers and non-compliance with the established zero-tolerance levels may lead to penalties, *e.g.* in export business. R-Biopharm developed a 5 in1 sample preparation method to extract chloramphenicol and the 4 nitrofuran metabolites (AOZ, AMOZ, AHD, SEM) from shrimp simultaneously. Subsequent ELISA analysis can be performed manually or automated by ChemWell® bioanalyzer, where chloramphenicol and nitrofuran metabolites are analyzed in parallel. Sample preparation and analysis can be conducted on only 1 single day. The detection limit and capability are below the minimum required performance limits (chloramphenicol: 300 ng kg^{-1}, nitrofuran metabolites: 1000 ng kg^{-1}).
The new method offers a sensitive and legislation compliant approach to screen shrimp for residues of prohibited substances. In routine analysis, it offers a time and labour saving alternative to separate single analysis. For producers, exporters and processors of seafood, it helps to prevent penalties from violation of zero-tolerance levels. Overall, the new method improves general food safety and consumer protection.

Introduction

Chloramphenicol (CAP) and nitrofuran antibiotics are broad-spectrum antibiotics, which are frequently used in animal husbandry or aquaculture due to their excellent antibacterial and pharmacokinetic properties. Residues of these antibiotics have carcinogenic and mutagenic characteristics and thus bear a health risk for consumers, when contaminated food is consumed. For this reason, the use of CAP and nitrofuran antibiotics for the treatment of animals used for food production is prohibited. Zero tolerance levels with minimum required performance limits (MRPL) of 300 ng kg^{-1} for CAP and 1,000 ng kg^{-1} for nitrofurans were established. Non-compliance with legislation may lead to severe penalties in export business. While the analysis of CAP focusses on the detection of the parent compound, the analysis of nitrofurans is more difficult. The parent compounds furazolidone, furaltadone, nitrofurantoin and nitrofurazone are metabolized very rapidly after treatment. The tissue bound nitrofuran metabolites AOZ, AMOZ, AHD and SEM however are present for a long time after administration and usually are used to detect nitrofuran abuse. LC/MS represents the official reference method, but this technique is expensive, labour intensive and in need of skilled analysts. The aim of this study was to develop a simple, time and labour saving screening method for routine analysis which is capable to handle large sample numbers. In general, enzyme immunoassays (ELISA) fulfil these criteria, but so far separate sample preparations had to be performed for each analyte and parallel pipetting of five ELISAs is labour intensive und error-prone.

Consequently, R-Biopharm developed a 5-in-1 sample preparation method to extract CAP and the 4 tissue-bound nitrofuran metabolites AOZ, AMOZ, AHD and SEM from shrimp simultaneously in only one procedure. Furthermore, a procedure for ChemWell® bioanalyzer was developed, which allows automated test implementation of several ELISAs in parallel.

Materials and Methods

Reagents

All reagents were obtained from Sigma-Aldrich in analytical grade.

10 mM 2-nitrobenzaldehyde in dimethylsulfoxide (DMSO) was prepared freshly for every analysis by dissolving 7.6 mg of 2-nitrobenzaldehyde in 5 mL DMSO.

Preparation of prawn samples

20 different shrimp samples were homogenized completely using a blender. Tissue bound nitrofuran metabolites (AOZ, AMOZ, AHD, SEM) were then hydrolysed from tissue and derivatised into nitrophenyl-derivatives metabolites (NP-AOZ, NP-AMOZ, NP-AHD, NP-SEM). For this, 4 mL of demineralized water, 0.5 mM 1 M HCl and 100 µL 10 mM 2-nitrobenzaldehyde in dimethylsulfoxide was added to 1 g of homogenized sample. After vortexing, the samples were incubated at 50°C for 3 h in a water bath. Nitrofuran metabolites and CAP were extracted by adding 5 mL 0.1 M K_2HPO_4, 0.4 mL 1 M NaOH and 5 mL ethyl

acetate and vortexing for 30 s. For improved phase separation, samples were warmed up to 50°C in a water bath. Samples were then centrifuged for 10 min at 3,000 g at room temperature (20 – 25°C). 2.5 mL of the supernatant was transferred without disturbance of the interface into a new glass vial and evaporated at 60 °C and under a mild nitrogen stream to dryness. After reconstitution of the dried residue in 1 mL of *n*-hexane, 1 mL of sample buffer from RIDASCREEN® Nitrofuran ELISA was added and samples were vortexed. After centrifugation for 10 min at 3.000 g at room temperature (20 – 25°C), the lower aqueous phase was transferred into a new vial.

Fortification of samples
For fortification, RIDA® Chloramphenicol Spiking Solution (Art. No. R1599) and RIDA® Nitrofuran Spiking Solutions AOZ (Art. No. R3798), AMOZ (Art. No. R3799), AHD (Art. No. R3796) and SEM (Art. No. R3797) were used.

If necessary, spiking solutions were further diluted in methanol. Prawn samples were spiked by adding the respective volume to 1 g of homogenised sample. After spiking, sufficient time was given for evaporation of methanol.

Manual test procedure of RIDASCREEN® ELISAs
RIDASCREEN® Chloramphenicol (Art. No R1505) is a competitive enzyme immunoassay from R-Biopharm AG, Darmstadt, Germany. All reagents required for the ELISA are contained in the test kit. ELISA was performed in accordance to instructions for use of the manufacturer. Briefly, 50 µL of standard or sample were added to separate duplicate wells. Then 50 µL of enzyme conjugate was added and incubated for 1 h at room temperature. After washing of microwells with 250 µL of washing buffer, 100 µL of substrate/chromogen was added and incubated for 15 min at room temperature. 100 µL stop solution were added and absorbance was read immediately at 450 nm on a microtiter plate spectrophotometer. Results were evaluated using RIDA® SOFTWIN.net software from R-Biopharm AG.

RIDASCREEN® Nitrofuran AOZ (Art. No. R3703), AMOZ (Art. No. R3711), SEM (Art. No. R3715) and AHD (Art. No. R3713) are competitive enzyme immunoassays from R-Biopharm AG, Darmstadt, Germany. All reagents required for the ELISA are contained in the test kits. ELISAs were performed in accordance to instructions for use of the manufacturer. Briefly, 50 µL (100 µL for SEM) of standard or sample were added to separate duplicate wells. Then 50 µL of enzyme conjugate and 50 µL of antibody was added and incubated for 1 h at room temperature. After washing of microwells with 250 µL of washing buffer, 100 µL of substrate/chromogen was added and incubated for 15 min at room temperature. 100 µL stop solution were added and absorbance was read immediately at 450 nm on a microtiter plate spectrophotometer. Results were evaluated using RIDA® SOFTWIN.net software from R-Biopharm AG.

Automated test procedure of RIDASCREEN® ELISAs on ChemWell® 2910 Analyser
The ChemWell® 2910 Analyser from Awareness Technology Inc. is a one-plate ELISA processor including fully automated pipetting with integrated software to calculate results. Automated test implementation of RIDASCREEN® Chloramphenicol and Nitrofuran ELISAs on ChemWell were performed using a special protocol developed by R-Biopharm, which is available on request. Results were evaluated using RIDA® SOFTWIN.net software from R-Biopharm AG.

Calculation of statistical and analytical parameters
Limit of Detection (LOD) = Mean Blank samples + 3 * standard deviation of blank samples

Recovery = (measured concentration / spike concentration) * 100%

Cut-Off Factor (Fm)= Mean spiked samples - 1,64 * standard deviation of spiked samples

Technical Threshold (T) = Mean blank samples + 1,64 * standard deviation of blank samples

Results

Determination of Limit of Detection (LOD) and Recovery
Twenty blank shrimp samples were collected and homogenized. 1 g of each samples was left blank, 1 g of each sample was spiked. Samples were spiked with 150 ng kg^{-1} CAP (= 1/2 of the MRPL), 1,000 ng kg^{-1} AMOZ, AHD and SEM (=MRPL) and 200 ng kg^{-1} nitrofuran AOZ (=1/5 of the MRPL). Samples were prepared as described in the methods section and analysed in duplicate with RIDASCREEN® enzyme immunoassays on ChemWell® Analyser.

Measured concentration values of blank samples were found under Standard 2 x dilution for CAP, AOZ and AHD. Mean of blank samples for AMOZ and SEM were 87 ng kg^{-1} and 157 ng kg^{-1}, respectively. Consequently, the LOD of the enzyme immunoassays for CAP and all four nitrofuran metabolites is below the MRPL of 300 ng kg^{-1} for CAP and 1000 ng kg^{-1} for the nitrofurans. Recovery ranges from 69% for AOZ to 123% for SEM. Coefficient of Variation is < 20% in all 5 enzyme immunoassays (Table 1).

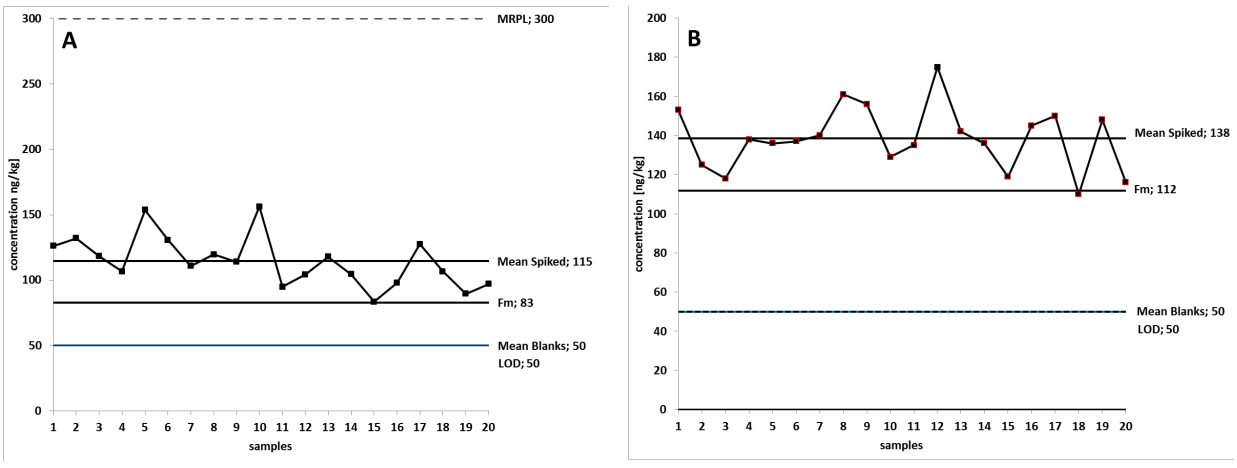
Table 1. Determination of limit of detection (LOD) and recovery.

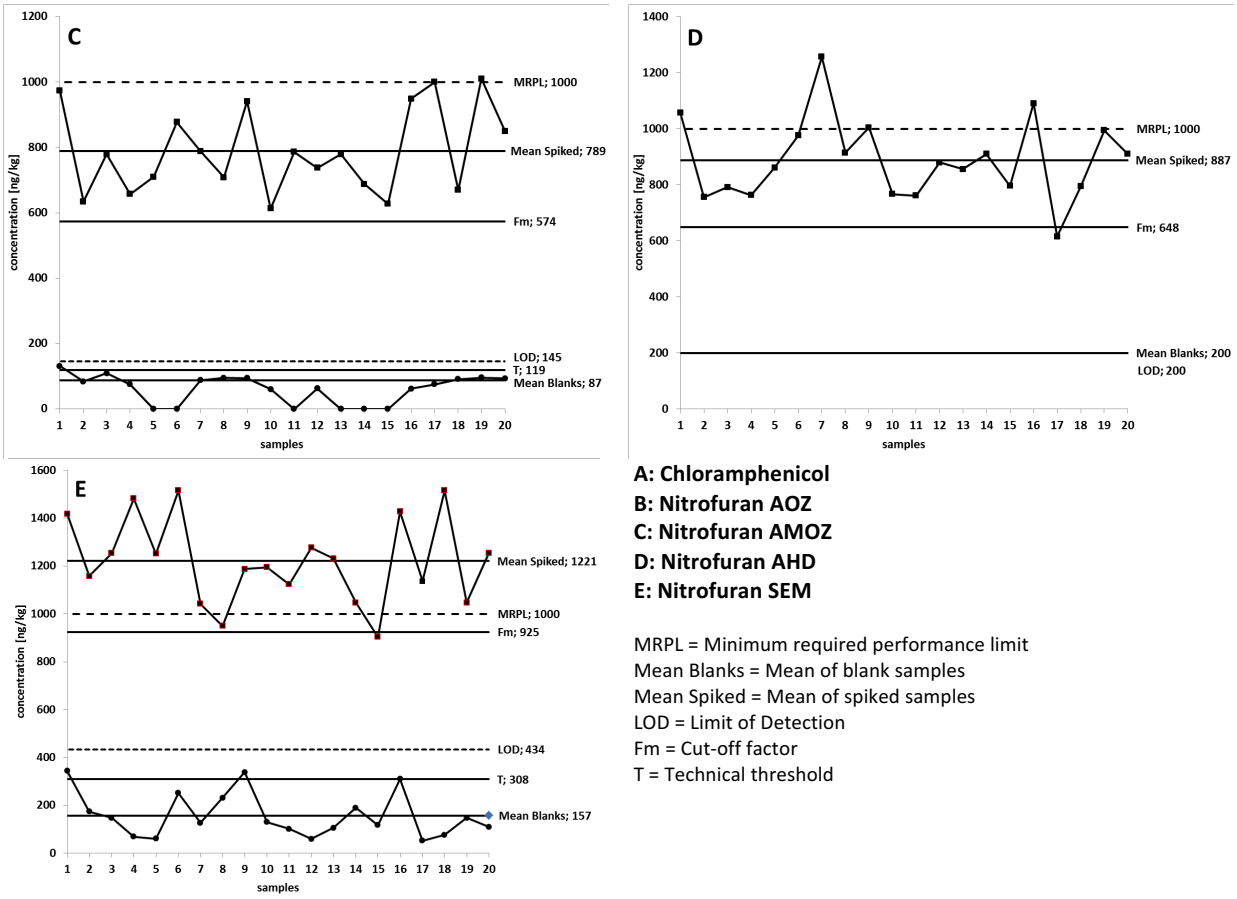
	RIDASCREEN® Chloramphenicol			RIDASCREEN® Nitrofuran AOZ			RIDASCREEN® Nitrofuran AMOZ			RIDASCREEN® Nitrofuran AHD			RIDASCREEN® Nitrofuran SEM		
	blank	spiked 150 ng kg^{-1}		blank	spiked 200 ng kg^{-1}		blank	spiked 1000 ng kg^{-1}		blank	spiked 1000 ng kg^{-1}		blank	spiked 1000 ng kg^{-1}	
Sample	**conc. [ng kg^{-1}]**	**conc. [ng kg^{-1}]**	**recovery [%]**	**conc. [ng kg^{-1}]**	**conc. [ng kg^{-1}]**	**recovery [%]**	**conc. [ng kg^{-1}]**	**conc. [ng kg^{-1}]**	**recovery [%]**	**conc. [ng kg^{-1}]**	**conc. [ng kg^{-1}]**	**recovery [%]**	**conc. [ng kg^{-1}]**	**conc. [ng kg^{-1}]**	**recovery [%]**
1	< Std 2	126	84	< Std 2	153	77	131	973	97	< Std 2	1056	106	343	1418	142
2	< Std 2	132	88	< Std 2	125	63	84	634	63	< Std 2	756	76	174	1158	116
3	< Std 2	118	79	< Std 2	118	59	109	779	111	< Std 2	791	81	148	1254	142
4	< Std 2	107	71	< Std 2	138	69	75	657	66	< Std 2	763	76	69	1482	148
5	< Std 2	154	102	< Std 2	136	68	< Std 2	710	71	< Std 2	861	86	60	1251	125
6	< Std 2	131	87	< Std 2	137	69	< Std 2	877	88	< Std 2	976	98	251	1516	152
7	< Std 2	111	74	< Std 2	140	70	88	788	79	< Std 2	1256	126	127	1042	104
8	< Std 2	120	80	< Std 2	161	81	95	708	71	< Std 2	914	91	231	949	95
9	< Std 2	114	76	< Std 2	156	78	94	941	94	< Std 2	1004	100	338	1187	119
10	< Std 2	156	104	< Std 2	129	65	60	613	61	< Std 2	767	77	130	1195	120
11	< Std 2	95	63	< Std 2	135	68	< Std 2	786	79	< Std 2	761	76	101	1124	112
12	< Std 2	104	69	< Std 2	175	88	63	737	74	< Std 2	879	88	59	1277	128
13	< Std 2	118	79	< Std 2	142	71	< Std 2	779	78	< Std 2	856	86	106	1231	123
14	< Std 2	104	70	< Std 2	136	68	< Std 2	688	69	< Std 2	910	91	189	1047	105
15	< Std 2	84	56	< Std 2	119	60	< Std 2	627	63	< Std 2	796	80	117	903	90
16	< Std 2	98	65	< Std 2	145	73	62	949	95	< Std 2	1090	109	310	1428	143
17	< Std 2	128	85	< Std 2	150	75	75	1000	100	< Std 2	615	62	52	1137	114
18	< Std 2	107	71	< Std 2	110	55	91	670	67	< Std 2	794	79	76	1516	152
19	< Std 2	90	60	< Std 2	148	74	96	1010	101	< Std 2	994	99	148	1047	105
20	< Std 2	97	65	< Std 2	116	58	93	850	85	< Std 2	910	91	110	1254	125
Mean	**< Std 2**	**115**	**76**	**< Std 2**	**138**	**69**	**87**	**789**	**81**	**< Std2**	**887**	**89**	**157**	**1221**	**123**
SD		**19**			**16**		**19**	**131**			**146**		**92**	**181**	
CV [%]		**17%**			**12%**			**17%**			**16%**			**15%**	
LOD	**50**			**50**			**145**			**200**			**434**		

conc. = concentration; SD = standard deviation; CV = coefficient of variation

Determination of detection capability (CCβ)

For determination of CCβ, concentration values of both blank and spiked samples were plotted over sample number. In accordance to guidelines from the community reference laboratories (CRLs) from 20/1/2010 for the validation of screening methods for residues of veterinary medicines, CCβ for a certain screening target concentration (STC) is valid, if there is no overlap between blank and spiked samples. Furthermore, a statistical confidence level of 95 % (β-error ≤ 5 %) is given, if no or only 1 out of 20 spiked samples is below the cut-off factor (Fm). At a STC of 300 ng kg^{-1} for CAP, 1000 ng kg^{-1} for AMOZ, AHD and SEM and 200 ng kg^{-1} for AOZ, there is a clear discrimination between blank and spiked samples with no overlap. Furthermore, no spiked sample was found below Fm for CAP and AMOZ and only 1 spiked sample for AOZ, AHD and SEM. Consequently, the CCβ for all 5 analytes is below the respective MRPLs. The False-positive rate is < 5 %, because Fm is larger as the technical threshold (T) (Figure 1).





A: Chloramphenicol
B: Nitrofuran AOZ
C: Nitrofuran AMOZ
D: Nitrofuran AHD
E: Nitrofuran SEM

MRPL = Minimum required performance limit
Mean Blanks = Mean of blank samples
Mean Spiked = Mean of spiked samples
LOD = Limit of Detection
Fm = Cut-off factor
T = Technical threshold

Figure 1. Graphical determination of detection capability (CCβ)

Discussion and Conclusion

The simultaneous 5-in-1 sample preparation method in combination with automated multiplex analysis by ChemWell® analyser offers a sensitive and legislation compliant method to screen shrimp for residues of the prohibited nitrofuran antibiotics and of chloramphenicol. In routine analysis, it offers a time and labour saving alternative to separate analysis of single antibiotics. For producers, exporters and processors of seafood, it can help to prevent penalties from violation of zero-tolerance levels. Overall, the new method improves general food safety and consumer protection.

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A UNIFIED APPROACH TO IDENTIFY BIOACTIVE SUBSTANCE IN COMPLEX MATRICES: A CASE STUDY OF A B-AGONIST IN URINE

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Abstract

The annotation of unknown bioactive compounds in various matrices is a real challenge. Using our experience in this area, a unified approach to find and identify unknown bioactive compounds in complex matrices was developed. The feasibility of this approach was investigated in a case study with an "unknown" bioactive compound in a male human urine sample. This sample taken from a fictive patient was offered to the laboratory to identify the unknown bioactive compound. The only supporting information provided, was a short case description with symptoms observed in this patient. Based on the case description a yeast estrogen, yeast androgen and β-agonist assay were applied to see if the sample contained bioactive compounds. Both the androgen and β-agonist assay showed activity. The androgen assay was triggered by testosterone present in the sample, which is not surprising due to the male origin of the sample. For identification and confirmation of a compound to explain the activity as found in the β-agonist assay, the routine used method based on UHPLC-MS/MS for β-agonists was applied first, but this was without success. Searching further to appoint the unknown bioactive, the sample was UHPLC fractionated. Therefore, ten fractions of 3 min each were collected. All fractions were tested on activity using the β-agonist bioassay and in parallel all fractions were analysed by UHPLC-(Q)-Orbitrap-MS. Two fractions (7 and 8) were positive in the bioassay and in order to find the responsible bioactive compound(s), orthogonal partial least squares discriminant analysis (OPLS-DA) was used to separate the non and active fractions. By use of a S-plot the masses that separate the groups were identified. The all ion fragmentation MS/MS spectra from the orbitrap experiment in combination with structure elucidation tools indicated the presence of bromochlorobuterol. By comparison with a commercial available standard, the identity of bromochlorobuterol was then confirmed as the unknown bioactive compound.

Introduction

The detection of unknown or unexpected bioactive compounds is of importance for various fields of research, *e.g*. finding new drugs. For food and feed safety the presence of unknown or unexpected bioactive compounds may lead to severe risks for consumers. In addition, after a human or animal intoxication incident inspection services will try to elicit the source or the compound causing the intoxication. In order to protect consumers and prevent intoxications, food and feed monitoring programs are established. Compounds included in these programs are often prescribed in designated legislation, like EU directive 96/23 for various types of compounds in products of animal origin (CD 96/23/EC 1996). Compounds included are certain natural toxins like mycotoxins and marine toxins, contaminants like dioxins and pesticides, and compounds registered for certain applications, like antibiotics, while other compounds may be forbidden for application, like growth-promoting agents such as steroid hormones and β-agonists.

In order to monitor for these compounds, targeted analytical methods, such as LC-MS/MS, are established (Pizzutti *et al*., 2014; Schneider *et al*., 2015). The drawback of these chemical methods is that compounds should be defined before the analysis can be performed. And even with untargeted measurement techniques, such as high-resolution mass-spectrometry, the number of compounds is restricted (Ates *et al*., 2014). At best a large library may be used, but even then new emerging hazardous compounds which are not present in the library may be missed.

A wide variety of rapid screening bioassays have been developed for various compound classes in order to detect unknown hazards. By use of these bioassays as a first screening, the number of samples that need to be investigated by mass-spectrometric analysis are reduced, as only the samples screened as suspected need to be investigated further for additional conformation and identification of the responsible compound(s). Screening assays include immunological methods such as an ELISA, receptor binding assays and cell based bioassays (Haasnoot *et al*., 1998; Bovee *et al*., 2004; Bovee *et al*., 2007). While immunological methods are limited with the regard of the number of compounds that can be detected in one test, receptor binding and, especially, cell-based bioassays have the advantage to be able to detect all compounds with a similar mode of action. However, as biosensors and bioassays are only able to determine activity of a sample and an analytical chemical method such as LC-MS is only able to identify the compound, a combination of both is perfect and of vital importance to determine if there is a potential unknown risk present in a sample, and if yes, which bioactive compound is responsible (Rijk *et al*., 2009).

Approaches to identify unknown compounds in suspect screened extracts may be done by bioassay guided LC fractionation, *i.e*. an identification approach using high-resolution mass-spectrometric methods to detect the compound in the active fraction. This approach is often successful for relatively simple matrices and with the active compound at a relative high concentration, such as an active compound in a single plant family or pharmaceutical mixture (Nielen *et al*., 2004; Uhlig, *et al*., 2014;

Yang *et al*., 2015). For the more complex matrices such as urine, animal feed or food supplements, these approaches are still hampered by the complexity and the variety of the matrix. Moreover, in feed and urine, the concentrations of the responsible compounds are often relatively very low, hampering the identification of the responsible compound between all non-relevant masses detected from the sample matrix. In metabolomics studies, often a control group is compared with a treated group. When the groups are compared, certain metabolic changes between the untreated control and treated groups may be observed. By applying powerful statistical tools such as multi-variate analysis, marker compounds may be discovered which are a result of the metabolic changes. This approach can also be used for the identification of unknown actives in fractions and in our workflow we mark the non-active fractions as control group and the active fractions as "treated".

In the present study, a general approach for bioactive driven identification of unknowns was explored, using a simple artificial case description that, together with a prepared sample, was send to various research groups within our Institute, *i.e*. the biosensor & bioassay group (BB) and an analytical group specialized in chemical analysis of veterinary drugs. After the screening in the BB group, this group collaborated with the analytical group, using a bioassay guided LC fractionation approach for identification and confirmation of the active fraction, using high-resolution mass-spectrometry in combination with powerful statistical and structure elucidation tools.

Experimental

Chemicals and Reagents

Bromochlorobuterol, clenbuterol, PBS buffer, ammonium formate and formic acid were purchased from Sigma-Aldrich (Darmstadt, Germany). Sodium acetate, acetic acid, ammonium hydroxide and chloroform were purchased from Merck (Darmstadt, Germany) and acetone, ethyl acetate and acetonitrile from biosolve (Valkenswaard, The Netherlands). 3H-dihydroalprenolol was obtained from GE Healthcare Europe GmbH (Eindhoven, The Netherlands) and methanol, water and acetic acid MS grade from Actu-All (Oss, The Netherlands). All chemicals were of pro-analyse grade or better.

Preparation of the blind artificial urine sample

A blank urine sample was obtained from a male volunteer; 10 mL of this urine was spiked at a level of 100 ng mL^{-1} bromochlorobuterol. In order to have unbiased results, this blind artificial sample was prepared by a technician who was further not involved in this work.

Bio-assay analysis

For testing the sample on the presence of a steroid hormone, control urine samples were prepared by spiking another blank urine sample with either 1 ng mL^{-1} estradiol or 5 ng mL^{-1} testosterone. Sample clean-up and screening the sample extracts in the yeast estrogen and yeast androgen bioassays were performed as described (Bovee *et al*., 2005; Bovee *et al*., 2009).

For testing the sample on the presence of a β-agonist in the competitive radioligand binding assay, a positive control urine sample was prepared by spiking 2 mL of another blank urine sample with clenbuterol at a concentration of 250 ng mL^{-1}. Following the addition of 3 mL 0.25 M sodium acetate buffer pH 4.8 and 10 µL of β-glucuronidase/arylsulphatase, enzymatic deconjugation was carried out in a water bath at 55°C for 2 h. The hydrolysed sample was subjected to SPE on a Strata X-C column (Phenomenex), previously conditioned with methanol and sodium acetate buffer. The vacuum-dried SPE column was washed subsequently with 1 mL 1 M acetic acid, 6 mL methanol and 2 mL of acetone/chloroform (1:1). Finally, the contents of the dried SPE column were eluted with 7.5 mL of 3% ammonia in ethyl acetate and the eluate was evaporated to dryness under a stream of nitrogen gas at 40°C. The residue obtained was dissolved in 50 µL acetonitrile and subsequently 450 µL PBS buffer was added. Screening the sample extract in the competitive radioligand binding assay, using radioactive 3H-dihydroalprenolol as a label, was performed as described before (Boyd *et al*., 2009). At the end, the radioactivity on the filter is determined with a liquid scintillation counter (Tri-carb 2300TR, Perkinelmer, Groningen, The Netherlands) and the measured amount of radioactivity is inversely proportional with the amount of β agonists in the extract.

LC-Fractionation

The fractionation system consisted of a Waters Acquity UPLC (Millford, MA, US) coupled to two Gilson 234 autosamplers (Middleton, WI, US) which were used as fraction collectors. UPLC separation was performed using a Waters Acquity BEH C18 (100 × 2.1 mm ID, 1.7 µm) column and a mobile phase consisting of (A) water and (B) 90/10 v/v% acetonitrile/water. At a flow rate of 400 µL min^{-1} a gradient was started at 40% B for 1 min and was then increased linearly to 100% B in 24 min. The mobile phase composition was kept at 100% B for 2 min and returned to 40% in 0.5 min. An equilibration time of 2.5 min was allowed. The column effluent was split in a 50:50 ratio and diverted towards the two fraction collectors. The software used for the modification of the autosamplers to fraction collectors was kindly donated by Dr. M. Giera (Giera *et al*., 2009). Furthermore, the autosampler hardware was modified, the original injection needle was replaced by a fused silica capillary and the original sample tray was modified in such a way that 24, 96 and 384-well plates could be placed on the tray. During the

UPLC high resolution fractionation each well was sampled during 3 min which corresponds to 600 µL. After fractionation bioactivity screening was performed in order to identify the bioactive fractions of interest.

LC-MS analysis

The LC fractions collected were diluted 1:1 with water and 10 µL was injected on the LC-MS system. The LC-MS system consisted of an Ultimate 3000 LC system coupled through a HESI II electrospray source to an Q-Exactive Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA). For LC separation, a 100 × 3 mm ID, 3 µm Atlantis T3 column from Waters (Milford, MA, USA) was used. The mobile phases consisted of water (A) and methanol:water 95:5 (B) both containing 2 mM ammonium formate and 0.002% formic acid. The LC eluent gradient was as follows: 0.1 min at 100% A, then a linear gradient to 45% B in 1.9 min, and a linear gradient to 100% B in 6 min. The final composition was held for 6.5 min after which the gradient returned to its initial conditions in 0.5 min which was then kept for 5 min. The flow rate was set at 300 µL min^{-1} and column temperature was maintained at 40°C.

The electrospray source was operated in positive mode, the electrospray settings were as follow, electrospray voltage 3.5 kV; sheath gas 47.5 a.u.; auxiliary gas 11.5 a.u. The probe-heater was set at 412.5°C, and the heated capillary was set at 320°C. Data were acquired by continuously alternating scan events: first a full-scan (135-1,000 amu) followed by five all-ion fragmentation events (*m/z* 150±110Da, *m/z* 250±110 Da, *m/z* 350±110 Da, *m/z* 450±110 Da and *m/z* 750±510 Da (Figure 1). The fragmentation normalised collision energy was stepped from 14, 40, 80 %. The resolving power was 70,000 for the full-scan event and 35,000 for the all-ion-fragmentation events (defined at full width half maximum at *m/z* 200). The other parameters for the mass spectrometer were automatically tuned with the tuning and calibration procedure. Before each series of analysis, the mass calibration of the mass spectrometer was checked and re-calibrated if needed.

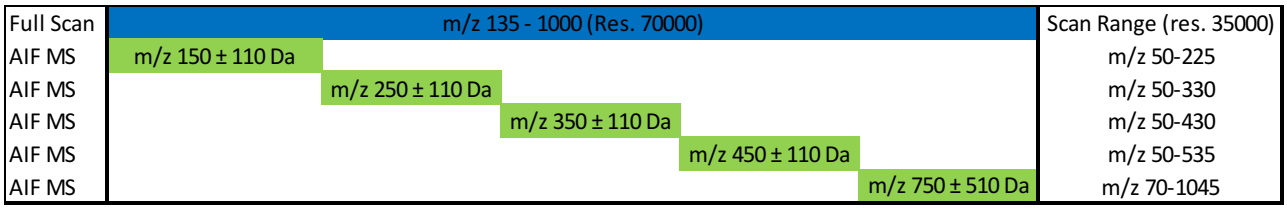

Figure 1. Various scan events applied with normalised collision energies of 14, 40 and 80%.

Statistical analysis

After chemical analysis, statistical analysis was performed to determine which *m/z* values causes the differences between the bioactive and non-bioactive fractions, i.e. which *m/z* values are mainly present in the active fraction only and could thus be responsible for the observed activity in the biosensor assay. First, the data-size of the obtained raw data was reduced and retention times were aligned with Metalign software (Lommen, 2009). The reduced and aligned data output consisted of *m/z*, retention-time, and intensity. These data were subsequently imported into Simca (version 13; Umetrics). Next, statistical analysis was performed by first log transform and Pareto scale the data and then data were examined using principal component analysis (PCA) to study if the data were equally distributed over the plane and possible outliers are identified. The data files were divided in a positive group (the bioactive fraction) and a negative group (samples before and after the bioactive fraction). The two groups were then separated using orthogonal partial least squares discriminant analysis (OPLS-DA). Validation of the model was performed using cross validation and a permutation test. The *m/z* values contributing to the separation, *i.e*. *m/z* values that are probably related to the bioactive compound, were identified using a S-Plot. The top ten upregulated *m/z* signals in the S-Plot were further examined by extracting the exact mass traces and used to reconstruct the co-eluting fragmentation spectra in the original raw data. Element compositions were determined from the mono-isotope mass and isotope pattern. Finally, the identity was elucidated by combining the obtained fragmentation pattern and the element composition by use of Metfrag (Wolf *et al*., 2010).

Results and Discussion

As an example of the unified approach to detect unknowns in various matrices a fictive case was created with an unknown β-agonist in urine. The case description in short: a healthy young male person suddenly had itches over his full body and visited a medical doctor. The doctor observed that the patient's urine was rather lightly coloured compared to normal urine. The young male had a healthy life style and was also visiting the gym at least 5 times a week. Based on this case description, the BB group decided to use a panel of in vitro (bio)assays to screen this person's urine. A yeast estrogen, yeast androgen and a β-agonist assay were applied (Bovee *et al*., 2004; Bovee *et al*., 2007; Boyd *et al*., 2009). The urine sample was negative in the yeast estrogen bioassay, but both the androgen and β-agonist assay showed activity. Especially, the latter triggered the possibility that the patient had been taken a β-agonist unawarely, *i.e*. via consumption of a food supplement. In order to investigate the suspicious urine sample, a unified approach was applied for the identification of the causative compound in both assays (Figure 2).

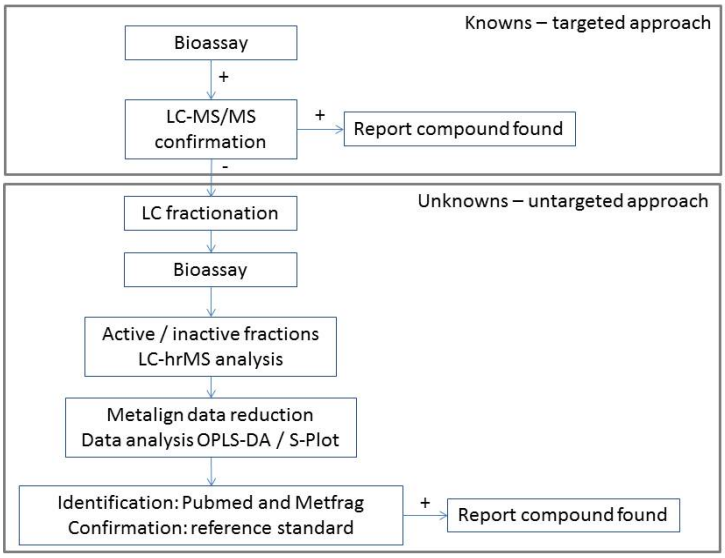

Figure 2. Unified approach to detect unknowns in complex matrices.

To determine which compound or compounds caused the observed activity in the yeast androgen bioassay and the radiolig- and competitive β-agonist binding assay, the urine sample was both analysed by our routine LC-MS/MS analyses for steroid hormones and β-agonists. The analysis for steroid hormones revealed the presence of testosterone levels that could fully explain the observed response in the yeast androgen bioassay. Routine LC-MS/MS analyses for β-agonists did not reveal a hit, *i.e*. no known β-agonist from the list of β-agonists analysed in our routine method was present in the urine. As a clear activity was found in de competitive binding-assay for β-agonists, this triggered both the BB and the analytical group to further investigate the sample.

To identify which compound or compounds are causing the activity in the β-agonist binding assay, the sample was first fractionated to make analysis less complex. Normally, a high resolution fractionation is preferred and applied at our laboratory using 96 or 384 well plates (Rijk *et al*., 2009). However, in this case, due to the use of a rather expensive receptor protein, a decision was made to perform a low resolution fractionation of 10 fractions of 3 min each first. Probably, these fractions still contain multiple non-relevant matrix compounds which will complicate the identification, but then there is still an option to increase the resolution by performing multiple fractionations in sequence. *I.e*., after the first 10-step fractionation a second 10-step fractionation can be performed by separating the active fraction from the first 10-step fractionation. In this way, only 20 biosensor measurements are needed and a resolution of 100 (10x10) is theoretically obtained. Within this study the first 10-step fractionation was first fully analysed, after which it can still be decided if the additional 10–step fractionation of the active fraction is needed. Figure 3 shows the results of the first 10 fractions as obtained in the competitive β-agonist binding assay.

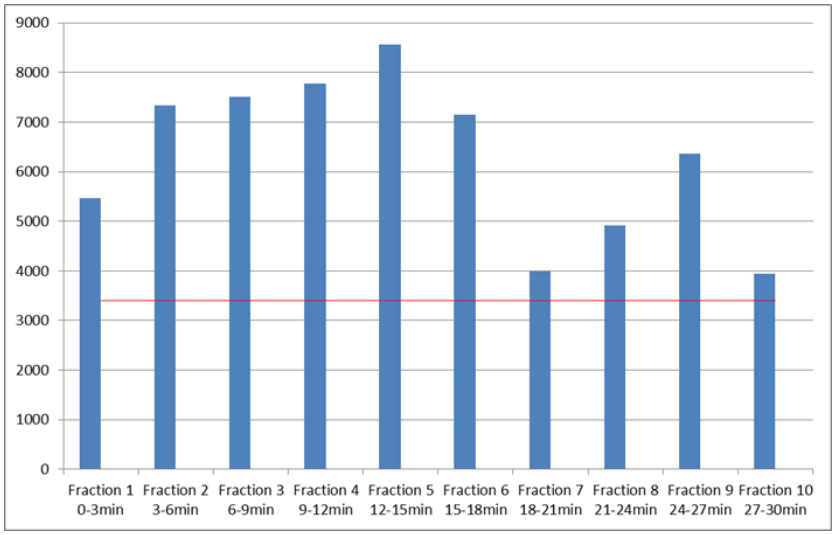

Figure 3. Activity of the various fractions as obtained in the competitive radioligand β-agonist binding-assay.

The first ten fractions were analysed with the Q-Exactive Orbitrap mass spectrometer. The LC method applied with the Orbitrap MS was not a fast UHPLC method in order to allow multiple scan events with a total cycle time of approximately 1.75 s. If UHPLC would have been applied and the average peak width is 8-10 seconds, only 4-6 data points would have been collected, which is not sufficient for a good chromatographic peak description. The conventional LC method we used now resulted in peak widths of 14-18 s, corresponding to respectively 8-10 data points, which allowed multiple scan events. The various scan events consisted of one full-scan event and multiple all ion fragmentation events with different isolation windows (Figure 1). By applying these various fragmentation events, additional information is obtained of the detected molecules. The scan event of *m/z* 230-330 for example means an isolation window of 100 Da what was used and only molecules in this window are fragmented. This means fragments obtained originate from precursor ions with a *m/z* 230-330. This strongly reduces the number of possible molecules and makes identification less complicated, since the MS/MS spectra will contain less interfering fragment ions from other masses.

The activity in three out of the first ten fractions was clearly inhibited (fractions 7, 8 and 10). Seeing the pattern, it was decided to treat fractions 7 and 8 as suspected (class 2) and the other fractions as not suspected (class 1). The ten fractions were subsequently analysed with the Q-Exactive Orbitrap mass spectrometer as described above and in the experimental section. In order to deal with the obtained large MS data files (~280 Mb each), data was reduced and aligned using Metalign software. In the end, approximately 19,000 unique *m/z* values were present in each of the 10 data files. In order to determine which masses were responsible for the differences between the fractions a OPLS-DA analysis was performed (Figure 4).

From the corresponding S-plot the top ten masses could be extracted that contribute the strongest to the separation of classes 1 and 2 in OPLS-DA. The largest contribution was caused by *m/z* 321.036 and *m/z* 323.034. Just by having a look at the isotope pattern and also by calculation, it was clear that both *m/z* values belong to a chlorinated or brominated compound. Next, the raw data files were used to extract these masses and a clear peak was observed. The full-scan spectra before and after this peak were then extracted from the spectra on top of the peak. Based on the isotopic pattern and the exact mass a tentative elemental composition could be determined: $C_{12}H_{18}ON_2BrCl$.

Subsequently, a databases search was applied on this element composition in Pubchem, but this resulted in 92 possibilities. However, thanks to the strategy used, we also have fragmentation data available and it was decided to use *in silico* fragmentation identification tools, *i.e*. in this study the online MetFrag software (http://msbi.ipb-halle.de/MetFrag/)(Wolf *et al*., 2010), to further tentatively identify this compound. Of all the masses that were present in the fragmentation spectra, 13 fragments could be correlated to a structure which ultimately gave the highest score.

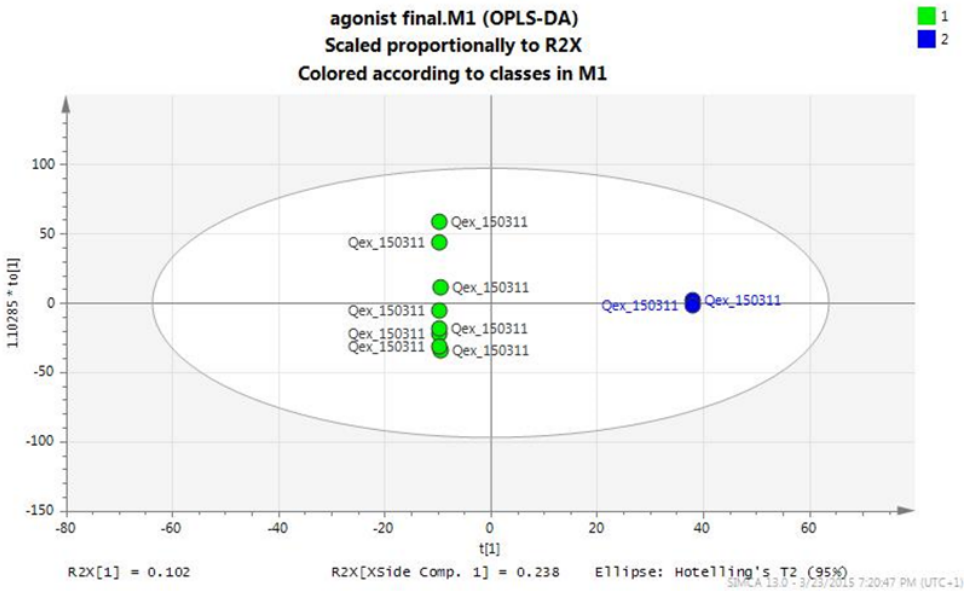

Figure 4. OPLS-DA of various fractions analysed by LC-Orbitrap MS.

The name of the compound that was related with this structure was bromochlorobuterol. Bromochlorobuterol is a clenbuterol-like compound and is also known to be a β-agonist. Still identification using this type of software tools is tentative. Both retention time and spectra of a standard reference compound should be matched with the suspected peak or NMR should be performed. However, as often in these cases, concentrations are relatively low (low µg mL^{-1} concentration) and the amount of sample is limited, NMR is not possible as a confirmation technique. In this specific case the compound was positively confirmed by comparison with a commercial available standard of bromochlorobuterol using LC-MS/MS.

Conclusions

The present work describes an approach to identify unknown bioactive compounds in complex matrices. The strengths of both biological effect or binding-assays and analytical chemistry in combination with powerful statistical tools and *in silico* fragmentation prediction prove that identification of unknown actives at low concentration levels in complex matrices is possible.

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DEVELOPMENT AND VALIDATION OF A CHIRAL HPLC-MS/MS METHOD FOR THE IDENTIFICATION OF CHLORAMPHENICOL ISOMERS AND QUANTIFICATION OF (R,R)-PARA-CAP IN URINE

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Abstract
Chloramphenicol (CAP) is a broad-spectrum antibiotic that is banned from human and animal medicinal use in Europe with the exception of eye drops for occasion treatment of conjunctivitis. There are some severe side effects that can occur from ingesting of CAP. These side effects are the reason that CAP has been banned in products of animal origin. To regulate the zero tolerance, a chiral HPLC-MS/MS method is used to determine the concentration of CAP in different matrices. Just one of the eight isomers of CAP is considered as the antimicrobial active substance namely (R,R)-para-CAP.
The aim of the study was to create a robust and cost-efficient method for the identification of the different isomers and the quantitative confirmation of (R,R)-para-CAP. Three different sample preparation methods were compared in duplicate. To determine the preferred method, three different parameters were compared: sensitivity, peak width and shape of the two daughter peaks.
The best method was validated according to the commission decision 2002/657/EC (1). Seven blanks were spiked at three levels for three different days (0.15 µg L^{-1}, 0.30 µg L^{-1}, 0.45 µg L^{-1}). Accuracy, decision limit (CCα), detection limit (CCβ) and other parameters were calculated using ResVal (2). It was concluded that the method applied is suitable for analysing large numbers of samples.

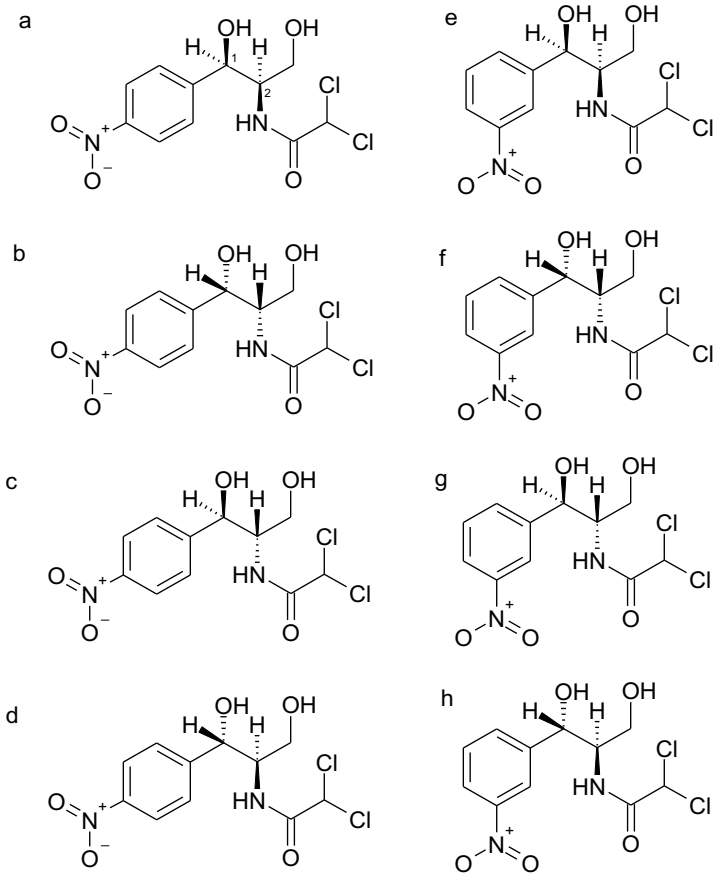

Figure 1. Molecular structures of eight CAP-isomers: a (R,R)-para-CAP, b (S,S)-para-CAP, c (R,S)-para-CAP, d (S,R)-para-CAP, e (R,R)-meta-CAP, f (S,S)-meta-CAP, g (R,S)-meta-CAP, h (S,R)-meta-CAP.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic that is banned from human and animal medicinal use in Europe with the exception of eye drops for the occasional treatment of conjunctivitis. CAP occurs in eight isomeric configurations, four meta- and four para-configuration (Figure 1). In general, the term CAP is used for the (R,R)-para-CAP isomer as this is the isomer with antimicrobial properties. Other para-CAP isomers are reported to be biologically active, but their antimicrobial properties are debated. For the quantification and conformation of CAP, mainly reversed-phase LC-MS/MS is used. These analytical columns can not discriminate the different CAP-isomers. A chiral stationary phase analytical column can separate the different CAP-isomers. Therefore, the method is suited to unequivocally confirm the identity of the individual CAP isomers. A paper of Berendsen *et al*. (3) is used as backbone for this study. Chromatography, selectivity and sensitivity were optimized by adjusting a few parameters and using a different sample clean-up.

Materials and Methods

Chemicals

Analytical standards (Table 1) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and Witega (Berlin, Germany). Water for sample preparation was deionised and passed trough a Millipore Milli-Q water purification system (Billercia, MA, USA). Acetonitrile (Ultra LC-MS), methanol (absolute) and ethylacetate (*p.a.*) were purchased at Biosolve (Valkenswaard, The Netherlands). *Helix pomatia* juice (SHP glucuronidase/ arylsulphatase) is purchased from Brunschwig (Amsterdam, The Netherlands). Acetic Acid glacial was purchased from Merck (Darmstadt, Germany).

Table 1. Different standards and suppliers

Standard	Supplier	Alternative name
(R,R)-para-CAP	Sigma-Aldrich	Levomycetin
(R,R)-para-CAP-d5*	Sigma-Aldrich	Threo-para-chloramphenicol-d5
(S,S)-para-CAP	Witega	Dextramycin
(R,S)-para-CAP-d5 en (S,R)-para-CAP-d5	Witega	Erythro-chloramphenicol-d5
(R,R)-meta-CAP en (S,S)-meta-CAP	Witega	Threo-meta-chloramphenicol
(R,S)-meta-CAP en (S,R)-meta	Witega	Erythro-meta-chloramphenicol
(R,S)-meta-CAP en (S,R)-meta-CAP	Witega	Erythro-meta-chloramphenicol

** Used as internal standard*

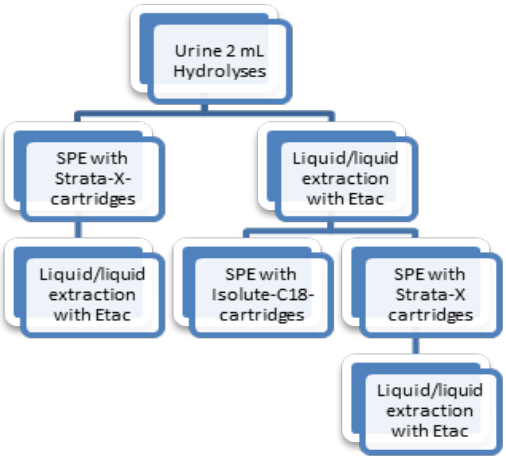

Figure 2. Three different extraction methods.

Sample preparation final method

Three different extraction methods were examined (Figure 2). In the final method, 2 mL urine was transferred into a 15-mL polypropylene (PP) centrifuge tube and the pH was adjusted to 4.5-5.0. Overnight hydrolysis was performed by adding 20 µL *Helix pomatia* juice. After the pH was adjusted to 6.8-7.0, the urine was extracted with 6 mL ethylacetate. After centrifugation (3,600 *g*, 10 min, 4°C.), the upper layer was pipetted into a 15-mL PP centrifuge tube. The solvent was evaporated under a gentle stream of nitrogen at 45°C until dry. The residue was dissolved in 50 µL methanol (MeOH) and 2 mL MilliQ water (H_2O). An OASIS HLB 6 mL, 200 mg (Waters, Milford, MA, USA) SPE cartridge was conditioned with 5 mL MeOH followed by 5 mL H_2O. The sample extract was applied onto the cartridge. The OASIS HLB cartridge was washed with 6 mL 1% acetic acid in MeOH/H_2O (40/60 $^{v}/_{v}$%) and 6 mL 1% ammonia in MeOH/H_2O (10/90 $^{v}/_{v}$%). After the cartridges were dried under vacuum a

clean test tube was placed under the SPE cartridge and the components were eluted from the cartridge using 3 mL MeOH/H_2O (80/20 $^{v}/_{v}$%). The solvent was evaporated under a gentle stream of nitrogen at 45°C until dry. The residue was dissolved in 250 µL 1 mM ammoniumacetate in 2% acetonitrile in water and transferred into a LC-MS/MS vial.

Table 2. Isocratic LC-conditions

LC-system	Agilent 1290 (Agilent Technologies, USA)
Column	CHIRALPAK AGP 5 µm, 2 x 150 mm (Daicel Chemical Industries, USA)
Injection volume	5 µL
Sample temperature	10°C.
Needle level	0.00 mm
Flow	0.40 mL min^{-1} (constant)
Stop time	10 min
Solvent A	1 mM ammonium acetate in 2% ACN/H_2O pH 6
Min pressure	500 psi
Max pressure	2,100 psi

Table 3. ESI^- MS-conditions

Mass spectrometer	Sciex 6500 (Sciex, Framingham, MA 01701, USA)
Acquisition	Scheduled MRM (sMRM)
MRM detection window	90 s
Curtain Gas (CUR)	35
Collision Gas (CAD)	Medium
IonSpray Voltage (IS)	-4,500
Temperature (TEM)	500
Ion Source Gas 1 (GS1)	60
Ion Source Gas 2 (GS2)	65
Min pressure	500 psi
Max pressure	2,100 psi

Table 4. sMRM settings and transitions

Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	ID	DP (V)	EP (V)	CE (V)
320.9	236.9	5.0		-25	-10	-18
320.9	206.9	5.0		-25	-10	-22
320.9	84.0	5.0		-25	-10	-40
320.9	151.9	2.8	(R,R)/(S,S)-meta-CAP (R,R)/(S,S)-meta-CAP	-15	-10	-22
320.9	257.0	2.8	(R,R)/(S,S)-meta-CAP (R,R)-para-CAP	-15	-10	-16
325.9	156.9	2.7	(R,R)-para-CAP	-20	-10	-24
320.9	206.9	3.4	(R,R)-para-CAP-d5	-20	-10	-20
320.9	179.0	3.4	(R,S)/(S,R)-meta-CAP (R,S)/(S,R)-meta-CAP	-20	-10	-30
320.9	156.9	2.3	(R,S)/(S,R)-para-CAP (R,S)/(S,R)-para-CAP	-30	-10	-24
320.9	262.0	2.3	(S,S)-para-CAP	-30	-10	-16
320.9	151.9	5.0	(S,S)-para-CAP	-25	-10	-22
320.9	121.0	5.0	(R,R)/(S,S)-meta-CAP (R,R)/(S,S)-meta-CAP	-25	-10	-42
320.9	257.0	8.1	(R,S)/(S,R)-meta-CAP (R,S)/(S,R)-meta-CAP	-25	-10	-16
320.9	176.0	8.1	(R,S)/(S,R)-para-CAP	-25	-10	-25
320.9	206.9	6.1		-20	-10	-20
320.9	179.0	6.1		-20	-10	-30
325.9	157.0	4.0		-30	-10	-24
325.9	262.0	4.0	(R,S)/(S,R)-para-CAP	-30	-10	-16

Ultra Performance Liquid Chromatography (UPLC)

UPLC was performed on an Agilent 1290 system (Agilent Technologies, USA) equipped with a CHIRALPAK HPLC AGP column (AGP, 150 x 2 mm, 5 µm). Mobile phase was 2% acetonitrile with ammonium salt. An isocratic flow rate of 0.4 mL min^{-1} was

used and runtime was 10 min. The column temperature was kept at 30°C and 5 µL extract was injected onto the analytical column (Table 2).

Mass spectrometric detection

The LC system was directly interfaced with a Sciex 6500 QTRAP mass spectrometer (Sciex, Framingham, MA 01701, USA) equipped with an electrospray ionization interface operating in the negative mode (ESI^-). Ion acquisition was operated at unit mass resolution. See Tables 3 and 4 for the MS-conditions and scheduled MRM settings and transitions. See Figure 3 for the structural formulas and different fragments of all CAP isomers.

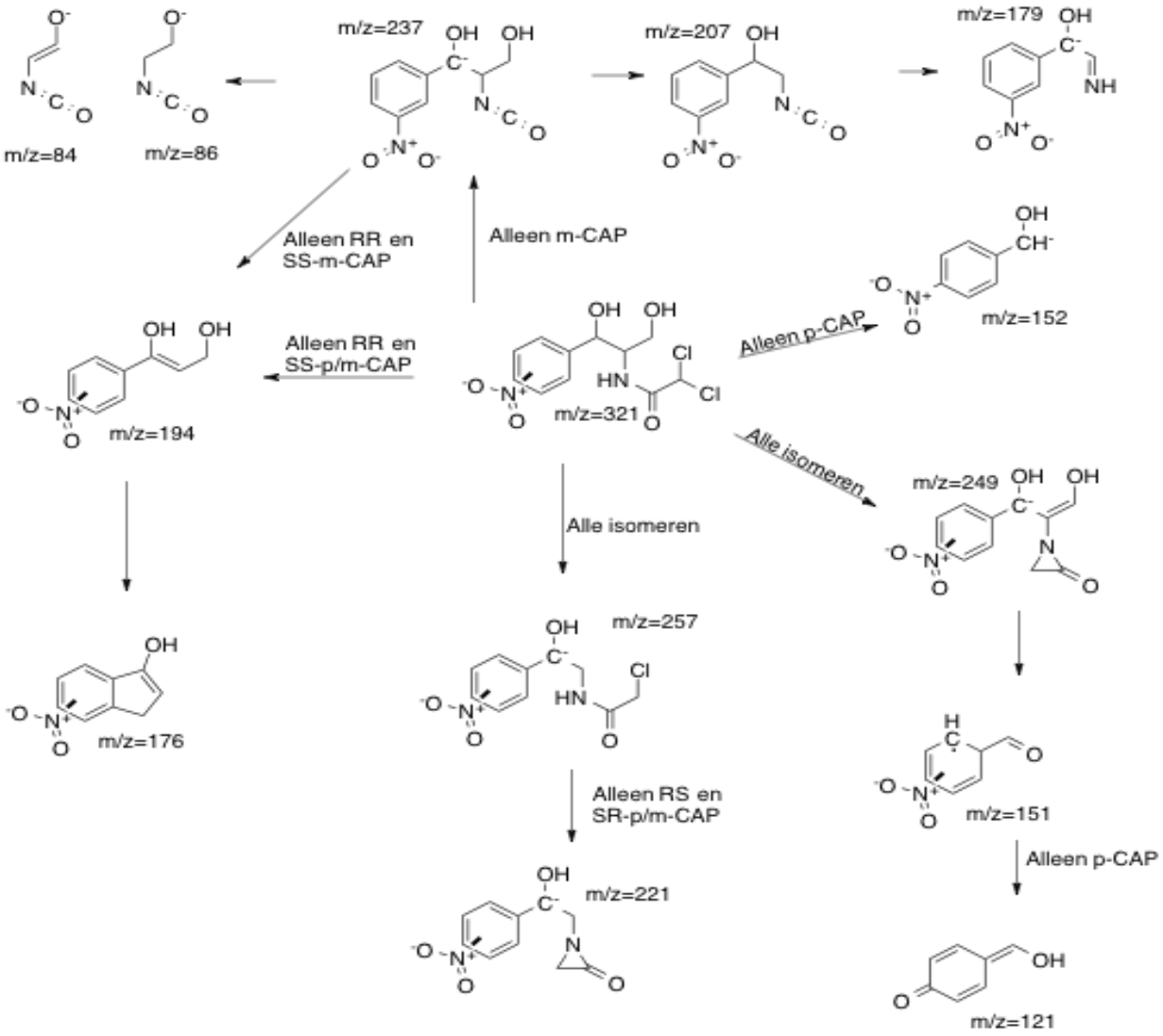

Figure 3. Structural formulas and different fragments of all CAP isomers.

Results

Sensitivity, peak width and shape from the three extraction methods were compared to each other (Figure 4). Each method earned a decreasing amount of points representing best to worst. For (R,R)-para-CAP this ended up in a column chart representation as found in Figure 5.

The first set of chromatograms of the different daughter peaks of (R,R)-para-CAP that were scored are shown in Figure 5. The chromatograms of 0.3 µg L^{-1} spiked urines were compared with respect to peak width, shape and size. Because of the best peak width and shape of both daughter peaks for (R,R)-para-CAP method 3 was used for validation. Strata-X (Phenomenex) SPE columns in the experiment were exchanged with OASIS-HLB SPE-columns for cost-efficient reasons while both columns gave equal results. Chromatographic separation was achieved by using the isocratic LC-conditions listed in Table 2 (Figure 6).

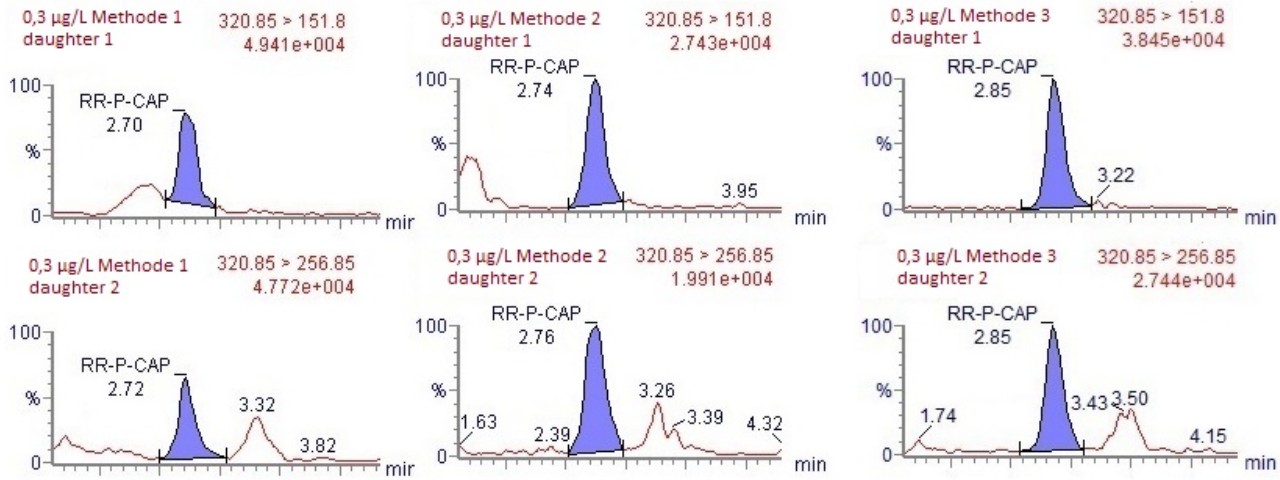

Figure 4: chromatograms of the daughter peaks of (R,R)-para-CAP for the three different methods.

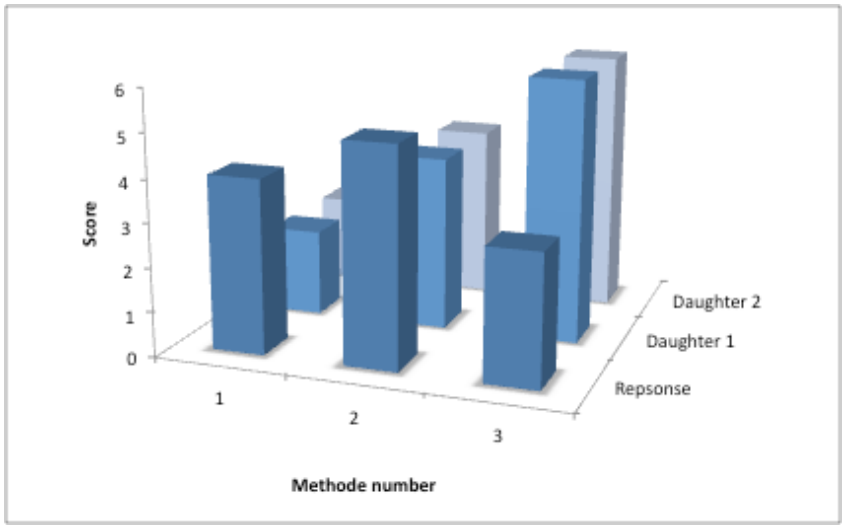

Figure 5. Column chart representation of the compared methods for (R,R)-para-CAP.

Table 5. Validation results (ResVal 3.5)

Parameter	(R,R)-para-CAP
Recommended concentration (µg L^{-1})	0.3
CCα (µg L^{-1})	0.08
CCβ (µg L^{-1})	0.16
Accuracy (%)	103.8
Measurement of Uncertainty (%)	35.1
Specificity	Passed
Relative standard deviation Reproducibility (RSDrl) (%)	15.7
Standard deviation Repeatability (Sr)	0.048
Relative standard deviation Repeatability (RSDr) (%)	15.6
Repeatability	0.136
Standard deviation BL-reproducibility (Srl)	0.049
BL-reproducibility	0.136

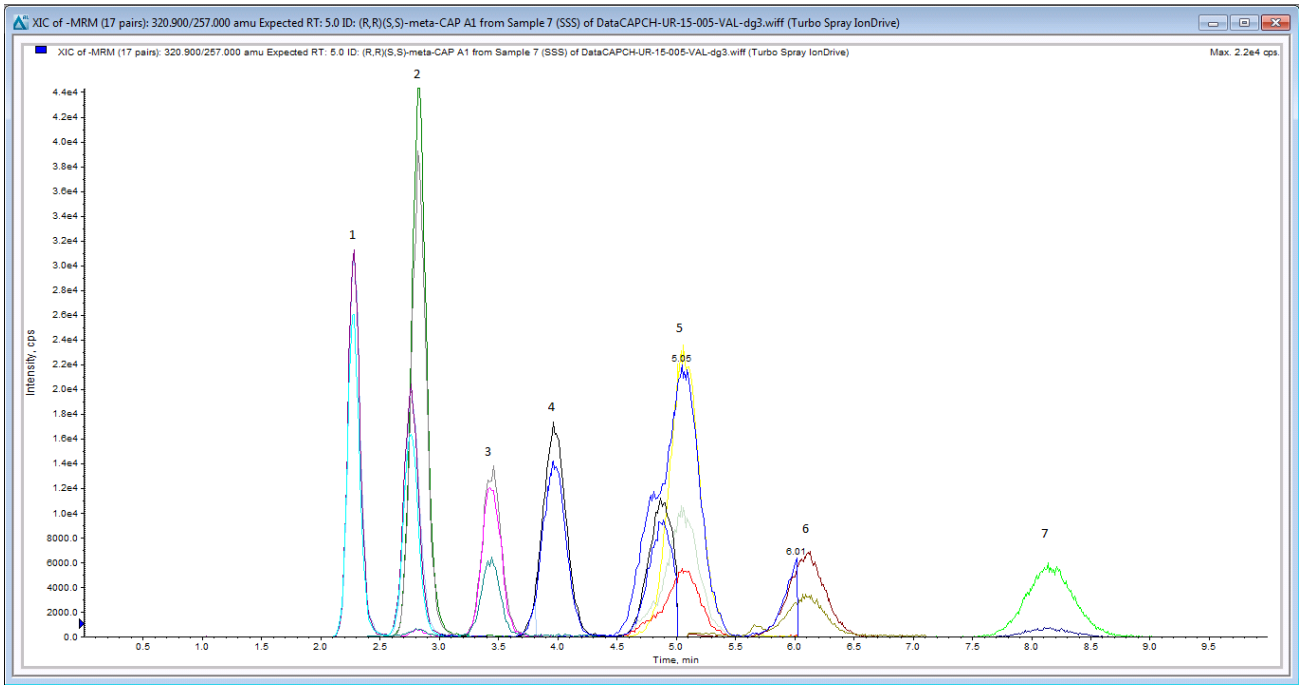

Figure 6. Total ion current of all 8 CAP isomers, Sciex 6500 QTRAP;
***1** RS/SR-p-CAP-d_5, **2** RR-p-CAP, **3** RS/SR-m-CAP, **4** RS/SR-p-CAP-d_5, **5** SS-p-CAP + RR/SS-m-CAP, **6** RS/SR-m-CAP, **7** RR/SS-m-CAP*

Conclusion

A quantitative chiral HPLC-MS/MS confirmation method was developed for (R,R)-para-CAP and identification of all other CAP isomers in urine. The method was validated according to the Commission Decision 2002/657/EC. The CCα of (R,R)-para-CAP is 0.08 µg L^{-1} and the CCβ is 0.16 µg L^{-1}.

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(035)

VALIDATION OF A METHOD FOR THE DETERMINATION OF RESIDUES OF ß-LACTAM ANTIBIOTICS IN MILK BY LC-MS/MS

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Abstract
The presented method is able to quantify and confirm the penicillins amoxicillin, ampicillin, penicillin G (benzylpenicillin), cloxacillin, dicloxacillin, penicillin V (phenoxymethylpenicillin), nafcillin, oxacillin and the cephalosporins cefalexin, cefquinome, ceftiofur, desfuroylceftiofur (DFC), cefapirin, desacetylcefapirin (DAC), cefoperazone, cefalonium and cefazolin in cow milk between 0.5 and 2.5 MRL. Applying a factor-comprehensive in-house validation concept, merely eight runs on different concentration levels need to be analysed in order to gain comprehensive knowledge about the reliability, robustness and performance of the method. After protein separation with acetonitrile and extraction with buffer, the clean-up is performed by way of SPE. The determination of the antibiotics is effected by means of a C18 column for LC separation and a triple quadrupole mass spectrometer in the positive ESI mode to measure the transitions of the substances in the MRM mode. The quantitation of the analytes is performed by way of linear matrix calibration curves. The reported validation parameters CCα, CCβ, recovery (96 - 109 %), the relative repeatability RSD_r (3.9 – 10.1 %) and the relative within-laboratory reproducibility RSD_{wR} (4.2 – 12.6) are in accordance with Commission Decision 2002/657/EC.

Introduction
The ß-lactam antibiotic groups penicillins and cephalosporins are prevalently used in the therapy of infectious diseases of animals. However, a long-time consumption of low-level doses of antibiotics, *e.g*. in milk or other food, can lead to the formation and distribution of drug-resistant micro-organisms. Maximum residue limits (MRLs) in different food matrices were established by European Regulation (EC) No 470/2009 and subsequent modifications to ensure human food safety.

With regard to this aim, methods for the determination of antibiotics should be developed in the range of the MRL. A method to investigate a number of different groups and substances in one analytical run is particularly efficient. This means that a growing quality is needed in the residue control of food of animal origin. Commission Decision 2002/657/EC requires the calculation of validation parameters in default limits in the framework of method validations for drugs authorised for food-producing animals.

On the basis of a method for other antibiotics, a method was validated which is fit for purpose to analyse ß-lactams in milk.

Materials and Methods

Chemicals, standards and solutions
All solvents (Fisher Scientific, Schwerte, Germany) and chemicals (Merck, Darmstadt, Germany) were of HPLC or analytical-grade quality, respectively. The ß-lactam antibiotic standard substances were purchased from Ehrenstorfer (Augsburg, Germany), SIGMA (Deisenhofen, Germany), Chemos GmbH (Regenstauf, Germany), Santa Cruz Biotechnology (Heidelberg, Germany) and Ribbon (Milano, Italy). Stock solutions of the individual substances with a concentration of 1 mg mL^{-1} and further intermediate standard solutions of the substances as well as working mix solutions were prepared by the use of the mixture water/methanol (90/10, v/v). The extraction solution was composed of McIlvaine buffer (0.1 M citric acid x H_2O/0.2 M Na_2HPO_4 x 2 H_2O = 60/40 v/v, pH 4.0) and 0.1M Titriplex III (Na_2EDTA x 2 H_2O). The SPE cartridges HLB OASIS (200 mg, 6 ml) were obtained from Waters (Eschborn, Germany). Component A of the HPLC mobile phase consisted of water with 0.1% formic acid and component B of the HPLC mobile phase consisted of acetonitrile with 0.1% formic acid.

Extraction procedure and clean-up
The analytical samples (2 g of cow milk) are weighed into 50 mL centrifuge tubes. Then the internal standard solution (penicillin G-d7, amoxicillin-d4) and the standard mixture of the analytes are added to the analytical sample. The extraction procedure begins 10 min after the spiking step. Six mL of acetonitrile are added to the milk sample. The mixture is carefully vortexed for approximately 1 min and shaken for 10 min. Subsequently, the mixture is centrifuged (3800 g, 5 min, 5°C) and the supernatant of acetonitrile is collected.

The acetonitrile phase is evaporated to dryness under a weak nitrogen stream in a Turbo-Vap evaporator at 40°C. The residue is dissolved in 5 mL of the extraction solution.

Table 1. Substance-specific LC-MS/MS parameters for QTRAP 6500.

Substance	Retention time [min]	Transition Q1→Q3 [*m/z*]	DP[a] [V]	EP[b] [V]	CE[c] [V]	CXP[d] [V]
Penicillins						
Amoxicillin	2.2	366→349	45	10	13	14
		→208			19	11
Ampicillin	4.5	350→160	49	10	19	4
		→79			65	4
Penicillin G	9.0	335→176	61	10	19	4
		→114			41	4
Penicillin V	9.9	351→160	43	10	20	4
		→114			44	4
Oxacillin	10.3	402→160	49	10	21	4
		→144			40	4
Cloxacillin	11.0	436→277	48	10	16	4
		→160			20	4
Nafcillin	11.3	415→239	81	10	35	4
		→182			58	4
Dicloxacillin	11.9	470→160	56	10	23	4
		→311			18	4
Cephalosporins						
DAC	1.8	382→152	58	10	31	4
		→226			28	4
Cefapirin	3.3	424→292	49	10	23	4
		→124			64	4
Cefquinome	4.9	529→134	41	10	75	4
		→324			21	4
Cefalexin	4.4	348→158	39	10	16	4
		→106			41	4
Cefalonium	4.8	459→337	51	10	15	4
		→163			29	4
DFC	5.4	430→126	61	10	40	7
		→227			29	8
Cefazolin	5.9	455→323	53	10	15	4
		→156			25	4
Cefoperazone	7.0	646→143	47	10	47	4
		→148			65	4
Ceftiofur	7.9	524→241	61	10	24	4
		→126			47	4
Int. standards						
Penicillin G-d7	8.9	342→98	75	10	59	4
		→160			27	4
Amoxicillin-d4	2.2	370→353	43	10	12	14
		→212			17	11

[a] *DP=Declustering Potential;* [b] *EP=Entrance Potential;* [c] *CE=Collision Energy;* [d] *CXP=Cell Exit Potential*

The OASIS HLB cartridge is conditioned with 6 mL of methanol followed by 6 mL of water. Next, the buffer extraction solution (5 ml) can be applied onto the cartridge. The cartridge is washed with 6 mL of the water/methanol mixture (95/5, v/v) and dried for 10 min in an air stream. After the drying step 6 mL of methanol are added to elute the analytes into the tube. The eluate is evaporated to dryness under a weak nitrogen stream in a TurboVap evaporator at 40 °C. Then the residue is dissolved in 0.5 mL of the water/acetonitrile mixture (90/10, v/v) and centrifuged. The supernatant is transferred into the LC vial. This solution is either injected directly into the LC-MS/MS system or stored for 12 h in a freezer at -25 °C before injection.

LC-MS/MS measurement

The LC-MS/MS system consists of an LC 1190 instrument from Agilent Technologies (Waldbronn, Germany) with a binary pump, an autosampler, a degasser, a column oven and a system controller. The LC is coupled to a QTRAP 6500 triple mass spectrometer from SCIEX (Darmstadt, Germany). As analytical column for the separation of the analytes, a C18 column ("Aqua" from Phenomenex, 150 mm x 2 mm, 3 µm particle size) with adequate guard is used. The separation of the analytes is performed by applying a gradient of components A and B at an oven temperature of 30°C and a flow rate of 0.3 mL min^{-1}. The injection volume is 10 µL. The samples are kept in the autosampler at a temperature of 10°C. The gradient starts with 90% of component A and decreases to 40% within 12 min.

This composition (40/60) is kept for 3 min. Then component A increases to 90% within 1 min, so that with the following equilibration time of 6 min, the resulting total run time is 22 min. The instrument parameters for the mass spectrometry measurement with the help of the "Analyst" software, version 1.6.2, are as follows: ESI+; scan type = MRM; dwell-time scheduled; resolution Q1 and Q3 = unit; gas = nitrogen; gas 1 = 70 psi; gas 2 = 70 psi; curtain gas = 30 psi; collision gas = high; ion spray voltage = 5500 V; source temp. = 400°C.

The mass spectrometry parameters applied for the transitions from precursor to product ions are shown in Table 1.

Validation procedure

Commission Decision 2002/657/EC requires a validation around the MRL for authorised drugs in food-producing animals. Following this demand the validation study was performed with concentration levels of 0.5, 1.0, 1.5, 2.0 and 2.5 MRL for each substance. The substances were chosen in accordance with the German residue control plan. In the case of pen V, where no MRL for cow milk is established, the level of interest of 10 µg kg^{-1} for the calculation of the critical concentrations was used.

The preparation of the specific study design and the calculation of the validation experiment data were effected with the help of the InterVal software (QuoData GmbH, Dresden, Germany) on the basis of an in-house concept with factor-level combinations. Different influencing factors have to be selected according to the conditions of the samples and the laboratory. It is necessary to select relevant factors and to consider major changes which may occur during routine analysis in the laboratory. For this validation study the six factors 'storage time of sample', 'lot of SPE cartridge', 'status of LC column', 'duration before measurement', 'LC-MS/MS MRM mode' and 'operator' were chosen and varied on two levels (see Table 2). For each run the analyses were performed at the above-mentioned concentration levels around the MRL and additionally on a matrix blank sample at zero level. This resulted in a total number of 48 analyses for the study.

Table 2. Factor-level combinations (runs) with six factors.

Run	Storage time of sample	Lot of SPE cartridge	Status of LC column	Duration before meas.	LC-MS/MS MRM mode	Operator
01	2 days	B	old	directly	sche-adv	G
02	2 days	B	new	directly	sche	M
03	2 days	A	old	after 12 h	sche-adv	G
04	2 days	A	new	after 12 h	sche	G
05	1 day	B	old	after 12 h	sche	G
06	1 day	B	new	after 12 h	sche-adv	M
07	1 day	A	old	directly	sche	M
08	1 day	A	new	directly	sche-adv	G

Results

The measurements of the study were carried out with LC-MS/MS by means of multiple reaction monitoring (MRM-Schedule and MRM-Scheduled advanced) of two fragment ions in a triple quadrupole mass spectrometer after HPLC separation. The validation study was spread over two months. The analytical system can be considered as being stable for this time period at least, because the samples for quality assurance were in the correct range. All substances were measured with the necessary sensitivity for qualification and quantification in one LC-MS/MS run. The verification of the confirmation with the correct ratio of the intensities of the two transitions and with the retention time was successful for all substances. Quantification was effected by means of matrix calibration curves. Only for the substances amoxicillin and pen G the suitable internal standards were used in addition. The MRL is the level of interest and was used as basic concentration level for the calculation of the validation parameters. For pen V, a substance without MRL for milk, the concentration of 10 µg kg^{-1} was used as the level of interest.

The InterVal software allows to calculate the validation parameters, *e.g*. the decision limit CC-alpha, the detection capability CC-beta, the repeatability RSD_r, the within-laboratory reproducibility RSD_{wR} and the recovery according to Commission Decision 2002/657/EC.

Table 3. Validation parameters of ß-lactam antibiotics in milk.

Substances	MRL (µg kg^{-1})	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	RSD$_r$ * (%)	RSD$_{wR}$ ** (%)	Recovery (%)
Penicillins						
Amoxicillin	4	4.33	4.89	3.9	4.2	99
Ampicillin	4	4.81	6.42	7.0	10.0	100
Penicillin G	4	4.44	5.18	5.8	5.8	98
Cloxacillin	30	33.8	40.6	5.1	6.2	104
Dicloxacillin	30	36.3	48.7	7.8	10.1	102
Nafcillin	30	35.5	45.6	7.4	9.0	109
Oxacillin	30	35.3	46.0	5.9	8.6	109
Penicillin V	10***	11.8	15.3	7.0	8.8	102
Cephalosporins						
Cefalexin	100	121	160	6.6	10.1	100
Cefquinome	20	24.7	33.1	8.8	11.1	99
Ceftiofur	100	121	160	6.1	9.9	98
DFC	100	126	183	10.1	12.6	99
Cefapirin	60	69.2	85.2	6.5	7.4	103
DAC	60	73.4	101	9.2	11.5	99
Cefoperazone	50	58.5	74.0	8.0	8.4	104
Cefalonium	20	22.7	27.4	6.2	6.7	96
Cefazolin	50	56.8	68.7	6.4	6.7	99

) RSD$_r$ = repeatability; **) RSD$_{wR}$ = within-laboratory reproducibility; *) no MRL, level of interest.*

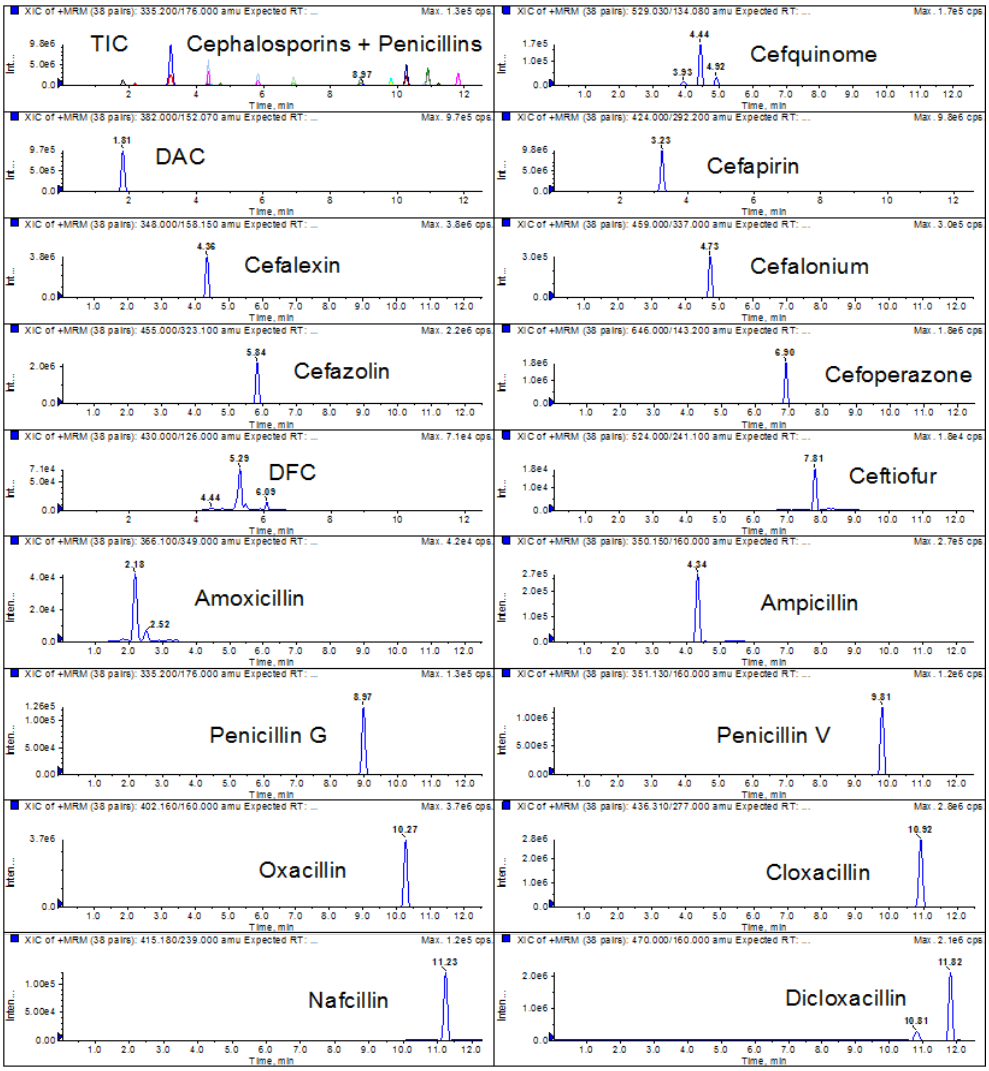

Figure 1. LC-MS/MS chromatograms (MRM, TIC and product ions) of ß-lactam antibiotics at MRL.

The calculated values of the validation study were in the required ranges (see Table 3). The TIC and the product ion chromatograms of the substances in matrix samples (see Figure 1) resulting from the MRM measurements were satisfying with regard to the peak shape and the separation of the peaks. No interferences were observed in the range of the peaks of interest for the analysed blank samples.

Conclusions

The robustness and the applicability of the method were checked successfully by using different factors, which had no significant influence on the validation parameters. The quantification was performed by means of matrix calibration curves and for two substances additionally by using internal standards. All the results of the calculated validation parameters were satisfying and were in the required ranges of Commission Decision 2002/657/EC.

The method is fit for purpose to analyse ß-lactams in cow milk in the concentration range around the MRLs in the framework of official residue control according to Council Directive 96/23/EC.

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(036)

VALIDATION OF A METHOD FOR THE DETERMINATION OF RESIDUES OF TRIPHENYLMETHANE DYES IN AQUACULTURE PRODUCTS BY LC-MS/MS

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Abstract
Triphenylmethane dyes (TMP) have been used in aquaculture to treat and prevent fungal and protozoal infections. These dyes are extensively metabolised to their reduced leuco forms in exposed fish. Due to concerns about the carcinogenicity and mutagenicity of TMPs and their metabolites, they are not allowed as veterinary medicinal products in the EU. A minimum required performance limit (MRPL) of 2 µg kg^{-1} for the sum of malachite green and leuco malachite green has to be met by analytical methods.
A simplified method is described for the determination and confirmation of TMPs and their corresponding leuco forms in aquaculture products. The analytes were extracted with acetonitrile without any further clean-up procedure. Each analyte was quantified with the help of its corresponding deuterated standard. The method was validated in accordance with Commission Decision 2002/657/EC by applying an in-house concept. Four factors (species, duration between sample extraction and analysis, age of column, operator) were varied on two levels, yielding a total of eight different factor-level combinations (runs). For each run, matrix samples were fortified in a concentration range between 0.5 and 2.0 µg kg^{-1}. For all monitored compounds the performance characteristics of the method (CCα, CCβ, repeatability, reproducibility and recovery) are reported.

Introduction
Aquaculture is a strongly growing sector of food production, but suffers from a bad image because of the overuse or illegal use of veterinary drugs. Triphenylmethane (TPM) dyes like malachite green (MG) had been successfully used as medicinal products in aquaculture for many years. However, due to their possible carcinogenic, mutagenic and teratogenic effects, these substances are no longer authorised for use in food-producing animals.

Residue control needs simple and economic methods with high accuracy and trueness. There is a minimum required performance limit (MRPL) of 2 µg kg^{-1} for the sum of MG and leuco malachite green (LMG) [1]. For the particular case of MG and LMG, it is recommended that each individual CCα be less than ½ MRPL, *e.g.* 1.0 µg kg^{-1} [2].

A simplified LC-MS/MS method was developed in accordance with the method of the EURL Fougères [3], which can simultaneously analyse residues of the dyes MG, crystal violet (CV) and brilliant green (BG) as well as of their corresponding leuco forms LMG, leuco crystal violet (LCV) and leuco brilliant green (LBG). The aim of the method development was to reach CCα and CCβ values of less than 1 µg kg^{-1} for all substances.

Materials and Methods

Chemicals, standards and solutions
All solvents and chemicals were of HPLC and analytical-grade quality respectively. Malachite green oxalate, leuco malachite green, crystal violet chloride, leuco crystal violet and brilliant green hydrogen sulphate of analytical grade were purchased from Sigma–Aldrich (Steinheim, Germany). Malachite green-D5 picrate, leuco malachite green-D5, crystal violet-D6 chloride, leuco crystal violet-D6 and brilliant green-D5 hydrogen sulphate were purchased from Witega (Berlin, Germany). Leuco brilliant green was custom-made by Atlanchim Pharma (Nantes, France).

Stock solutions of the individual substances in a concentration of 1 mg mL^{-1} were prepared with acetonitrile. Stock mixtures of standards and internal standard compounds were prepared in a concentration of 10 µg mL^{-1}. The colour substance mixtures with MG, CV and BG were prepared separately with acidic acetonitrile. The leuco substances were diluted only with pure acetonitrile. The working mixtures had a concentration of 10 ng mL^{-1} and were prepared fresh every day. All the standard solutions, stock and working mixtures were stored at -20°C.

The extraction solution was composed of acetonitrile and a solution of ascorbic acid (1 %; 100/1, v/v). Component A of the HPLC mobile phase consisted of an ammonium formate buffer (50 mM, pH 4.5). Component B of the HPLC mobile phase consisted of acetonitrile.

General sample preparation

The analytical sample (1.00 g of muscle of an aquaculture product) was weighed into a 50-mL centrifuge tube. Then 500 µL of hydroxylamine solution (9.5 g L^{-1}) and the standard mixtures were added to the analytical sample. After an incubation time of 10 min, 8 mL of extraction solution and 1 g of anhydrous magnesium sulphate were added to the sample. The mixture was vortexed for 1 min and agitated in an overhead shaker (100 rpm, 10 min). Subsequently the extraction mixture was centrifuged (3,400 g, 5 min, 5°C). A part of the supernatant was centrifuged again (20,000 g, 5 min), transferred to LC vials and used for analysis.

Matrix calibration

Blank tissue samples were fortified with all analytes at levels corresponding to 0.0, 0.25, 0.5, 1.0, 1.5 and 2.0 µg kg^{-1} by adding the respective volumes of standard working mixtures. The volume differences were evened out with extraction solution. 100 µL of both internal standard working mixtures were added to each sample. After sample preparation und analysis the calibration curves were calculated as a function between the analyte concentration and the ratio of the peak area of the analyte to the peak area of the internal standard.

Standard calibration

Additional solutions for standard calibration were prepared with the same levels of analytes and internal standards as the matrix calibration samples. The volume was adjusted to 1 mL with extraction solution.

Recovery samples

A second lot of blank tissue samples was spiked with all analytes at levels of 0.5, 1.0, 1.5 and 2.0 µg kg^{-1} by adding the corresponding volumes of standard working mixtures. The volume differences were evened out with extraction solution. A volume of 100 µL of both internal standard working mixtures was added to each sample. The concentrations of each analyte were calculated with the ratio of the peak area of the analyte to the peak area of the corresponding internal standard and the corresponding matrix calibration and standard calibration, respectively. No internal standard has been available for LBG until now. Therefore, it was quantified with LCV-D6.

Liquid chromatography–mass spectrometry analysis

The LC equipment was an HPLC binary solvent delivery system (Agilent Technologies, 1290 Series) equipped with a reversed-phase C-18 analytical column of 100 mm length × 2.0 mm I.D. and 3.5 µm particle size (Symmetry C18 , Waters). The LC gradient started with 20% of B. After 1 min B was linearly increased to 90% within 2 min. For 7 min the mobile-phase composition was maintained at 90% of B. After a decrease to 20% (1 min), the re-equilibration time was 3 min. The resulting run time was 14 min. The volume of injection was 10 µL. The HPLC system was connected to a triple quadrupole mass spectrometer system (5500 QTRAP® LC/MS/MS, SCIEX) with an electrospray interface (ESI). It was operated in positive ionisation mode. The source operated at 600°C, with the capillary voltage set at 5,500 V, curtain gas 20 and 50 psi for GS1–GS2. Nitrogen was used as nebulizer gas, curtain gas and collision gas.

Table 1. LC-MS/MS parameters for each compound.

Compounds	Retention time (min)	Precursor ion (*m/z*)	DP	MRM1	CE1	CXP1	MRM2	CE2	CXP2
Malachite green	4.5	329.0	156	313.2	49	14	208.2	57	16
Crystal violet	5.1	372.1	66	356.1	55	28	340.2	73	26
Brilliant green	5.7	385.1	216	341.2	55	20	297.2	71	12
Leuco malachite green	6.4	331.1	131	239.2	45	36	223.2	71	26
Leuco crystal violet	6.5	374.1	131	358.2	43	30	239.2	49	10
Leuco brilliant green	9.2	387.4	101	358.2	31	24	281.3	45	18
Internal standards									
Malachite green-D5	4.5	334.1	101	318.2	51	26			
Crystal violet-D6	5.1	378.2	71	362.3	57	28			
Brilliant green-D5	5.7	390.3	136	302.2	57	26			
Leuco malachite green-D5	6.4	336.1	121	239.3	41	18			
Leuco crystal violet-D6	6.5	380.2	91	364.3	45	6			

DP: declustering potential (V); CE: collision energy (eV); CXP: collision exit potential; MRM1: quantitation; MRM2: confirmation

In order to obtain maximum sensitivity for the identification and detection of the target compounds, a careful optimisation of all MS parameters (declustering potential (DP), entrance potential (EP) for precursor ions, collision energy (CE) and collision cell exit potential (CXP) for product ions) was performed by flow injection analysis (FIA) in the spectrometer. Table 1 shows the values of the parameters optimised and the MRM transitions used for the confirmation and quantification of all compounds studied. The MS operated in multiple reaction monitoring (MRM) mode with 100 ms of dwell time per ion transition. For the confirmation of the analytes, the following criteria were used: the acquisition of two MRM transitions for each compound, retention time and the monitoring of the MRM ratio (which is the relationship between the abundances of transitions selected for identification and for quantification). The most intensive MRM transition was selected for quantitation purposes (see Table 1). Data acquisition and processing was carried out using commercial software (Analyst 1.6.2, MultiQuant 2.1.1, SCIEX).

Table 2. Factor-level combinations (runs) with 4 factors.

Run	Species	Storage of extract	Age of column	Operator
Run 01	trout	24 h	old	Mö
Run 02	trout	24 h	new	Ho
Run 03	trout	0 h	old	Ho
Run 04	trout	0 h	new	Mö
Run 05	shrimp	24 h	old	Ho
Run 06	shrimp	24 h	new	Mö
Run 07	shrimp	0 h	old	Mö
Run 08	shrimp	0 h	new	Ho

Validation procedure

The validation was performed in accordance with Commission Decision 2002/657/EC [4], which recommends for the particular case of malachite green and leuco malachite green that each individual CCα be less than ½ MRPL (SANCO 2004/2726-rev 4-December 2008) [2]. This means a validation around ½ MRPL (1 µg kg^{-1}). The study performed for the different matrices was accomplished with concentration levels of 0.5, 1.0, 1.5 and 2.0 x ½ MRPL for each substance. The selection of the substances was performed in accordance with the German residue control plan.

Different influencing factors had to be selected according to the conditions of the samples and the laboratory. The preparation of the specific study design on the basis of an in-house concept with factor-level combinations and the calculation of the validation experiment data were carried out with the help of the InterVal software (QuoData GmbH, Dresden, Germany). For the design of the validation study, it is necessary to select relevant factors (maximum: seven factors) and to consider major changes which may occur during routine analysis. For this validation study, the four factors 'species', 'storage of extracts', 'age of LC column' and 'operator' were varied on two levels (see Table 2). Each run included 7 quality assurance samples: two instrument performance control samples (Q1), a reagent blank sample (Q2), a matrix blank sample (Q3) and three recovery control samples (Q4). Furthermore, the runs contained five samples for recovery, six for matrix and six for standard calibration. This resulted in a total of 24 injections.

Results and Discussion

The different steps of sample preparation, like the extraction with acetonitrile containing a solution of ascorbic acid, the omittance of the evaporation step and the reconstitution step and a second high-speed centrifugation in place of filtration, proved to be advantageous. The analyses were performed by means of LC-MS/MS with multiple reaction monitoring (MRM) of two fragment ions in a triple quadrupole mass spectrometer after HPLC separation. The separation of the analytes was satisfying, as shown by the chromatograms of a representative trout sample which had been spiked with all analytes at a level of 1 µg kg^{-1} (Figure 1).

The validation study was performed within four weeks, hence the analytical system can be considered as being stable for at least this time period. It was possible to measure all substances with the necessary sensitivity for qualification and quantification in one LC-MS/MS run. The confirmation with the correct ratio of the intensities of the two transitions and with the retention time was successful in all cases. Quantitation was effected on the basis of matrix calibration curves with the help of the internal standards. Figure 2 shows the correlation between the spike levels of each analyte and the calculated concentrations on the basis of a matrix calibration. For all analytes with a corresponding internal standard a very narrow confidential interval can be observed. In contrast to that, the values of LBG show a greater variance, because no deuterated standard was available and LCV-d6 was used instead.

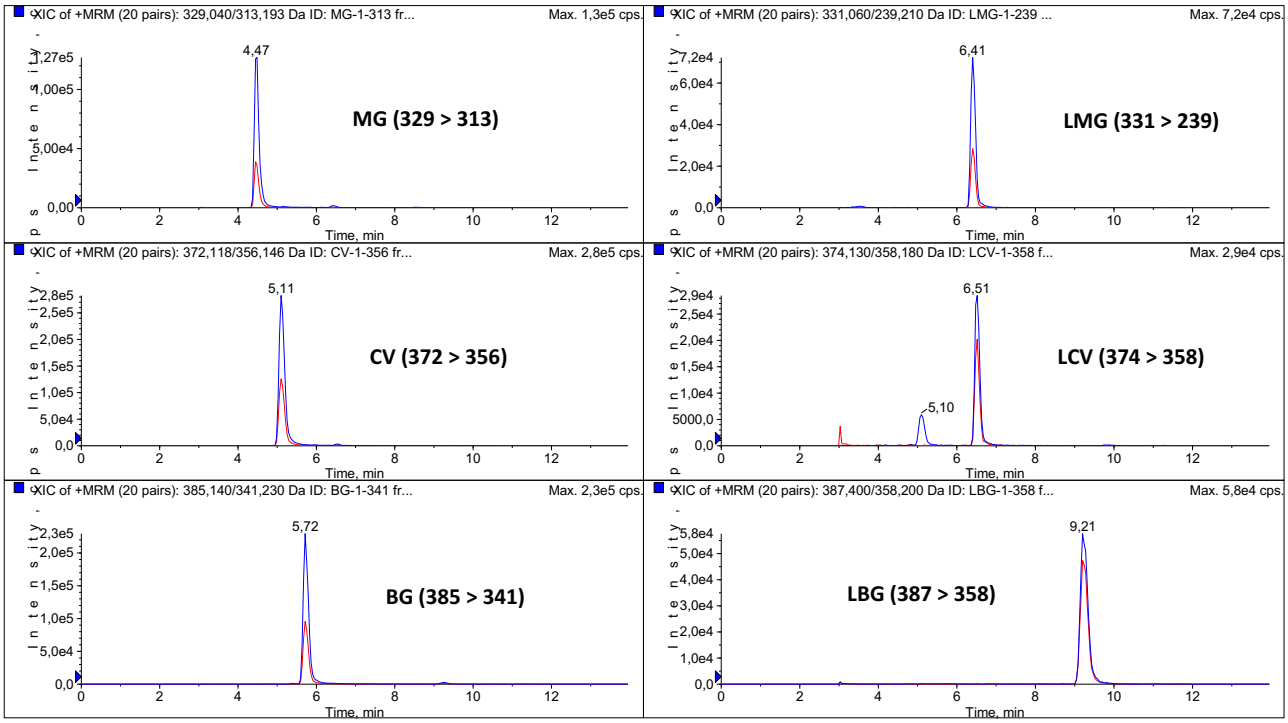

Figure 1. Chromatograms of two transitions of analytes in a trout muscle sample fortified at a level of 1 µg kg^{-1}.

The InterVal software allowed to calculate the validation parameters. The validation parameters like the decision limit CCα, the detection capability CCβ, the repeatability RSD_r, the within-laboratory reproducibility RSD_{wR} and the recovery were calculated according to Commission Decision 2002/657/EC [4]. The performance characteristics of the validation study were in the demanded range on the basis of a matrix calibration as well as on the basis of a standard calibration with the exception of LBG, which did not have a direct internal standard (Table 3). The analysed blank samples showed no interferences in the range of the peaks of interest.

Table 3. Performance characteristics of the analytical method at a concentration level of 0.75 µg kg^{-1} by matrix calibration or standard calibration.

	Matrix calibration					Standard calibration				
Analyte	RSD_r* [%]	RSD_{wR}** [%]	Recovery [%]	CCα [µg kg^{-1}]	CCβ [µg kg^{-1}]	RSD_r* [%]	RSD_{wR}** [%]	Recovery [%]	CCα [µg kg^{-1}]	CCβ [µg kg^{-1}]
MG	5.9	5.9	97.8	0.64	0.73	6.4	6.4	106.7	0.64	0.74
LMG	7.1	8.3	100.2	0.67	0.80	7.0	9.3	99.9	0.69	0.83
CV	5.2	5.2	99.0	0.62	0.70	5.5	5.5	103.5	0.62	0.71
LCV	10.1	10.1	98.7	0.72	0.86	10.5	10.5	103.0	0.72	0.86
BG	5.4	8.3	97.7	0.70	0.82	5.2	5.2	106.9	0.61	0.69
LBG	13.8	17.2	102.0	0.83	1.15	14.2	23.6	123.7	0.87	1.27

** RSD_r: repeatability; ** RSD_{wR}: within-laboratory reproducibility*

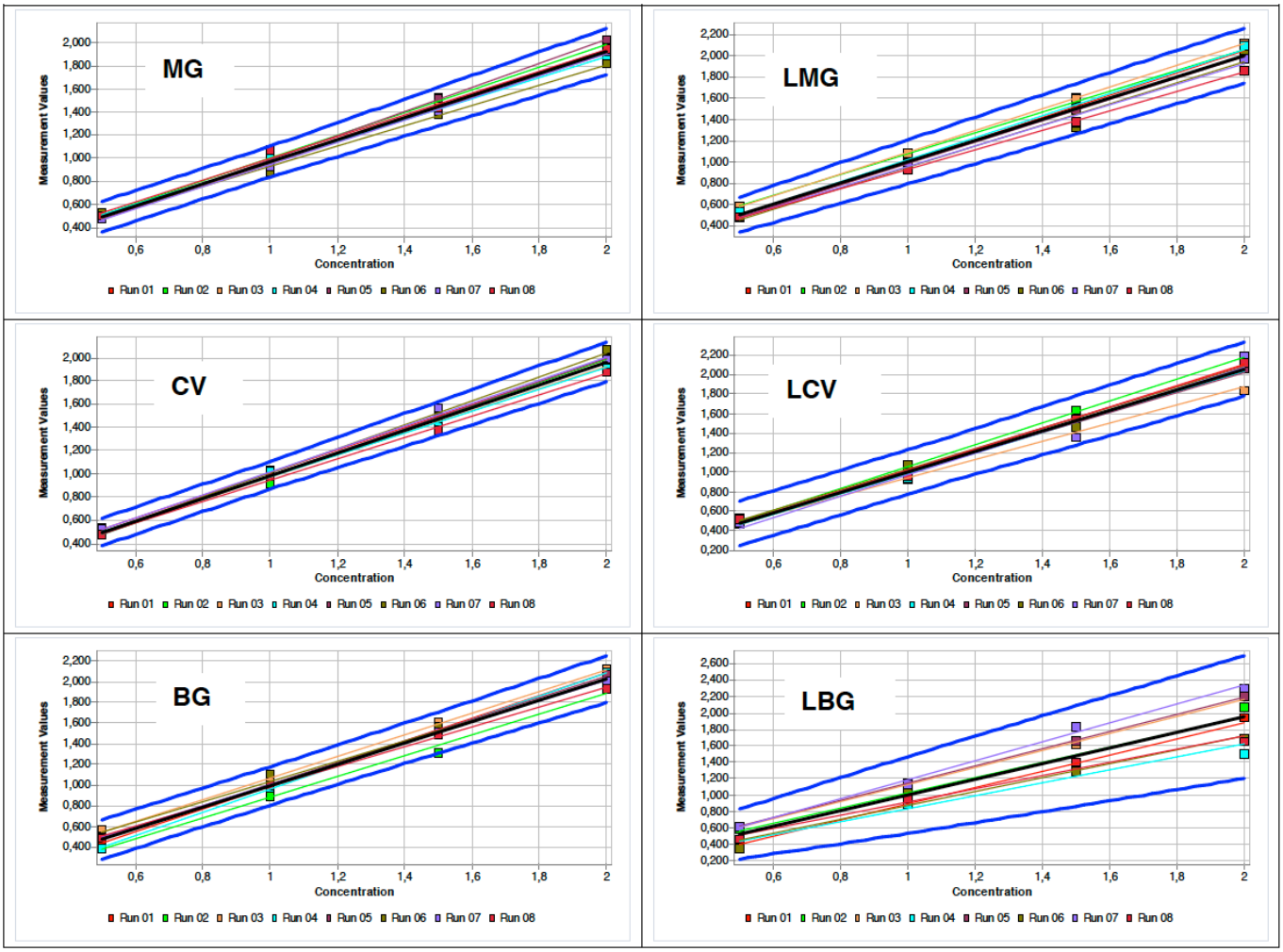

Figure 2. Single curves (run 1-8), overall curves (black) and prediction intervals (blue) of the measurement values of the analytes MG, CV, BG, LMG, LCV and LBG in the recovery samples of the 8 factor-level combinations of the validation

Conclusions

The verification of different factors was successful regarding the applicability and the ruggedness of the method. Their influence on the validation parameters was not significant. Quantitation was effected on the basis of matrix calibration curves as well as standard calibration. All validation parameters were satisfying and lay in the required ranges with the exception of CCβ for LBG. The recommendation of document SANCO/2004/2726-rev4 for CCα to be less than 1 µg kg^{-1} (½ MRPL for the sum of MG and LMG) was fulfilled. This was true also for the other substances. The method can be applied for muscle of aquaculture (fish and crustaceans) in a concentration range around 1 µg kg^{-1}, and thus for official residue control according to Council Directive 96/23/EC [5] and Regulation (EC) No 882/2004 [6].

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DEVELOPMENT AND VALIDATION OF A CONFIRMATORY METHOD FOR THE DETERMINATION OF AMPHENICOLS IN MUSCLE AND KIDNEY OF SEVERAL ANIMAL SPECIES

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Abstract

A rapid and reliable method using liquid chromatography (UHPLC) tandem mass spectrometry (MS/MS) to identify and quantify chloramphenicol (CAP), thiamphenicol (TAP), florfenicol (FF) and its marker residue florfenicol amine (FFA) has been developed and validated. FF and TAP are both allowed substances with different MRLs depending on the combination matrix/species and CAP is a well-known forbidden substance with a MRPL of 0.3 µg kg^{-1} in all matrices.

It was relevant to develop a confirmatory method to quantify simultaneously the four amphenicols in kidney and muscle of all species, in order to make the official control programme for this group of substances more effective. The variety of levels of interest to be studied for the allowed compounds and the different chemical behaviour of FFA have been the main difficulties in the development and validation of the method.

The method has been validated in *bovine*, *caprine*, *equine*, *porcine* and aquaculture species, rabbit and poultry muscle and kidney (except aquaculture species, rabbit and poultry). Performance criteria have been calculated in accordance with the Commission Decision 2002/657/EC.

The proposed procedure is being applied for the official control in the Valencian Region and it has been recently successfully evaluated by ENAC (Spanish national body of accreditation).

Introduction

Amphenicols are broad spectrum antibiotics that have been widely used in food-producing animals for treatments of various infections. Chloramphenicol (CAP) is forbidden in the European Union since 1994 by the Commission Regulation 1430/94 of 22 June 1994 (1). Florfenicol (FF) and Thiamphenicol (TAP) are allowed for treatment of different animal species followed by the appropriate withdrawal time. According to Regulation 37/2010 (2), FF determination must include FF and Florfenicol amine (FFA) quantitation, as a marker residue. There are different maximum residue limits (MRLs) for many combinations of matrix/species, which are shown in Table 1.

To ensure an harmonized implementation of the Council Directive 96/23/EC of 29 April 1996 (3) and the same level of protection for consumers in all EU member states, the European Commission established a minimum required performance limit (MRPL) of the analytical method for the determination of CAP in different animal products of 0.3 µg kg^{-1} (Commission Decision 181/2003/EC of 13 march 2003) (4). Therefore, an effective surveillance and monitoring of these substances are hardly required in all species of food producing animals.

Various analytical methods have been reported for the analysis of some of the amphenicols investigated in this paper (5-16). These approaches are usually developed for one matrix (ex. muscle) in one or two species (5-7, 9-12) and the determination of FFA has been included only in a few studies (9, 10). Moreover, those methods were not fit for official control purpose because the MRL of FF and TAP were not taken into account for the validation to calculate the limit of decision (CCα) and the detection capability (CCβ) according to Decision 657/2002/EC (17).

The aim of this study was to develop and validate a method for the determination of all the amphenicols including FFA as a marker residue of FF considering the wide range of concentration of the different MRLs established in Regulation 37/2010 (2) and setting a CCα for CAP lower than its MRPL.

To our knowledge, no work has previously been reported for the complete set of amphenicols in muscle and kidney in all food-producing animal species according to the European Regulations (37/2010 (2), Decision 657/2002/EC (17) and 181/2003/EC (4)). This new approach results in a simplification of the current laboratory methodologies and a considerable improvement for the official control purposes.

Table 1.- MRLs of the amphenicols investigated.

Analyte	Species	MRL in muscle (µg kg^{-1})	MRL in kidney (µg kg^{-1})
Florfenicol (FF and FFA)	Broiler	100	750
	Bovine, *ovine* and *caprine*	200	300
	Porcine	300	500
	Fish	1,000	-
	Others (rabbit, *equine*, …)	100	300
Tiamphenicol	All species	50	50

Materials and Methods

Standards

CAP, TAP, FF, FFA and chloramphenicol-d5 (CAP-D5, internal standard) were purchased from Sigma®. Stock standard solutions of individual amphenicol were prepared in methanol at concentrations of 1,000 µg mL^{-1} and were stored at -20°C. Intermediate solutions were also prepared in methanol for CAP, TAP, FF and FFA at 10, 20, 80 and 80 µg mL^{-1} respectively, and they were stored at 5°C. Working solutions of CAP were prepared daily by diluting 100- and 800-fold the intermediate solution in water. The working solution of TAP, FF and FFA was prepared daily by diluting 500-fold the intermediate solution in water.

Reagents and chemicals

All chemicals (anhydrous sodium sulphate, ammonia (25%)) were of analytical-reagent grade. Methanol used as mobile phase was hypergrade quality from Merck®. Acetonitrile and ethyl acetate used for sample preparation were HPLC grade from Merck®. HPLC grade water was in-house produced using a MilliQ system (Millipore). QuEChERS fatty dispersive-SPE EN kit (15 mL polypropylene tube containing 150 mg PSA, 150 mg C18 and 900 mg $MgSO_4$) used for sample preparation, were obtained from Agilent technologies.

Apparatus

The UHPLC system consisted of a Dionex Ultimate 3000 and TSQ quantiva triple quadrupole-mass spectrometer of Thermo Scientific Corporation. Separation was obtained at 50°C under gradient conditions using a 50 mm x 2.1 mm, 2.6 µm Kinetex Biphenyl 100 A column from Phenomenex®, with water (solvent A)/methanol (solvent B) at 0,600 mL min^{-1} as mobile phase. The gradient program was 0.0 min 100% A, 0-4.0 min 70% A, 4.0-4.15 min 30% A, 4.15-5.15 min 0% A, 5.16-8.0 min 100% A. The injected volume was 10 µL. The entire LC flow was directed into the MS detector between 1.5 and 5.0 min using the divert valve.

Acquisition parameters for ESI source were capillary voltage: 3.5 Kv; sheath gas: 30 a.u.; auxiliary gas: 10 a.u.; sweep gas: 1 a.u.; ion transfer tube temperature: 342°C and vaporizer temperature: 292°C. Switching negative-positive electrospray ionization (ESI^-/ESI^+) and selected reaction monitoring (SRM) mode were used for amphenicols detection and confirmation. Table 2 shows the particular conditions and transitions for each analyte.

Meat and kidney samples

Samples were collected by Spanish veterinary Inspectors of the Public Health Authorities at slaughterhouses, bullrings and fish markets. Samples were received in frozen conditions and were stored at -20°C until analysis.

Porcine, *bovine*, *ovine*, *caprine*, *equine*, aquaculture species, rabbit and broiler muscle samples and *porcine*, *bovine*, *ovine*, *caprine* and *equine* kidney samples were used during the validation of the proposed method.

Sample preparation, extraction and clean-up

A 2-g portion of minced and homogenised muscle or kidney samples was weighed into a 50 mL centrifuge tube and 20 µL of working internal standard solution was added. Then, 5 mL of ethyl acetate with ammonia 2% and 4 g of anhydrous sodium sulphate were added. The mixture was thoroughly homogenised using a vortex. After mixing, the homogenate was centrifuged at 5,000 rpm for 5 min at 15°C and the supernatant was transferred into a 15 mL polypropylene tube.

The extraction step was repeated by the addition of 5 mL of ethyl acetate with ammonia 2%, shaken and centrifuge again under the same conditions. The resulted supernatant was merged with the previous in the 15 mL polypropylene tube and the mixture was evaporated to dryness in a TurboVap® (Zymark®) under a nitrogen stream at 50°C.

The residue was then dissolved in 5 mL of acetonitrile and the extract was placed into the 15 mL QuEChERS fatty dispersive-SPE EN polypropylene tube and vortexed for one minute. The mixture was centrifuged at 4,000 rpm for 4 min at 15°C. The supernatant was evaporated to dryness in a TurboVap® (Zymark®) under a nitrogen stream at 50°C. Finally, the residue was reconstituted in 500 µL of water and filtered by 0.2 µm PVDF syringeless filters and 10 µL were injected into the UHPLC-MS/MS system.

Table 2. Mass spectrometry conditions and range of relative intensities for each compound.

Analyte	Polarity (+/-)	Precursor ion	Collision energy (V)	Product ion	Range of relative intensities (%)
CAP	(-)	321	20	152	Quantifier ion.
			15	194	[16-55]
			33	121	[14-48]
TAP	(-)	354	15	290	Quantifier ion.
			22	185	[35-45]
			33	119	[5-8]
FF	(-)	356	12	336	Quantifier ion.
			21	185	[91-106]
			33	119	[18-24]
FFA	(+)	248	12	230	Quantifier ion.
			24	130	[59-66]
			44	91	[13-16]
CAP-D5	(-)	326	19	157	

Validation process

The method validation was carried out according to the criteria specified in the 2002/657/EC European Decision.

The performance characteristics to be determined in quantitative methods are decision limit (CCα), detection capability (CCβ), trueness, precision (repeatability and within-laboratory reproducibility), selectivity/specificity and applicability (analytes, concentration range).

Trueness and within-laboratory reproducibility were determined by analysing spiked samples at the levels of interest at different days by different operators. Repeatability experiment was performed by analysing sample sets (at different days) from two to eight replicates (processed in the same day) of identical spiked samples at the levels of interest. Repeatability and within-laboratory reproducibility were evaluated by calculating the coefficients of variation (CV).

The CCα was established according to the requirements for identification plus quantification. For authorized substances, the corresponding concentration at the MRL for each substance plus 1.64 times the standard deviation of the within-laboratory reproducibility equals the CCα. The same standard deviation of mean measured content at the decision limit and at MRL was assumed to determinate the CCβ. The corresponding concentration at the value of the CCα plus 1.64 times the standard deviation of the within-laboratory reproducibility equals the CCβ.

For CAP, CCα was experimentally estimated after spiking several samples at decreasing concentration level from the MRPL level. The tentative CCα level was selected when the less abundant ion was detected with a signal-to-noise near three and the confirmation was possible in 50% of the cases. The CCα level was then verified by spiking 20 samples at the estimated concentration through the confirmation of CAP at least in the 50% of the cases. The corresponding concentration at the CCα for CAP plus 1.64 times the standard deviation of the within-laboratory reproducibility equals the CCβ.

Results and discussion

Extraction and clean up

The aim of this study was to develop a method for the simultaneous extraction and determination of FFA, FA, CAP and TAP. Up to now, common protocols for all amphenicols determination have not been able to extract quantify and confirm FFA with appropriate quality parameters, mainly due to the FFA chemical attributes. For this reason, the first step was the extraction with ethyl acetate basified with ammonia. After that, the extract was evaporated to dryness and injected directly into the UHPLC-MS/MS system. This one-step procedure (without clean-up) resulted in a poor performance for FFA and CAP probably because of the combination of matrix effects and the low concentrations of the levels of interest to achieve. A clean-up step was then added in order to increase method sensitivity. Furthermore, considering that the final evaporation stage is dependent on the water content of each matrix (muscle is more time-consuming than kidney) anhydrous sodium sulphate was added to standardize this step.

Two strategies were checked for the performance of the clean-up of the ethyl acetate extract: 1) mixed-mode strong cation-exchange SPE cartridge and 2) QuEChERS fatty dispersive-SPE EN/AOAC method. First option was developed with oasis MCX cartridges as described in scientific literature (5,11). In view of the results the second strategy was selected for the purification step. Better analytes signals were obtained at the low concentration level for CAP when using the EN Kit than AOAC Kit or MCX cartridge.

Column and mobile phase selection

The Kinetex Biphenyl 100 Å column was chosen due to its ability to separate FFA and amine containing components derived from matrix samples. Figure 1 contains the chromatograms of the amphenicols investigated spiked in a blank *bovine* muscle (A) and blank *bovine* kidney (B) spiked at the limit of quantitation (LOQ).

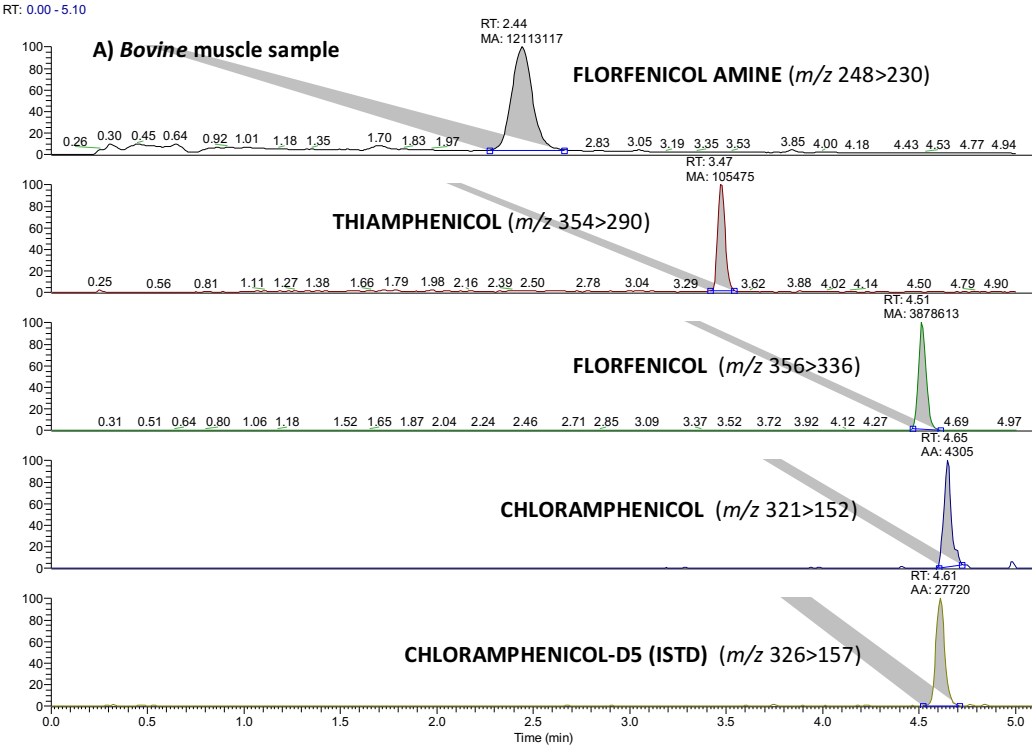
For the improvement of the chromatographic analysis an extensive study was carried out. The goal was to optimize the LC method to reach the maximum signal for CAP at low concentrations without detrimental of the others amphenicols responses. Different compositions were investigated for the mobile phase including acetonitrile or methanol as organic phase and water with formic acid, acetic acid, ammonium acetate, ammonium formate or trifluoroacetic acid as aqueous phase. When using mobile phase in acidic conditions an increase of the FFA signal was observed, on the other hand worse responses of CAP, TAP and FF were obtained. Finally, a chromatographic gradient of water/methanol without the use of modifiers provided the best results to determine all amphenicols at once.

Validation process

The specificity/selectivity of the method was demonstrated by analysing 35 blank samples of *equine*, *bovine*, *porcine*, *ovine*, *caprine*, *lapine*, aquaculture species and broiler muscle and 25 blank samples of *equine*, *bovine*, *porcine*, *ovine* and *caprine* kidney. Samples were either free of interference signals (no interference was found in the retention time of the compounds) or presented interferences at levels below 30% of the LOQ. The linearity of the developed method was evaluated for all analytes by procedural matrix-matched calibration curves. The concentration range were 0.10-4.00 µg kg^{-1} for CAP, 25-1500 µg kg^{-1} for FF and FFA and 6.3-250 µg kg^{-1} for TAP. Regression coefficients $R^2 > 0.97$ were obtained.

Due to the lack of Certified Reference Materials (CRM) trueness of the measurements was determined by fortifying blank samples at 1/3x MRPL, MRPL and 13x MRPL for CAP. For TAP, FF and FFA trueness was calculated at the LOQ, different MRLs and the upper limits. This was performed in 35 replicates at each level on different days and with different operators. As can be deduced from Table 3, the calculated trueness fulfils the criteria established in the Decision 2002/657/EC (17) for each mass fraction.

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were estimated. The results summarized in Table 3 indicate the good precision of the method. Repeatability was lower than within-laboratory reproducibility. The obtained coefficients of variation (CV's) were in accordance with the Decision 2002/657/EC (17) stating that CV's obtained for mass fraction lower than 100 µg kg^{-1} should be as low as possible, for mass fraction between 100 and 999 µg kg^{-1} should be less or equal 23 and for mass fraction higher than 1,000 µg kg^{-1} should be less or equal 16. CCα and CCβ were calculated as indicated above and the results are presented in Table 3. Identification was also validated by monitoring two ion ratios per molecule. Table 2 shows the relative intensities ranges of the qualifier ions for the different analytes. Three transitions were monitored for all analytes. Therefore, 5.5 IPs (1.0 precursor + (3 x 1.5 product)) were achieved for each analyte providing to the method a very high selectivity and a great confidence in the results.



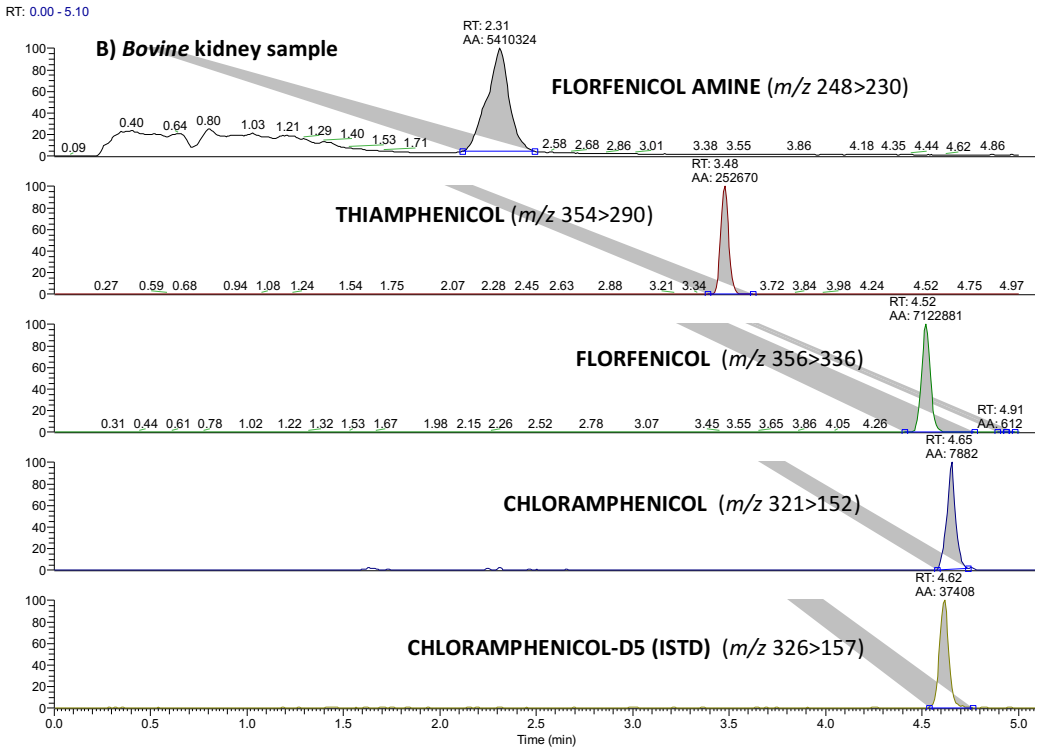


Figure 1. Chromatograms of spiked bovine muscle (A) and bovine kidney (B) at the limit of quantitation.

Table 3.-Validation result of amphenicols in animal muscle and kidney.

Analyte/concentration (μg kg^{-1})	Trueness (%)	Repeatability (CV%) n=35	Within-laboratory reproducibility (CV%) n=35	CCα (μg kg^{-1}) n=35	CCβ (μg kg^{-1}) n=35
CAP (0.10)	101	12	16	0.10	0.13
CAP (0.30)	98	6	7		
CAP (4.00)	104	7	8		
FF (25.0)	94	13	16		
FF (100.0)	106	9	13	121.8	143.7
FF (200.0)	106	10	12	241.7	283.5
FF (300.0)	110	7	9	347.4	394.8
FF (500.0)	108	6	6	555.5	610.9
FF (1000.0)	92	9	15	1217.8	1435.6
FF (1500.0)	101	9	13		
FFA (25.0)	107	13	21		
FFA (100.0)	81	11	20	126.5	153.0
FFA (200.0)	96	16	19	261.0	322.0
FFA (300.0)	92	21	23	402.6	505.2
FFA (500.0)	88	13	23	663.6	827.1
FFA (1000.0)	92	14	16	1243.4	1486.7
FFA (1500.0)	105	11	14		
TAP (6.3)	95	13	19		
TAP (50.0)	102	7	13	60.7	71.5
TAP (250.0)	98	13	16		

Conclusions

A rapid, quantitative and confirmatory method was developed for the determination of all amphenicols at once. The presented method provides good validation data in accordance with the EU criteria (2002/657/EC) (17) and was successfully accredited under the ISO/IEC 17025:2005 system by the Spanish national body of accreditation (ENAC).

In order to select an appropriate sample preparation procedure for all amphenicols, two methodologies were studied. The QuEChERS method was selected as the best clean-up step for the suitable detection and identification of the investigated compounds (CAP, FF, FFA and TAP) at the levels of interest.

The validated method was further applied for the analysis of muscle and kidney tissues samples in the Spanish regulatory control programme.

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AMPHENICOLS ANALYSIS IN MEAT BY ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

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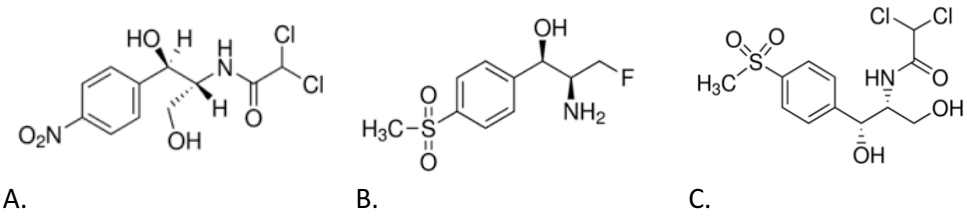
Abstract
A complete methodology was developed for the analysis of three amphenicols in meat comprising a specific sample preparation step and an ultra-high-performance liquid chromatography (UHPLC) coupled to tandem mass-spectrometry analysis. The methodology was validated and applied to real meat samples.

Introduction
Chloramphenicol (CAP), florfenicol (FLOR) and thiamphenicol (TAP) are broad-spectrum antibiotics from the family of amphenicols. CAP was first extracted from bacterium *Strepromyces venezualae* in the year 1947 and was approved by the US Food and Drug Administration in 1949 as the first broad-spectrum antibiotic (Schukla *et al.*, 2011). It was widely used in the treatment of infectious conditions ranging from bronchitis to bacterial meningitis. However, in the 1960s, toxicity of CAP was highlighted in terms of effects on bone marrow (Wiest *et al.*, 2012). Since 1990s, use of CAP in food was banned and countries have established a zero tolerance policy. FLOR and TAP are structurally related to CAP but do not present such toxicity (Figure 1).



A. B. C.

Figure 1. chemical structures of A. CAP, B. FLOR and C. TAP.

FLOR is *e.g.* proposed for treatment of *bovine* respiratory disease and TAP for the treatment and control of respiratory and intestinal infections in cattle and poultry (The European Agency for the Evaluation of Medicinal Products, London, UK). Maximum residue limits are available in European Union and Switzerland for these two substances (*e.g.* 200 and 50 µg kg^{-1} in muscle for FLOR and TAP, respectively). Considering this information, analytical methods have to be developed to detect and quantify the presence of these compounds in food and in particular in meat.

CAP, FLOR and/or TAP can be analysed by various analytical techniques, such as liquid-chromatography and capillary electrophoresis, hyphenated to UV, DAD or mass-spectrometry (Bol'shakov *et al.*, 2016; Amelin *et al.*, 2015; Souza *et al.*, 2016; Fedeniuk *et al.*, 2015). However, in complex matrices, such as food matrices, mass-spectrometry, due to its great selectivity and sensitivity, appears to be the best detection system. In the context of known targeted compounds, triple quadrupole mass spectrometer offers very good quantitation performance and unambiguous identification with high selectivity and specificity by the use of MRM transitions.

In order to reduce the matrix effect for trace analysis, sample pretreatment is needed. Liquid-liquid extraction (LLE), solid-phase extraction (SPE), matrix solid-phase dispersion (MSPD), and molecular imprinted polymer (MIP) extraction have been published for sample pretreatment prior to quantitative determination of CAP, FLOR, and/or TAP (Liu *et al.*, 2016).

In this work, a specific sample preparation for CAP, FLOR, and TAP in meat was developed, combining two liquid-liquid extractions followed by SPE purification and targeted screening by UHPLC-MS/MS. The method was validated and used for inspection of imported meats.

Materials and Methods

Chemicals
Chloramphenicol, florfenicol and thiamphenicol reference standards were purchased from Sigma-Aldrich (Buchs, Switzerland) as powder or standard solution. Chloramphenicol-d5 was purchased from Cambridge Isotope Laboratories (Tewksbury, USA). Stock solutions of 0.5 mg mL^{-1} for each substance were prepared by dissolving 5 mg of the pure analytical standard in

10 mL appropriate solvent. For each compound, diluted standard solution were prepared by appropriate dilutions to final concentrations of 10, 1 and 0.1 µg mL^{-1}. These solutions were used to prepare calibration samples and quality control (QC) samples. Acetonitrile and hexane were obtained from Biosolve (Dieuze, France). Methanol and sodium chloride were purchased from Sigma-Aldrich (Buchs, Switzerland). Water was purified with an Elix 3 and MilliQ apparatus from Millipore (Molsheim, France). Other chemicals were of HPLC or analytical grade and were used without any further purification. When not in use, all standard solutions were stored at −20°C in the dark.

Sample preparation

An amount of 5 g of meat was ground and weighed in one polypropylene (PP) tube and 50 µL 1 µg mL^{-1} internal standard (CAP-d5) was added. A QC sample was prepared by adding appropriate volume of the standard solutions (see Chemicals) to 5.0 g blank meat making sure that the spiking volume did not exceed 5% of the sample volume. Final concentrations in the QC sample were 1 µg kg^{-1} for CAP, 50 µg kg^{-1} for TAP and 200 µg kg^{-1} for FLOR, *i.e*. taking into account the specific maximum residue levels. QC samples were left standing for at least 30 min at room temperature before starting extraction.

a. First extraction: 10 mL of ethyl acetate were added to the tube. Samples were thoroughly vortexed for 5 min and then centrifuged at 2,700 rpm for 5 min. Samples were transferred in a conical glass tube and then evaporated under nitrogen until dryness. An oily residue was obtained. A portion of 500 µL methanol was added and the sample was vortexed for few seconds. The sample was transferred into a 15-mL PP tube.
b. Second extraction: The conical tube was rinsed with twice with 5 mL 4% NaCl which were then added to the 15-mL PP tube. 4.5 mL of hexane were added to the conical tube. The conical tube was then vortexed for 10 s and hexane was added to the 15-mL PP tube. The 15-mL PP tube was vortexed for 30 s and then centrifuged at 2,700 rpm for 5 min. The hexane layer was discarded. 4.5 mL hexane were added again to the conical tube and transferred to the 15-mL PP tube. The same steps were performed (vortex, centrifugation, hexane elimination). The aqueous phase was ready for the SPE.
c. Solid phase extraction: An OASIS HLB SPE cartridge (Waters, Milford, USA) was first conditioned with 2 mL methanol and 2 mL water. The sample was then charged at one drop min^{-1}. The cartridge was rinsed with 3 mL water and 2 mL methanol/ water (50/50). The cartridge was dried for 5 min under vacuum. The sample was finally eluted with 2 mL methanol in a 5-mL tube. Volume was adjusted to 5 mL with water. Finally, 1 mL was filtrated over a 0.45-µm nylon filter.

Liquid chromatography

An Acquity UPLC system (Waters Corp., Milford, USA) was employed for all experiments. 10 µL of each extract were injected. The chromatography was carried out on a Waters Acquity UPLC BEH C18, 1.7 µm 50 x 2.1 mm column protected with a pre-column VanGuard Acquity UPLC BEH C18, 1.7 µm 5 x 2.1 mm. The mobile phase consisted of H_2O/MeOH (98/2) (A) and MeOH (B). The chromatographic separation was performed in a gradient mode (A/B: 0 min: 95/5 v:v; 0.50 min: 95/5; 1.6 min: 10/90; 1.9 min: 10/90; 2.05 min: 95/5) at a flow rate of 450 µL min^{-1} for a total run time of 3.5 min. The column and autosampler were maintained at 40°C and 10°C, respectively.

Table 1: MS/MS transitions and conditions

MRM transition ESI-	Parent ion [*m/z*]	Product ion	Cone voltage [V]	Collision voltage [V]
Chloramphenicol 1	321	152	28	16
Chloramphenicol 2	321	257	28	12
Chloramphenicol 3	321	194	28	12
Chloramphenicol-D5	326	157	25	15
Florfenicol 1	356	336	22	10
Florfenicol 2	356	185	22	18
Florfenicol 3	356	119	22	26
Thiamphenicol 1	354	185	30	20
Thiamphenicol 2	354	290	30	12
Thiamphenicol 3	354	240	30	18

Tandem mass spectrometry

A XEVO-TQMS with an electrospray (ESI) interface (Waters Corp., Milford, USA) was employed. The capillary voltage was set at -4.0 kV and the source temperature at 150°C. The desolvation temperature was fixed at 500°C with nitrogen flow rates of 50 L H^{-1} and 1,000 L H^{-1} for the cone gas and desolvation gas, respectively. The 4.1 version of MassLynx software was used for instrument control and data acquisition. Used MRM transitions for the analytes and internal standard are listed in Table 1.

Results and discussion

Method development

Attention was paid on the extraction efficiency. An oily residue was obtained after the ethyl acetate extraction. To ensure a complete recuperation of the amphenicols that can be included in the remaining oil, hexane was tested for dissolving fat in the conical tube. In this context, different volumes of hexane and numbers of successive additions were tested. The most efficient option was to first use twice 5 mL of a solution of 4% NaCl and then to recuperate fat with successively twice 4.5 mL hexane. A second liquid-liquid extraction was then performed and thanks to the use of NaCl, a good separation between the aqueous and the hexanic phases was obtained. The fat dissolution with hexane permitted a best recovery of amphenicols.

The second part of investigation was the addition of a purification step by SPE to achieve the required limits of detection. With CAP being forbidden, the analytical method has to be able to detect very low concentrations. The SPE protocol was optimized in terms of conditioning solvents, rinsing solvents and elution solvents. The SPE permitted purification of the extracts. With these two steps, improved extraction and SPE purification, low concentration detection levels were obtained, *i.e.* ≤ 1 µg kg^{-1} for the three analytes.

Method performance

For verification of matrix effects, extraction was performed in triplicate at the levels of 1, 50 and 200 µg kg^{-1} for CAP, TAP and FLOR, respectively. One corresponding standard and one non-fortified sample were also prepared. UPLC-MS/MS responses were compared between the three fortified samples and the corresponding standard. Detected effects were attributed to the extraction and the ionisation yields. Generally, due to the relative important number of steps of the sample preparation procedure and the complexity of the meat matrix, matrix effects were found for each analyte (matrix effects comprised between 40 and 80%).

In order to deal with this aspect, an isotopically labelled standard for CAP was introduced and it could correct the matrix effect. For FLOR and TAP, no internal standards were available. Correction with the CAP standard was tested and was beneficial for TAP but not for FLOR. Quantitation in terms of trueness was not optimal for these last compounds due to the absence of a specific internal standard. Consequently, samples were first quantified with the QC sample. The QC sample was prepared from spiked meat and extracted using the same conditions as the field samples, as described in the Materials and Methods. For CAP, thanks to the excellent correction with the internal standard, results could be directly considered for quantitation. For FLOR and TAP, positive sample detected during this screening step will be precisely quantified by standard addition. Despite the loss of time, this quantitative confirmation ensures the result, correcting matrix effects.

The method was validated according to the directive EC/657/2002. Validation was conducted on three days with three concentration levels (1, 5 and 10 µg g^{-1} for CAP; 25, 50 and 75 µg g^{-1} for TAP; 150, 300 and 450 µg g^{-1} for FLOR) and four repetitions. Concentration levels were chosen according to the maximum residue limits of each amphenicol. CAP is namely forbidden and MRLs for TAP and FLOR in muscle are 50 and 200 µg kg^{-1}, respectively. Best results were obtained with the CAP-d5 correction for CAP and TAP and without any correction for FLOR. Table 2 presents the validation results in details.

Table 2: Validation results

Compound	Concentration levels (ng mL^{-1})	Trueness [%]	Repeatability (RSD; (%))	Intermediate precision (RSD; (%))	Confidence interval (t=1.83; (%))
CAP	1	111	13	16	30
	5	108	15	17	30
	10	110	5	10	20
FLOR	150	126	6	8	19
	300	127	5	5	12
	450	116	4	4	9
TAP	25	71	16	24	30
	50	76	11	14	20
	75	66	9	8	10

Limits of detection were set at 0.1, 1 and 1 µg kg^{-1} for CAP, TAP and FLOR, respectively. Limits of quantification were set at 1.0, 5 and 5 µg kg^{-1} for CAP, TAP and FLOR, respectively. These results are satisfying.

The validated methodology was then applied to 17 imported meats. The methodology was performed and all samples were found compliant. No amphenicol residues were detected.

Conclusion

A specific screening method was developed to analyze meats for the presence of CAP, FLOR and TAP residues. Matrix effects and quantitative performance were determined. They were found to be satisfying, dealing with the use of a QC sample in

matrix for the first quantitation and standard addition technique for ambiguous positive samples. The method was validated and applied to imported meat samples. No amphenicols were detected.

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DEVELOPMENT OF A VICH GUIDANCE FOR CONDUCT OF RESIDUE DEPLETION STUDIES IN AQUATIC SPECIES

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Abstract
VICH (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products) guidelines exist for study design recommendations to facilitate universal acceptance of residue data for veterinary products in food producing animals. Food-producing aquatic species were not originally included in Guidelines 46 (metabolism) and 48 (residue depletion). Global production from marine and freshwater aquaculture has increased by an average of 8.2% per year from 1970 to 2010. With the exception of Atlantic salmon in Europe, aquatic species are considered as minor species by many regulatory agencies. Study design recommendations for residue studies to generate data used to establish withdrawal periods and hence facilitate the approval of safe veterinary medicines are needed for products developed to treat diseases in aquatic species.
The VICH Metabolism and Residue Kinetics (MRK) expert working group met in June of 2014 to discuss extension of GL 48 to residue studies in food producing aquatic species. An update on the progress and unique challenges in developing this document will be presented.

Introduction

VICH
VICH is a trilateral (EU-Japan-USA) programme aimed at harmonising technical requirements for veterinary product registration. One of the aims of VICH is to establish and implement harmonized technical requirements for the registration of veterinary medicinal products in the VICH regions, which meet high quality, safety and efficacy standards and minimize the use of test animals and costs of product development [1].

VICH is composed of industry and regulatory agencies from the three regions, plus observers from Canada, Australia/New Zealand and South Africa. Expert working groups (EWGs) are tasked with developing draft guidelines, which are then circulated and approved by the various agencies.

Metabolism and Residue Kinetics (MRK) EWG
This EWG was formed in 2005 and took up work to develop guidelines to demonstrate the safety of residues in meat, milk and eggs. Four guidelines have been completed and adopted:

- GL 46 Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Metabolism study to determine the quantity and identify the nature of residues [2]
- GL 47 Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Comparative metabolism studies in laboratory animals [3]
- GL 48 Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Marker residue depletion studies to establish product withdrawal periods (including revision in 2015) [4]
- GL 49 Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Validation of analytical methods used in residue depletion studies (including revision in 2015) [5]

The guidelines, in particular GL 46 and GL 48, describe the recommendations for metabolism and residue depletion studies in mammals and birds.

Fish and other aquatic species
Fish and other (non-mammalian) aquatic species were not included in the previous guidances for various reasons, but the aquatic species are becoming more important as food producing animals as the demand for protein-based food increases globally; demand for seafood is also increasing because of perceived benefits to health.

Due to depletion of wild stocks, commercial farming of aquatic species is becoming more common. According to FAO, world aquaculture production of aquatic food-producing animals reached 70.2 million tonnes in 2013, worth USD 150 billion [6]. The contribution of aquaculture to total global fish production reached 43% in 2013. The global production of finfish (by weight and value) outstrips that of molluscs and crustaceans by at least three fold.

The rise of aquaculture is accompanied by a need for approved veterinary medicinal products for aquatic species in order to maintain animal health and welfare, maximizing productivity. Disease pressure will often increase when the huge numbers of animals are close proximity to each other, as observed in terrestrial intensive farming operations. The availability of approved medicines for aquaculture is limited and normally approval is granted in a similar manner as for mammalian and avian products, requiring establishment of safe residue levels and corresponding withdrawal periods.

Reliable residue depletion data are needed to establish withdrawal periods for such products and it is the task of the VICH MRK EWG to develop a simple guideline for generating such data that will be mutually acceptable in all the VICH regions.

MRK EWG Members Working on draft guidance for Aquatic Species

Topic co-chairs: Pam Boner (Zoetis, AHI) and Mary Smal (Elanco, AMA)

Members: K.K. Hamamoto (JMAFF) and Ryoji Koike (former member, JMAFF), Kazuo Fukumoto (JVPA), Satoru Nakano (JVPA)

Advisors: Julia Oriani (FDA/CVM) and Satoshi Miwa (JMAFF advisor)

Challenges with Developing the Aquatic Species Guidance

Terrestrial livestock species (generally limited to cattle, sheep, pigs and poultry) have been reared for centuries and are well understood, with uniform patterns of husbandry. They are typically regarded as major species for regulatory purposes.

1. Large number of diverse orders and species
   While some species are widely cultured through the world (for example, Atlantic salmon), numerous species are cultured only to a very limited extent in restricted geographical regions, under diverse husbandry conditions. Such very minor species still require medicinal products, but development costs are a barrier for sponsors.
2. Effect of water temperature on absorption kinetics and depletion rate
   Unlike birds and mammals, fish and other aquatic species do not regulate their body temperature, which is critical for absorption and depletion kinetics. Instead body temperature is regulated by that of the surrounding water, leading to faster absorption kinetics, particularly through the gills during immersion treatment, and also to faster depletion rate.
3. Non-uniform regulatory approaches for withdrawal period expression
   Since withdrawal period is governed by water temperature, some regulatory agencies have introduced the concept of degree days for withdrawal setting (for example, a WDP of 300 degree days would be equivalent to 30 days in waters at 10°C), while some agencies have set two different WDPs (in time units) for different water temperatures, and yet others simply use one WDP (in time units).
4. Consumption of types of tissues varies among VICH regions
   While fish muscle is uniformly consumed, the consumption of overlying skin and scales is variable based on structural make-up and cultural preferences. Additionally, the consumption of various body parts of molluscs and crustaceans and of fish offal varies due to cultural preferences.

Approach taken by EWG

In order to produce a simple guidance, ideally with only one residue depletion study being required, the following approaches have been taken

- Ideal to develop an approach to group fish – consider physiology, drug delivery, housing
- Extrapolating data from one representative fish to other fish within the same order
- Tissues for analysis in most cases would be skin with underlying muscle in natural proportions, and a mixture of offal.

Process for finalizing the draft guidance

After the draft guidance is developed by the MRK EWG and is approved by the VICH Steering Committee, the document is published for public comment. Through the support and network of the World Organization for Animal Health (OIE), countries that are not part of VICH are invited to provide comments about the draft guidance. After the comment period, the EWG will review the comments and finalize the guidance in consideration of the comments. The VICH Steering Committee approves the final guidance and proposes an implementation date to the regulatory authorities represented in the Steering Committee. Each VICH region implements the final guidance.

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METHOD FOR DETERMINATION OF NITROIMIDAZOLE RESIDUES IN POULTRY FEATHERS SAMPLES USING SUPELMIP SPE AND HPLC-MS/MS

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Abstract
Plasma, eggs, honey and muscle are the most common matrixes for the determination of nitroimidazole residues but recently the attention is also turned to pig hair and poultry feathers in which nitroimidazole residues also accumulate and are longer stable. This proceeding presents the method for the determination of nitroimidazole residues in poultry feathers by HPLC-MS/MS using SupelMIP SPE Nitroimidazoles cartridges after the extraction of analytes to acetonitrile. The main aim of this work was to develop a sufficiently sensitive and robust analytical method for the screening and confirmation of nitroimidazole residues in this still not so common matrix. The full validation according to EU-decision 2002/657 was done and its results are presented.

Introduction
Nitroimidazoles are a group of drugs with antibiotic and anti-coccidial activities. They have an activity against trypanosomes and they are also effective in the prophylactic and therapeutic treatment of histomoniasis and coccidiosis in poultry. Their use in veterinary practice is strictly forbidden in food-producing animals due to a suspected carcinogenicity and the presence of residues is to be monitored. On the other hand, the stability of nitroimidazoles in a biological matrix is one of the most limiting factors of residues analysis. It was investigated that the stability of nitroimidazole residues in a muscle is in the order of hours or the stability of nitroimidazole residues in a serum is in the order of days. The main benefit of feather analysis is significantly longer stability of nitroimidazole residues that enables their successful determination after months (Kindt and Poltze, EURL-NRL workshop, Berlin, 2014).

Materials and Methods

Sample preparation
Dry feathers were milled in a grinder and approx. 0.25 g of homogenized sample feathers was taken for analysis. The sample was spiked with internal standards (dimetridazole-d3, metronidazole-d3, ronidazole-d3, metronidazole-OH-d2, HMMNI-d3, ipronidazole-d3, ipronidazole-OH-d3) at the concentration of 80 ng g^{-1}. A volume of 8 mL of acetonitrile was added and the sample was thoroughly homogenized using a homogenizer. After this the sample was centrifuged for 10 min at 15 ºC, 1,100 g and the upper layer was separated and transferred into the evaporation vial. Then 2 mL of acetonitrile were added, the sample was mixed on a shaker and the extraction was repeated. Combined extracts were evaporated under a nitrogen stream at 40°C near to dryness.

A volume of 2 mL water was used for dissolution and the sample was loaded to a SPE cartridge SupelMIP conditioned with 1 mL toluene, 1 mL acetonitrile and 1 mL 0.01 mol L^{-1} ammonium acetate (pH 6.0) prior to use. After sample application, the cartridge was washed with 1 mL of deionized water and dried by applying high vacuum for 10 min. Then two portions of 1 mL hexane and 1 mL of the mixture heptane:toluene (3:1 v/v) were passed and the cartridge was dried under a strong vacuum for 10 min. The analytes were eluted with 2x 1 mL of a mixture acetonitrile:0.5 % acetic acid (3:2 v/v). The eluate was evaporated at 40°C near to dryness. The sample was re-dissolved in 200 µL of 0.05% formic acid:acetonitrile (7: 3 v/v) and used for analysis.

LC-MS/MS method
HPLC chromatograph Finnigan Surveyor (Thermo) connected with a mass spectrometer TSQ Quantum Discovery Max (Thermo) was used. Chromatographic separation was achieved using Symmetry C8, 3.9 x 150 mm, 5 µm, Waters column. The mobile phase consisted of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The column temperature was set at 30°C and the flow rate was set at 0.25 mL min^{-1}. The injection volume was 25 µL, chromatographic conditions are reported in Table 1 with an overall run time of 18 min.

MS/MS measurement of the eluent was carried out in the ESI+ mode using spray voltage 4 kV, capillary temperature 270°C, drying gas flow rate 20 units and source CID voltage 14 V. The multiple reaction monitoring (MRM) conditions for the analytes are given in Table 2.

Table 1. LC conditions. See text for composition of eluents A and B.

Time (min)	[A] (%)	[B] (%)	Flow rate (µL min^{-1})	Analytical column
0.00	85	15	250	Symmetry C8, 3.9 x 150 mm, 5 µm, Waters
11.00	5	95	250	
13.00	5	95	250	
13.01	85	15	250	
18.00	85	15	250	

Table 2. Retention times, MRM transitions, collision energies and internal standards.

Component	Retention time (min)	MRM transition 1	MRM transition 2	Internal standard
Dimetridazole (DMZ)	10.0	142 > 96 (12 eV)	142 > 81 (21 eV)	DMZ-d3
Metronidazole (MNZ)	8.7	172 > 82 (21 eV)	172 > 128 (9 eV)	MNZ-d3
Ronidazole (RNZ)	9.7	201 > 140 (6 eV)	201 > 55 (17 eV)	RNZ-d3
Metronidazole-OH (MNZOH)	7.7	188 > 123 (9 eV)	188 > 144 (7 eV)	MNZOH-d2
HMMNI	9.2	158 > 140 (10 eV)	158 > 110 (8 eV)	HMMNI-d3
Ipronidazole (IPZ)	12.6	170 > 124 (11 eV)	170 > 109 (22 eV)	IPZ-d3
Ipronidazole-OH (IPZOH)	11.0	186 > 168 (9 eV)	186 > 122 (18 eV)	IPZOH-d3
Carnidazole (CNZ)	12.2	245 > 118 (8 eV)	245 > 75 (24 eV)	DMZ-d3
Ternidazole (TNZ)	9.3	186 > 128 (12 eV)	186 > 82 (22 eV)	IPZOH-d3
Secnidazole (SNZ)	9.9	186 > 128 (12 eV)	186 > 82 (20 eV)	IPZOH-d3
Tinidazole (TIZ)	10.6	248 > 121 (12 eV)	248 > 128 (16 eV)	IPZOH-d3
Ornidazole (ONZ)	11.1	220 > 128 (11 eV)	248 > 82 (25 eV)	IPZOH-d3
Dimetridazole-d3 (DMZ-d3)	10.0	145 > 99 (11 eV)		
Metronidazole-d3 (MNZ-d3)	8.7	175 > 85 (21 eV)		
Ronidazole-d3 (RNZ-d3)	9.7	204 > 143 (7 eV)		
Metronidazole-OH-d2 (MNZOH-d2)	7.7	190 > 125 (9 eV)		
HMMNI-d3	9.2	161 > 58 (16 eV)		
Ipronidazole-d3 (IPZ-d3)	12.6	173 > 127 (12 eV)		
Ipronidazole-OH-d3 (IPZOH-d3)	11.0	189 > 125 (18 eV)		

Results and discussion

Validation study

The validation study was performed according to the Commission Decision 2002/657/EC requirements. Poultry feathers samples were spiked with standards of investigated nitroimidazoles at the concentration range of 0-120 ng g^{-1} and the concentration of internal standards was 80 ng g^{-1}. Six six-point calibration curves were prepared and measured within four days. Concentration levels 0.0; 40.0; 60.0; 80.0; 100.0 and 120.0 ng g^{-1} were used for all investigated analytes. Each sample was injected twice *i.e*. 72 values were statistically calculated. The CCalpha and CCbeta limits were calculated from this dataset. Results are shown in Table 3. The laboratory reproducibility was calculated using results of six spiked samples at the level of 60.0 ng g^{-1} that were measured within four days. Results are shown in Table 4. Linearity of relative responses is shown in Table 5 (six-point calibration curve; each sample was injected twice).

Table 3. Validation study - CCalpha and CCbeta limits.

Component	CCalpha limit (µg kg^{-1})	CCbeta limit (µg kg^{-1})
Dimetridazole (DMZ)	3.20	5.46
Metronidazole (MNZ)	2.16	3.68
Ronidazole (RNZ)	2.45	4.18
Metronidazole-OH (MNZOH)	3.83	6.52
HMMNI	3.36	5.72
Ipronidazole (IPZ)	1.85	3.15
Ipronidazole-OH (IPZOH)	2.18	3.72
Carnidazole (CNZ)	16.73	28.50
Ternidazole (TNZ)	5.46	9.30
Secnidazole (SNZ)	7.53	12.82
Tinidazole (TIZ)	6.53	11.13
Ornidazole (ONZ)	6.60	11.25

Table 4. Validation study - reproducibility

Component	RSD (%)	Component	RSD (%)
Metronidazol-OH	8.76	Secnidazol	22.19
Metronidazol	7.05	Tinidazol	19.01
HMMNI	13.27	Ipronidazol-OH	4.69
Ronidazol	7.37	Ornidazol	17.05
Dimetridazol	8.46	Carnidazol	50.88
Ternidazol	6.00	Ipronidazol	5.14

Table 5. Validation study - linearity of relative responses

Component	Intercept	Slope	Correlation coefficient R^2
Dimetridazole	0,0649	0,0118	0,9924
Metronidazole	0,0258	0,0115	0,9963
Ronidazole	0,0387	0,0130	0,9936
Metronidazole-OH	0,0016	0,0121	0,9954
HMMNI	0,3522	0,0710	0,9919
Ipronidazole	0,0153	0,0053	0,9991
Ipronidazole-OH	0,0929	0,0267	0,9866
Carnidazole	0,0282	0,0037	0,9643
Ternidazole	0,0024	0,0079	0,9875
Secnidazole	0,0292	0,0090	0,9831
Tinidazole	0,0140	0,0148	0,9945
Ornidazole	0,0307	0,0523	0,9804

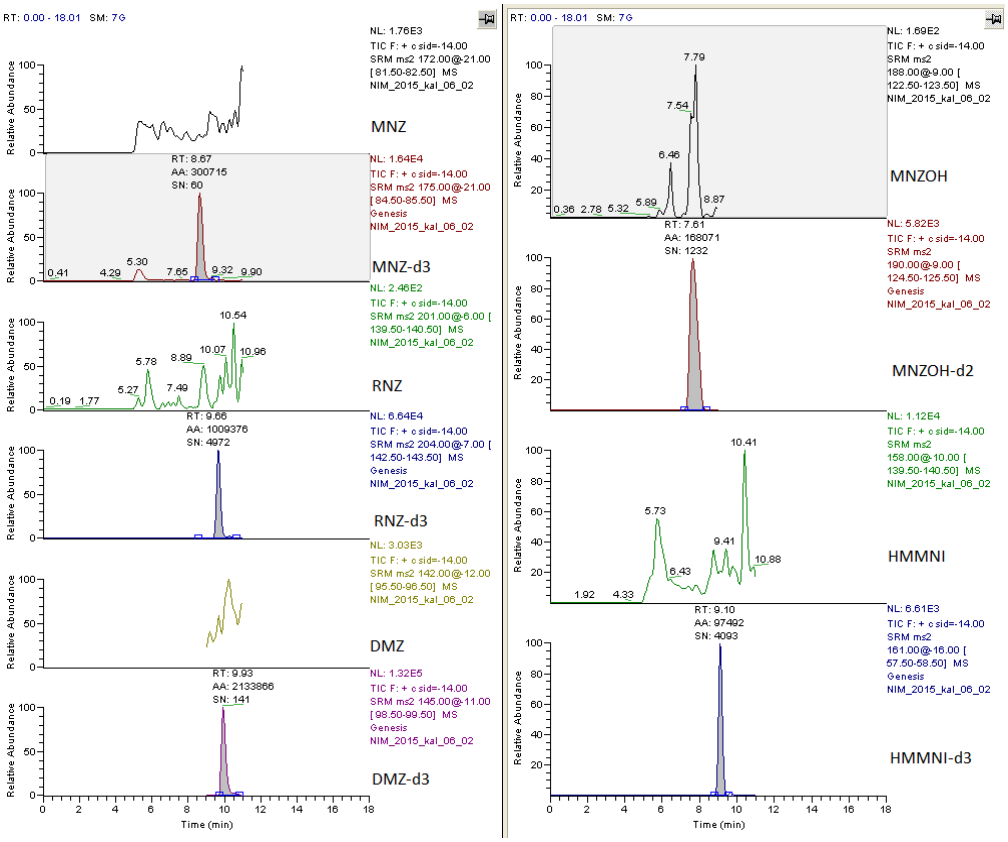

Figure 1. MRM chromatogram of a blank - hen feathers spiked with internal standards at the level of 80 ng g^{-1} (compounds MNZ, RNZ, DMZ, MNZOH, HMMNI).

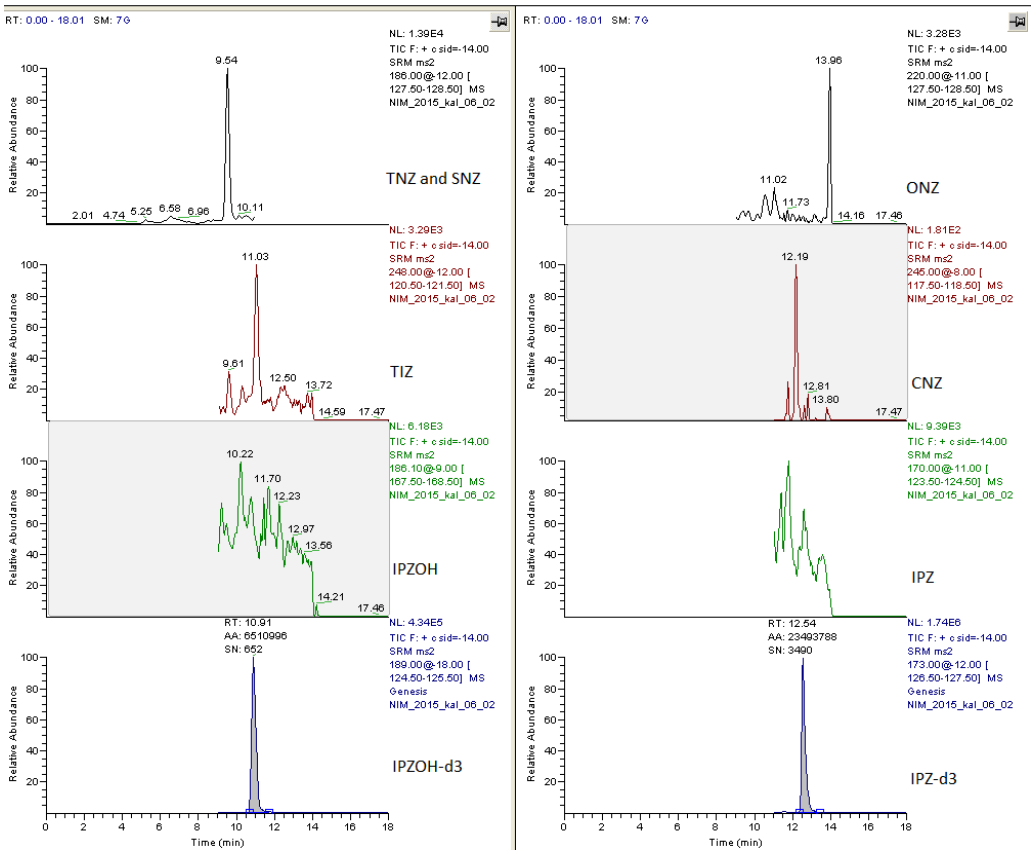

Figure 2. MRM chromatogram of a blank - hen feathers spiked with internal standards at the level of 80 ng g^{-1} (compounds TNZ, SNZ, TIZ, IPZOH, ONZ, CNZ, IPZ)

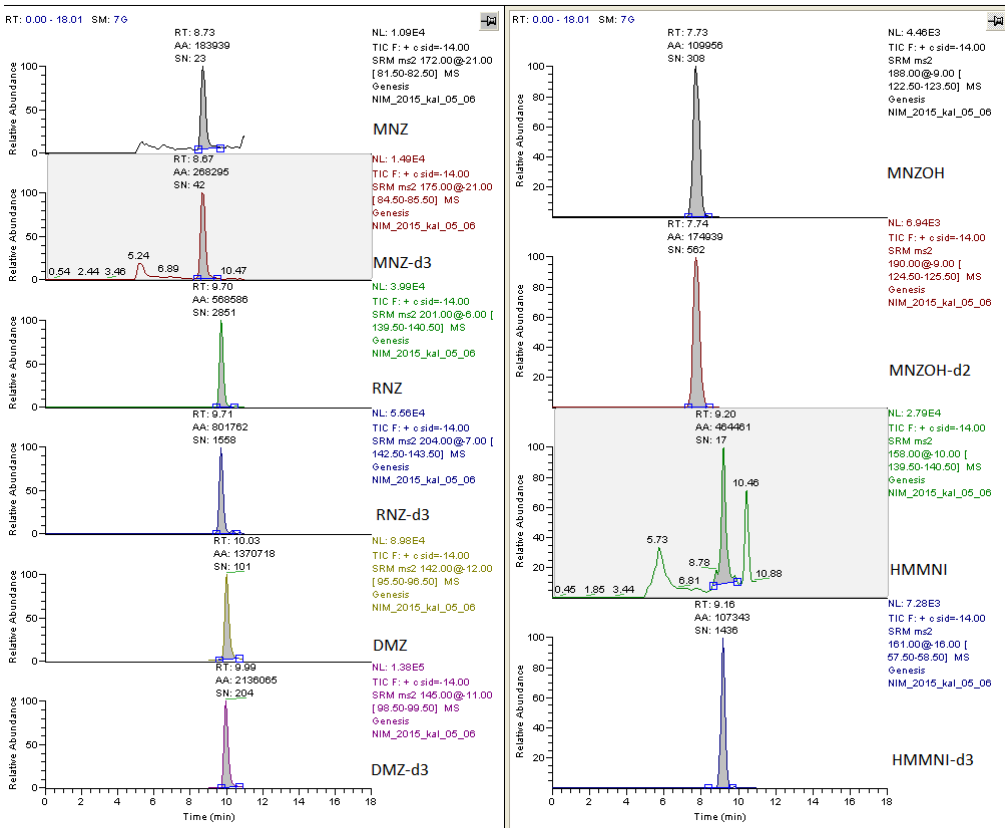

Figure 3. MRM chromatogram of fortified sample - hen feathers spiked with internal standards at the level of 80 ng g^{-1} and spiked with standards of nitroimidazoles at the level of 60 ng g^{-1} (compounds MNZ, RNZ, DMZ, MNZOH, HMMNI)

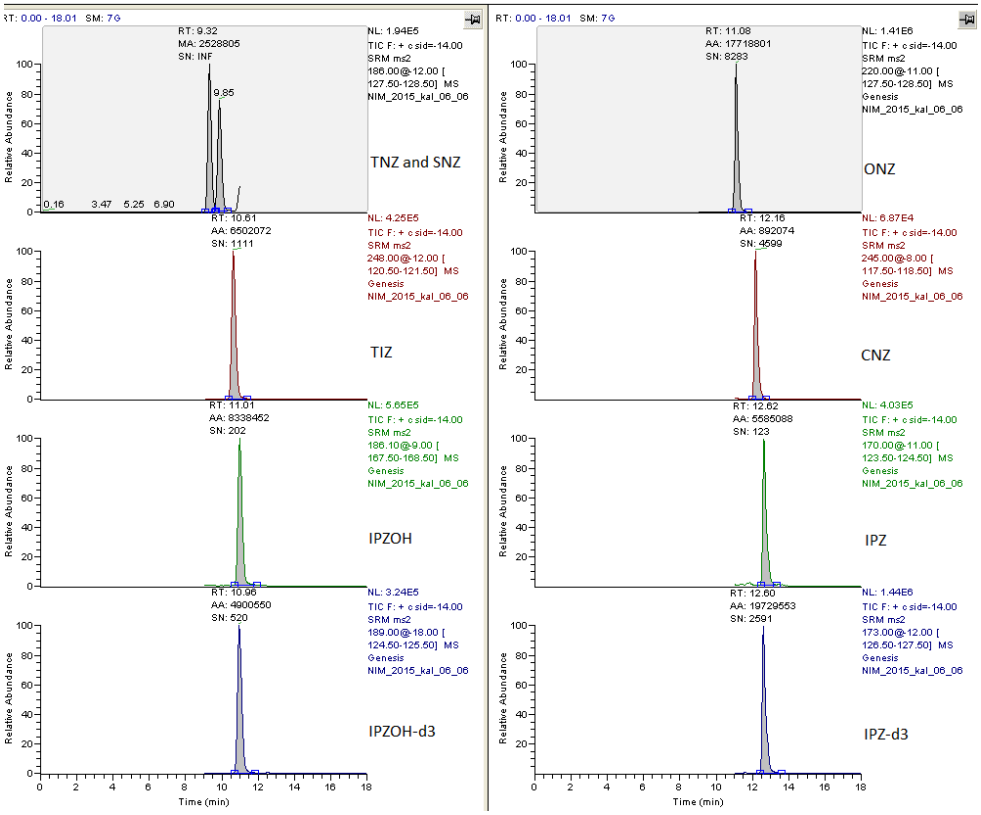

Figure 4. MRM chromatogram of fortified sample - hen feathers spiked with internal standards at the level of 80 ng g^{-1} and spiked with standards of nitroimidazoles at the level of 60 ng g^{-1} (compounds TNZ, SNZ, TIZ, IPZOH, ONZ, CNZ, IPZ).

Conclusions

The analytical method for the determination of nitroimidazoles in feathers was developed and described. The method has been also validated according to EU-decision 2002/657. Hen feathers, duck feathers and goose feathers were used during this validation. As it was shown, this analytical method provides sufficiently linear and reproducible responses of all investigated compounds. In addition, sensitivity is satisfactory. This method can be used for screening and confirmation of residues of nitroimidazoles in feathers.

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(041)

RAPID DETECTION OF THE RESISTANCE TO B-LACTAM ANTIBIOTICS IN GRAM-NEGATIVE BACTERIA BY INSTRUMENTAL ANALYSIS

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Abstract
The state of the art approach for rapid detection of compounds is based on advanced Mass Spectrometry (MS) techniques like MS/MS, HRMS and MALDI-ToF-MS. In this study a MALDI-ToF-MS assay was developed to detect β-lactam hydrolysis products produced by resistant bacteria. The use of MALDI-ToF-MS was compared to the use of MS/MS and a classical MIC (minimal inhibition concentration) assay. First, ampicillin and amoxicillin were used as the target antibiotics. Second, for cephalosporins the same set of techniques was used to develop an assay to specifically detect extended spectrum β-lactamase (ESBL) activity. The results of using advanced MS techniques for the measurement of bacterial β-lactam resistance and comparison with the MIC assay were very optimistic for amoxicillin and ampicillin. However, the use of MALDI-ToF MS for the detection of hydrolysis products (as a marker for resistance against an antibiotic) is less straightforward for most of the other selected β-lactam antibiotics. As soon as the set of hydrolysis/degradation products is well defined the use of MALDI-ToF MS (more general MS/MS) is very interesting alternative - due to its speed and selectivity - for the more traditional approaches for the measurements of resistance.

Introduction
The use of antibiotic was as mentioned before a medical revolution like no other in the treatment of infectious diseases. Nevertheless, a rapid appearance of a great number of bacteria presenting acquired resistance was observed, thus resulting in therapeutic fails. Six years after the introduction of penicillin in the market, the frequency of staphylococci resistance in some hospitals increases for specific compounds from 10% to 60% and today it is over 90% at world level. Therefore, the group of the β-lactams (including penicillins and cephalosporins) will be studied in this project.

For the classic way of detection of bacteria some time consuming procedures are necessary. Nowadays it becomes more important to start therapeutic treatment of infections as quick as possible. It is therefore necessary to know if there is any resistance. The use of more advanced analytical techniques is a possible approach to speed up the determination of resistance.

Analytical techniques, which are being used for the detection of small molecules (< 1,000 Da) are based on LC-MS/MS. In case large molecules have to be detected (>> 1,000 Da), such as proteins, the use of MALDI-ToF-MS is an interesting approach. MALDI-ToF-MS is widely applied in clinical laboratories for the identification of bacterial species (Camara *et al.* 2007; Matsumura *et al*. 2014). To test the use of MS for the detection of resistance it is necessary to use both techniques because it is not known on forehand which marker (and which molecule) is the best marker analyte. Furthermore, it is necessary to compare the use of an analytical chemical technique with the classical bioassay techniques.

Materials and Method

Hydrolysis assay for detection of resistant resistant pathogens
Based on the information found in literature (Wright *et al*. 2005) and contact with experts in the field, it was chosen to start with the development of a MALDI-ToF assay to detect β-lactam hydrolysis products converted by resistant pathogens. In Figure 1 two general mechanisms of hydrolysis of β-lactam antibiotics by β-lactamases are depicted. The first mechanism is mediated by a serine in Ser-β-lactamase that catalyses the hydrolysis of the β-lactam ring of the antibiotic (Figure 1A). The second mechanism depicted in Figure 1B is based on the catalysis of the hydrolysis by a metal ion in the active pocket of the metallo-β-lactamase.

The MALDI-ToF method was developed according to the method described by Sparbier *et al*. (2012). In Figure 2 the workflow of the method is depicted. For optimization of the hydrolysis and the MALDI-ToF detection method, an ampicillin resistant *E. coli* lab strain was used and a kanamycin resistant *E. coli* lab strain as a negative control. The two strains were grown overnight in a liquid culture in presence of ampicillin and kanamycin, respectively, to prevent loss of the plasmid containing the resistance gene. The overnight culture was washed to remove remnants of the antibiotics, added to an ampicillin solution of 10 mg mL^{-1} and incubated at 37°C. After 15, 30, 60 and 90 min samples were taken and spun down. The supernatant, containing the ampicillin and possible hydrolysis products, were analysed by MALDI-ToF.

For the study of resistance due to the use of cephalosporins the *E. coli* isolates with some widely spread and well-characterized reference ESBLs were chosen: TEM-52, CTX-M-1, CTX-M-14 and CTX-M-15. The selected antibiotics were the cephalosporins ceftiofur, cefuroxime, cefquinome, cefotaxime and ceftazidime.

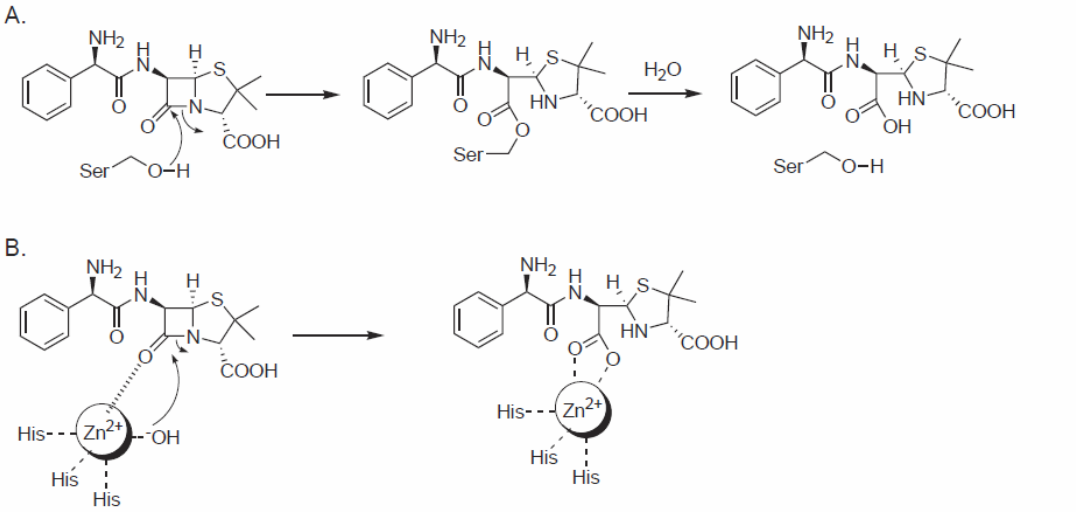

Figure 1. Two general mechanisms of β- lactamase. Hydrolysis of the β-lactam antibiotic amoxicillin catalysed by A) a Ser-β-lactamase and B) metallo-β-lactamase (adapted from Wright et al*. 2005).*

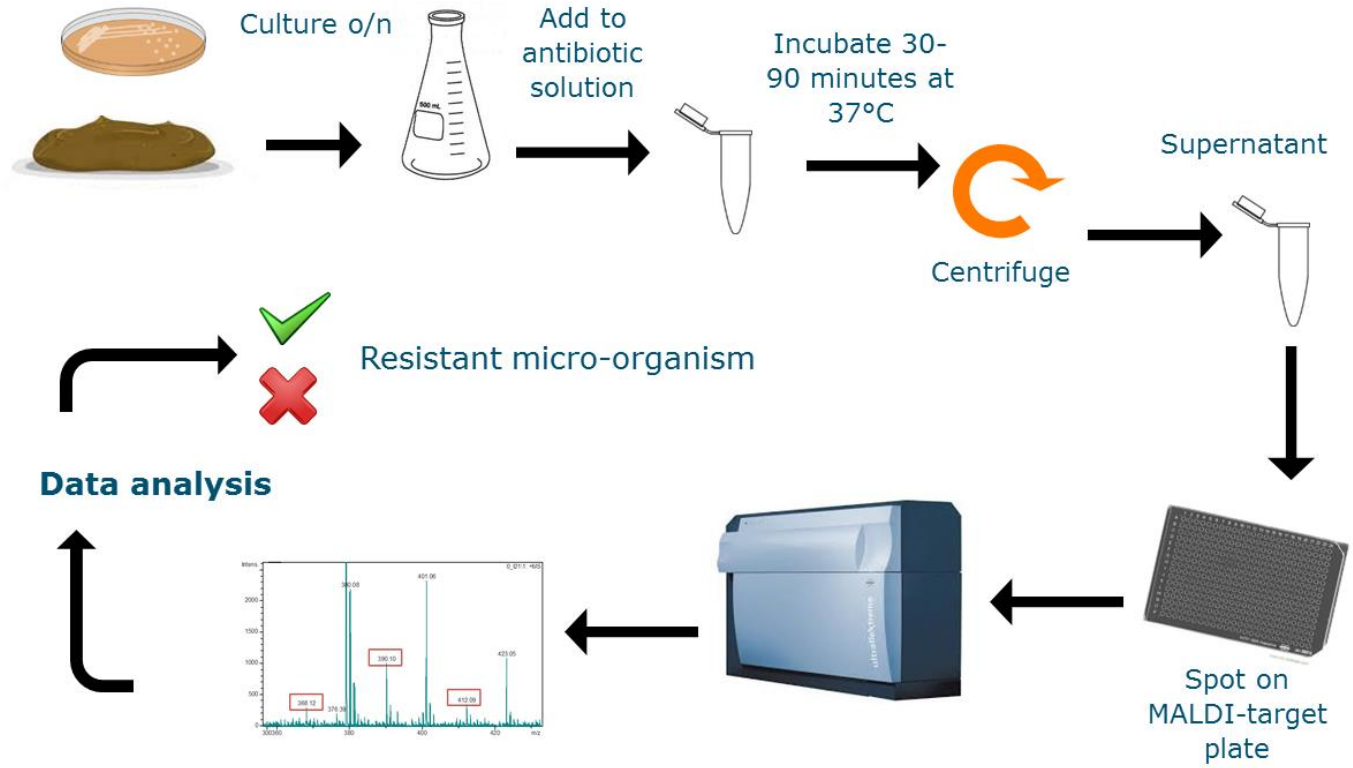

Figure 2. Workflow hydrolysis assay for detection of antibiotic resistant micro-organisms using MALDI-ToF.

Results and discussion

Ampicillin and amoxicillin hydrolysis assay for E. coli *and* S. typhimurium

In this study a MALDI-TOF based hydrolysis assay has been successfully developed for ampicillin- and amoxicillin-resistant *E. coli* and *S. typhimurium*. It is possible to identify resistant and sensitive strains, and the results correspond to the results obtained by a traditional microbiological MIC assay. To distinguish between strains with different MIC values, *i.e*. between the fully sensitive and intermediate groups, more method development is needed. A first approach to increase the sensitivity

could be a longer incubation of the strains with the antibiotic. This will result in more hydrolysis of the antibiotic, if resistance is obtained by expression of β-lactamase. Further characterization of the strains to determine the resistance mechanism, by *e.g*. PCR, might allow to explain the observed differences between the MIC assay and MALDI-ToF hydrolysis assay. Another way to increase sensitivity of the assay would be the use of LC-MS/MS rather than MALDI-ToF. LC-MS/MS allows to separate the molecules of interest from the background and has a higher sensitivity compared to MALDI-ToF. This makes it possible to detect low(er) amounts of hydrolysed antibiotic and thereby likely to identify the observed intermediate groups. One drawback of the use of LC-MS/MS would be the longer analysis time needed. Further optimization of the assay could be obtained by quantification of the signals in the MALDI-ToF spectra. The current method is qualitative and only the presence or absence of the antibiotic and its hydrolysis products decides the strain to be sensitive of resistant. The quality of the spectrum is very important, as was shown for the *E. coli* ampicillin assay. It is influenced by various factors, amongst which the sample composition. The addition of an internal standard to the sample allows to monitor these differences. If no signal for the internal standard is observed, the quality of the spectrum is not sufficient and no conclusions can be drawn. In addition, from the relative ratios of the intensities of the (hydrolysed) antibiotic to internal standard, the hydrolysis of the antibiotic can be followed more accurately and might allow to distinct different groups of β-lactam antibiotic resistant strains.

Penicillin G hydrolysis assay for S. aureus

Development of the MALDI-TOF hydrolysis assay for penicillin G resistant *S. aureus* has been partially successful. It is possible to discriminate between resistant and sensitive strains. However, some MIC classified resistant strains were not identified by the MALDI-ToF. It could be that the sensitivity of the assay is not sufficient due to the auto-hydrolysis of penicillin G. Another explanation could be that the strains that did not hydrolyse the penicillin G and employs a different resistant mechanism. Characterization of the strains by *e.g*. PCR should give more insight to explain these results. The use of other penicillin type of antibiotics is not likely to be helpful. For example, MALDI-ToF analysis of a penicillin V standard also showed hydrolysis of the antibiotic, even though less pronounced compared with penicillin G (data not shown). Other antibiotics will undergo another mechanism to *S. aureus* and can therefore not be used to replace penicillin G in the hydrolysis assay.

Comparison MALDI-ToF hydrolysis assay and MIC assay for penicillins

The MALDI-ToF hydrolysis assay corresponds mostly with the results obtained with the MIC assay. Some discrepancy could possibly be explained by a different resistance mechanisms of the strains studied or a lack in sensitivity of the current method. Both possibilities should be investigated further to be able to truly compare the two methods.

The goal of the project was to investigate the possibilities of a MALDI-ToF-based detection method for resistant pathogens and to develop a faster sensitive method that can accurately identify β-lactam resistant pathogens, compared to the traditional microbiological methods used, such as the MIC assay. The MALDI-ToF hydrolysis method described in this study includes an overnight culturing step to obtain sufficient bacteria to hydrolyse the antibiotics.

To reduce the time needed for the hydrolysis assay, (single) colonies could be picked from culture plates and mixed directly with the desired antibiotic. By omitting the overnight culturing step, the protocol will become one day faster compared with the MIC assay. Furthermore, if pathogens from infection sites need to be identified, it might be even possible to directly grow the isolate for some hours in medium, after which the hydrolysis assay is carried out. The pathogen is most likely the most abundant species and will outgrow any other micro-organism present and no clean colonies are needed. Further research will be necessary to find out if this would be a possibility to speed up the detection of resistant pathogens.

Detection method for cephalosporin resistance by MALDI-ToF

Although the cephalosporins standard solution were detected successfully by MALDI-ToF the detection of hydrolysis products was not easy. The hydrolysis procedure developed for penicillins was applied to the cephalosporins. However, no hydrolysis products were detected. The hydrolysis step was optimized but still no hydrolysis products (the expected M+18 Da ions) were detected. Then the research focussed on the search for alternative hydrolysis/degradation products. Although there were some positive results by applying HRMS instead of MALDI-ToF MS, the results obtained were not reproducible from one MS to the other (from HRM to MALDI-ToF MS). The final conclusion is that although the MALDI-ToF MS results show distinct spectra for susceptible and resistant bacterial strains for most cephalosporins, potential marker compounds identified from the HRMS approach could not always be observed in the MALDI-ToF MS spectra. This can be explained by the higher resolution and sensitivity of the HRMS method, but it should also be taken into account that the methods rely on different ionization techniques, which might favour ionization of method-specific sets of compounds with distinct relative abundance.

Comparison MALDI-ToF hydrolysis assay and MIC assay for cephalosporins

The results show an acceptable correlation between the HRMS results and the MIC determination. For ceftiofur the results cannot be compared since β-lactam hydrolysis was inhibited by the presence of methanol in the incubation. Assuming the TEM-52 and CTX-M strains are clear ESBL representatives, cefotaxime and ceftazidime (which are the compounds used in routine diagnostics for establishing expression of ESBLs) appear to be very straightforward indicator substrates in the HRMS

assay. From these results it could be concluded the pET21 β-lactamase is not an ESBL. Nevertheless, the MIC results show a somewhat subtler picture, showing different degrees of susceptibility in particular with ceftazidime. The differences can be explained by the fact that the underlying process is not fully identical. The HRMS assay essentially monitors a biochemical process, which only depends on the presence, activity and substrate specificity of β-lactamase enzymes, while in the MIC determination also includes bacterial growth.

For cefquinome and cefuroxime no resistance cut-off value has been defined, since these substrates are not specific for extended spectrum β-lactamases, but are also hydrolysed by "simpler" β-lactamases. This is illustrated by the hydrolysis of these compounds by the pET21 control.

Conclusions

The results of using advanced MS techniques for the measurement of bacterial β-lactam resistance and comparison with the MIC assay are very optimistic for amoxicillin and ampicillin. However, the use of MALDI-ToF MS for the detection of hydrolysis products (as a marker for resistance against an antibiotic) is less straightforward for most of the other selected β-lactam antibiotics. The specific set of hydrolysis products is very well established for the amoxicillin and ampicillin and therefor the used approach was very successful. As soon as the set of hydrolysis/degradation products is well defined the use of MALDI-ToF MS (more general MS/MS) is very interesting alternative - due to its speed and selectivity - for the more traditional approaches for the measurements of resistance.

Acknowledgements

Thanks to the Dutch Ministry of Economic Affairs and to the South-Korean Animal and Plant Quarantine Agency (QIA) for their financial support.

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(042)

FEATHER SEGMENTATION ANALYSIS FOR MONITORING OFF-LABEL USE OF ANTIBIOTICS IN THE POULTRY SECTOR

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Abstract

In the combat against bacterial resistance, policies for reduction of antibiotic use in livestock breeding require an up-to-date farmer registration indicating all treatments. To enforce such policies, tests are needed to detect antibiotic use during slaughter, even if applied just after hatching. Furthermore, antedating of administration and discriminating different types of treatment would be beneficial, so to prevent off-label use and the superfluous use of especially last resort antibiotics. It has previously been demonstrated that depletion of antibiotics from feathers is much slower than in matrices regularly used for MRL monitoring (e.g. urine, muscle and eggs). Therefore, feathers are a promising and non-invasive matrix to monitor antibiotic use in the poultry sector. For the first time, feather segmentation in combination with the differentiation of extractable and non-extractable residues was applied to discriminate among different oxytetracycline and enrofloxacin treatments. Based on this approach we are able to distinguish between a registered therapeutic oral treatment, an off-label spray treatment, and an illegal prolonged sub-therapeutic treatment. The outcomes also indicate that the presented approach is promising for antedating treatments.

Introduction

Antibiotics are widely used to treat microbiological infections in both human and veterinary medicine (Campoli-Richards *et al*.). In The Netherlands, additional policies were established to limit the use of antibiotics, with the focus on last resort antibiotics (fluoroquinolones and cephalosporins). These policies require an up-to-date farmer registration indicating all treatments. To enforce such policies, tests are needed to first detect and then antedate administration.

In 2013, a pilot study was done where deposition of oxytetracycline (OTC) to feathers was investigated (Berendsen *et al*.). In order to confirm the results of this pilot study, an additional study was done with enrofloxacin (ERF) in 2015 (Jansen *et al*.).

Here we assess the results of both studies. We advocate that feather segmentation is a strong tool to determine and prevent off-label or superfluous use of antibiotics in the poultry sector. Furthermore, it is our belief that this procedure can be applied to a wide range of antibiotics.

Materials and Methods

Animal treatment

In the pilot study, animals were exposed to three different treatments with OTC (see Figure 1). A therapeutic oral treatment administered via drinking water to 1-week-old chicks during 5 days (treatment A), a therapeutic oral treatment administered to older chicks (20 days) during 3 days (treatment B) and a sub-therapeutic oral treatment administered to 1-week-old chicks until day 30, being 8 days before slaughter (treatment C).

Code	Treatment oxytetracycline	D7	D8	D9	D10	D11	D12	...	D20	D21	D22	D23	...	D30	...	D38
																S
Treatment A	Therapeutic water 1	Start														2
Treatment B	Therapeutic water 2								Start							2
Treatment C	Sub-therapeutic water	Start														2

Figure 1. Schedule of different OTC treatments where 'start' is the first day of treatment, the coloured blocks indicate the period of administration, the S indicates the day of slaughter, and the '2' indicates the number of chickens that were slaughtered.

In this research a limited number of OTC-incurred poultry feather samples were obtained. A second study was carried out with improved feather sampling and a research setup more focused on the collection of feather samples. In this second study animals were exposed to three different treatments with enrofloxacin (see Figure 2): a therapeutic oral treatment (treatment D), a therapeutic spray treatment (treatment E) both administered to approximately 1-week-old chicks during 5 days, and a sub-therapeutic oral treatment (treatment F) administered to chicks of the same age, but continuous until the day of slaughter. In both studies, a control group was included. During the whole experimental setup, the animals belonging to these groups did not get in contact with OTC or ERF.

Code	Treatment enrofloxacin	D6	D7	D8	D9	D10	D11	D12	D13	...	D34
											S
Treatment D	Therapeutic water	Start									2
Treatment E	Therapeutic spray	Start									2
Treatment F	Sub-therapeutic water	Start									2

Figure 2. Schedule of different ERF treatments.

Sample preparation

In the OTC pilot study, a method for ground feather material was developed for this compound. Feathers were cut into four different segments. During the second study, a method was developed with the purpose of measuring multiple groups of antibiotics, including fluoroquinolones and thus enrofloxacin in feather material. Feathers were also washed to remove freely extractable antibiotics, so to measure the non-extractable fraction of residues. In this study feathers were cut into six different segments before analysis.

Results and Discussion

Control treatments were analysed and did not contain OTC or ERF (<LOD). In Figure 3 the results of both studies for treatment A – F are summarized. As pointed out in both studies, in order to discriminate between treatments, the amount of residues found per cm of feather should be compared.

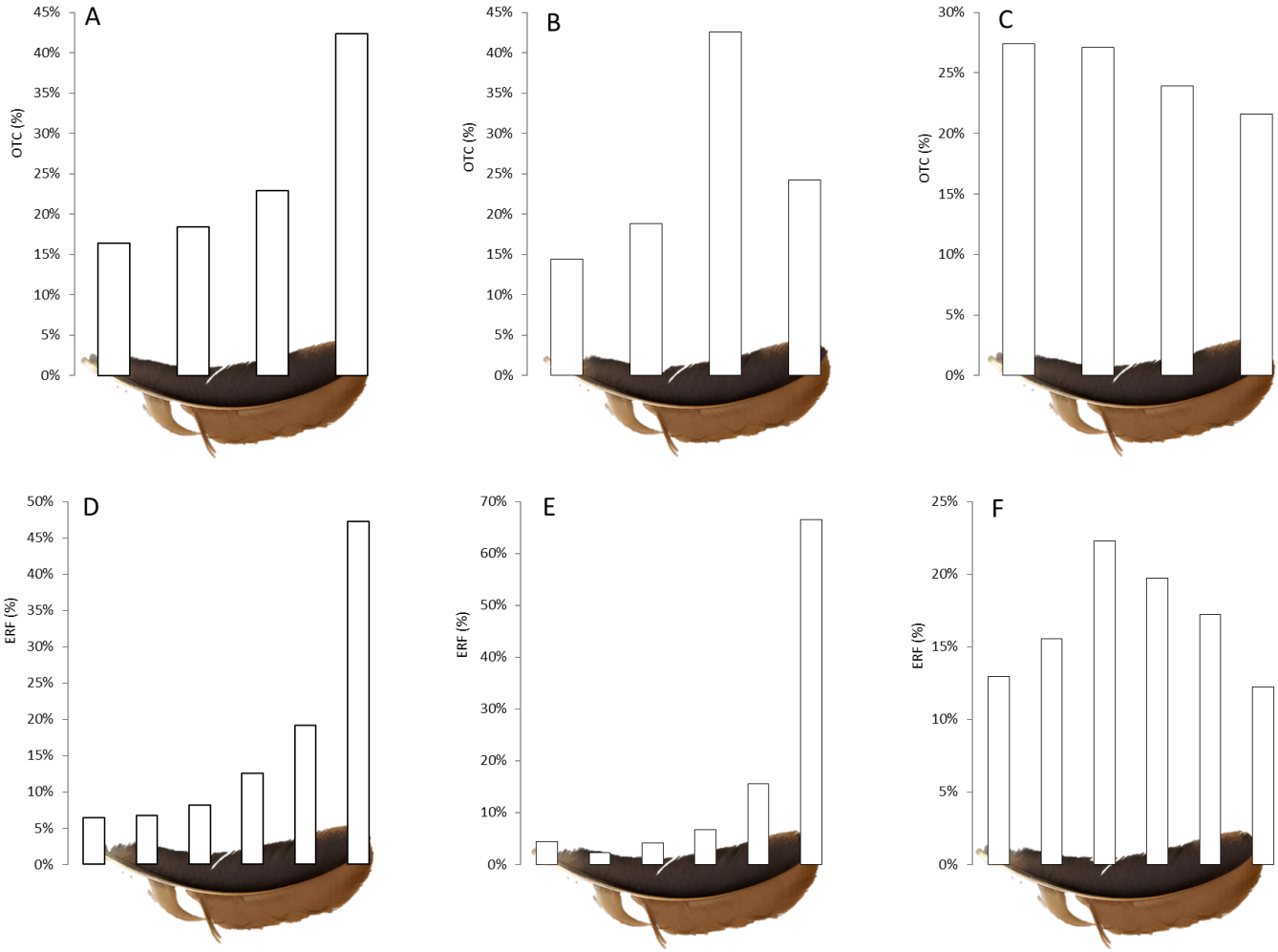

Figure 3. Amount of OTC (A-C) and ERF (D-F) distributed over feather segments for different treatments (A – F).

In Figure 3A-C, the OTC content in four segments is shown for different treatments. Based on the young age of the chicks during administration and short duration of the treatment, it is to be expected that the antibiotic concentration in the top segments of the feather is higher than in the lower segments. Figure 3A fits this theory perfectly. In figure 3D these results

are confirmed showing high levels of ERF in the top of the feather when treatment was carried out on 1-week-old chicks. Therefore, it is very likely that this pattern can be identified as a therapeutic treatment that was administered to young animals. The same can be said for the sub-therapeutic, continuous treatments. In graph 3C, an OTC pattern showing evenly deposition of OTC over all segments is observed. This was confirmed in the study with ERF in Figure 3F. This means that sub-therapeutic patterns can be identified and, in case of an unknown treatment, it is possible to discriminate between a single and a continuous treatment based on these patterns.

It is expected that treatment of older animals will become visible by a shift of the highest amount of antibiotic in lower feather segments. Figure 3B supports this: the pattern of an oral therapeutic OTC treatment administered to 20-day-old chickens shows the highest antibiotic content in the second highest segment. These results are very promising and are the proof of the possibility to antedate treatments using feathers.

A spray treatment was included in the experimental setup in the ERF study. As can be seen in Figure 3E, a distinction between a therapeutic spray treatment and a therapeutic oral treatment (Figure 3D) cannot be made on basis of the distribution patterns alone. We compare the extractable and non-extractable residues by analysing the feather segments with and without applying the washing procedure. Indeed, on basis of the relation between extractable and non-extractable residues, it was possible to discriminate between oral treatment and external treatment (or contamination).

Conclusions

Tetracyclines, quinolones and presumably other antibiotics, grow inside a feather after administration to chickens. This mechanism can be used to discriminate between treatments. In order to discriminate between a therapeutic oral treatment and a spray treatment, a comparison of extractable and non-extractable residues should be made. This approach is a new and strong tool in the enforcement of new policies in the fight against off-label and superfluous antibiotic use. Furthermore, this approach is promising for antedating treatments.

Acknowledgements

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STRATEGIES TO DISTINGUISH SYNTHETIC FROM NATURALLY PRODUCED CHLORAMPHENICOL

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Abstract

Chloramphenicol is a broad spectrum antibiotic, which is banned for use in food-producing animals, but is also produced by soil bacteria, *Streptomyces Venezuelae*. It is transferred to crops and can unintendedly be part of an animal's diet. As chloramphenicol is sometimes detected in animal urine, to enforce legislation, it is important to discriminate synthetic and naturally produced chloramphenicol. Strategies to discriminate natural from synthetic chloramphenicol were studied.

During this biosynthesis of chloramphenicol also intermediate products are formed. These products could be useful as a biomarker for the presence of natural biosynthesised chloramphenicol. Sterilized soil was incubated (Berendsen , 2013) with *Streptomyces Venezuela* in order to produce chloramphenicol. Soil was extracted using acetonitrile and extracts were analysed using UPLC-HRMS. Data was interpreted using Metalign Software (Metalign, RIKILT) and 49 possible biomarkers were selected. Additionally, straw samples containing chloramphenicol were analysed. In two out of the four tested straw samples, containing chloramphenicol in a range of 5-30 µg kg^{-1}, two of the selected possible biomarkers were detected. However, these biomarkers proved to be present at trace levels in straw and therefore characterisation of chloramphenicol is not possible at relevant levels.

A second study focussed on $^{12}C/^{13}C$ isotope ratios of chloramphenicol to discriminate between natural and synthetic produced chloramphenicol. Different types of sterilized soil were incubated with *Streptomyces Venezuelae* to produce natural chloramphenicol. Soil extracts containing naturally produced chloramphenicol and solutions of synthetic chloramphenicol were prepared and derivatised yielding chloramphenicol-di-TMS derivatives to allow Gas Chromatography Isotope Ratio Mass Spectrometry (GC-IRMS) to determine $^{13}C/^{12}C$ isotope ratio. Final results from these experiments will be published in a scientific paper in 2016.

Introduction

Chloramphenicol is a broad spectrum antibiotic which is a suspected carcinogen (Wongtavatchai, 2004). The use of chloramphenicol in food-producing animals is banned since 1990 within the European Union. The established reference point of action is 0.3 µg kg^{-1} in matrices from food-producing animals (EFSA J, 2014; 2005/34/EC). Besides synthetic production, chloramphenicol can be naturally produced by *Streptomyces Venezuelae* and can successively be allocated to crops. Through this route, chloramphenicol residues can unintendedly end up in food producing animals. This results in a debate related to the enforcement after detecting chloramphenicol residues in straw and animal matrices. With current analytical techniques it is not possible to discriminate between chloramphenicol of natural and synthetic origin as it is chemically the same substance. To distinguish between synthetic and naturally produced chloramphenicol, two studies were carried out.

First, it was examined whether by-products or intermediates of the biosynthesis of chloramphenicol are useful to characterise the origin of chloramphenicol. Soil extracts were analysed using HPLC-HRMS. Data obtained from these measurements were examined by using Metalign Software to trace for by-products from the biosynthesis of chloramphenicol.

The second study focussed on the $^{12}C/^{13}C$ isotope ratio by using Isotope Ratio Mass Spectrometry (IRMS). With this technique it was examined if the difference between natural and synthetic chloramphenicol is expressed in the $^{12}C/^{13}C$ isotope ratio. Several soil types were incubated with *Streptomyces Venezuelae* to produce biosynthesised chloramphenicol. Incubations were carried out in sterile soil by adding a suspension of *Streptomyces Venezuelae* of 2.0 x 10^9 CFU mL^{-1}. Soil extracts and solutions of synthetic chloramphenicol of different origin, were prepared with a dedicated method to isolate chloramphenicol. Chloramphenicol was subsequently derivatised using BSTFA containing 1% TMCS to obtain chloramphenicol-di-TMS derivatives allowing GCMS-IRMS analysis to determine the $^{13}C/^{12}C$ isotope ratio.

Materials and Methods

Sample material

Soil samples were obtained from Alterra Wageningen UR and straw and swine urine samples were obtained from previous monitoring studies

Chemicals and reagents

Streptomyces Venezuelae was obtained from strain DSM40230 and was in-house produced at a concentration of 2.0 x 10^9 CFU mL^{-1}. All used chemicals and standards were of analytical grade or higher purity.

Instrumental equipment

For the by-products determination, an Orbitrap mass spectrometer from Thermo Scientific, type Q-Exactive, equipped with an Ultimate 3000 LC system from Dionex, was used in several modes, e.g. Full scan (FS) and All Ion Fragmentation (AIF) (resolution 70,000), MS^2 (resolution 17,500). A linear gradient of ultrapure water and acetonitrile on a Kinetex 1.7u C18 chromatographic column, 100 x 2.1 mm was used for chromatographic separation. For the IRMS measurements, a gas chromatograph from Thermo Scientific, type trace GC Ultra using a VF-1MS, 60 m x 0.25 mm ID, film thickness 0.25 µm (Varian CP8916) GC column for chromatographic separation, equipped with an ultra-Automatic injector from Gerstel, type MPS2X-L was coupled to an isotope ratio mass spectrometer (Thermo Scientific, type MAT253) was used.

Sample preparation and extraction

In the analysis of by-products formed during the biosynthesis of chloramphenicol by *Streptomyces Venezuelae*, 100 g of sterile soil-2 (Table 2), containing 20% water, was incubated with 0.1 mL of 2.0 x 10^9 CFU mL^{-1} *Streptomyces Venezuelae* for 14 days at 28°C. Ten grams of incubated soil was extracted with acetonitrile for 30 min, then centrifuged and dried under nitrogen and reconstituted in 0.5 mL methanol. Full scan mass spectra obtained were reviewed using Metalign software. Using the software, blank soil data was subtracted from incubated soil data. Subtracted data, containing spectra including by-products from the incubation with *Streptomyces Venezuelae,* was used for detecting by-products. The most abundant by-products were selected for monitoring in targeted MS^2 mode with a resolution of 17,500 using the Q-Exactive Orbitrap. Additionally, four straw samples, containing chloramphenicol in the range of 5 to 30 µg kg^{-1}, were extracted according to the method used for soil-2. Data from these extracts were compared with data from the soil.

For discriminating natural from synthetic chloramphenicol using GC-IRMS different types of sterile soil (Table 2), *e.g*. clay, subsoil, peatland and sand were inoculated with *Streptomyces Venezuelae (*8 x 10^6 CFU g^{-1} soil) and incubated for 5 days at 25°C. Moisture content of the substrates was set at 20% using purified sterile water. The incubated samples were extracted using purified water followed by solid phase extraction (Strata-X cartridges,Phenomenex) followed by liquid-liquid extraction with ethyl acetate. Ethyl acetate, containing chloramphenicol residues, was dried under nitrogen and reconstituted in purified water and subsequently the extract was purified using a UPLC fraction collector. An aliquot of the extract was injected on an Acquity UPLC BEH chromatographic column (Waters) using a linear gradient of water and acetonitrile containing 0.1% formic acid. Chloramphenicol was fractionated within a narrow fraction window and collected fractions were dried under nitrogen and a chloramphenicol di-TMS derivative was formed using BSTFA + 1% TMS for 60 min at 60°C. Derivatives were dried under nitrogen, reconstituted in iso-octane and analysed for $^{13}C/^{12}C$ isotope ratio using GC-IRMS. Figure 1 shows the derivative of chloramphenicol-di-TMS. Additionally, straw samples and swine urine samples, containing chloramphenicol, were extracted using the method used for soil samples and analysed with GC-IRMS.

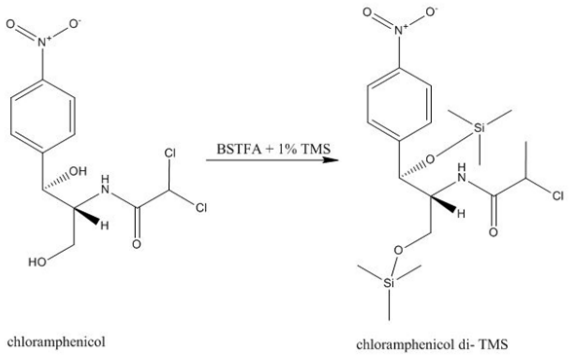

Figure 1. Derivatisation of chloramphenicol to produce chloramphenicol-di-TMS by BSTA +1%TMS.

Results and discussion

Analysis of by-products from the biosynthesis of chloramphenicol by Streptomyces Venezuelae

Besides chloramphenicol many by-products were formed during inoculated soil incubation. The most abundant by-products of the biosynthesis, a total of 49 molecular ions (25 ESI-, and 24 ESI+) were selected. Table 1 shows 20 of the most abundant molecular ions. Two out of four straw samples analysed contained two biomarkers which are also present in soil (see underlined in Table 1). Figure 2 shows a proposed structure of the by-product *m/z* 281 and its fragments. In straw, the concentration of both chloramphenicol and the by-product were lower compared to soil.

Table 1 Most abundant ions present in incubated soil, underlined ions also present in straw sample containing chloramphenicol.

m/z ESI-	*Retention time min*	*Proposed by-product*	*m/z* ESI+	*Retention time min*	*proposed by-product*
321.0052	9.03	$C_{11}H_{12}O_5N_2Cl_2$	357.2778	12.93	$C_{18}H_{38}ON_4P$
356.9820	9.03	$C_{11}H_{13}N_2O_5Cl_3$	<u>293.1482</u>	<u>9.37</u>	$\underline{C_{15}H_{21}O_4N_2}$
<u>281.1147</u>	<u>8.23</u>	$\underline{C_{13}H_{18}N_2O_5}$	297.1434	9.30	$C_{14}H_{21}O_5N_2$
317.0910	8.22	$C_{13}H_{19}N_2O_5Cl_1$	297.2566	12.93	$C_{18}H_{36}NP$
384.0008	9.03	$C_{11}H_{13}N_3O_8Cl_2$	331.2617	11.83	$C_{16}H_{36}ON_4P$
363.0161	10.38	$C_{13}H_{14}N_2O_6Cl_2$	573.3916	15.40	$C_{34}H_{48}N_6O_2$
364.9548	9.19	$C_{11}H_{12}N_2O_5Cl_1Br_1$	403.1349	7.80	$C_{14}H_{33}O_4NP_4$
295.1300	9.27	$C_{14}H_{20}N_2O_5$	663.4938	13.05	$C_{35}H_{55}N_{11}O_1$
331.1069	9.26	$C_{14}H_{21}N_2O_5Cl_1$	328.1061	7.35	$C_{11}H_{21}N_1O_8S_1$
385.2599	11.95	$C_{21}H_{38}O_6$	366.1758	7.61	$C_{10}H_{24}N_9O_4P_1$

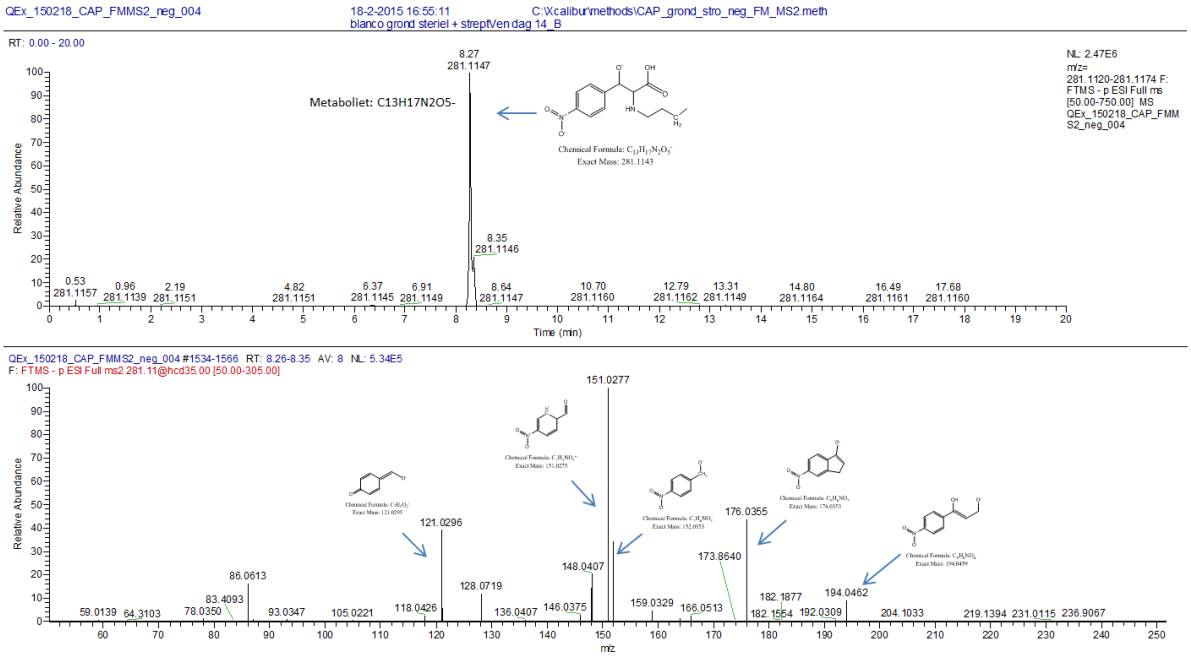

Figure 2. Extracted ion chromatogram of inoculated soil sample and the product ion spectrum of m/z 281.1147 (proposed molecular formula $C_{13}H_{17}N_2O_5^-$) in ESI-.

Discriminating natural from synthetic chloramphenicol using Isotope Ratio Mass Spectrometry on $^{12}C/^{13}C$ isotope ratio

Incubation of different types of sterilised soil with *Streptomyces Venezuelae* results in high concentrations of biosynthesised chloramphenicol. The produced amount of chloramphenicol varies depending on the soil type (see Table 2).

Table 2 Soil types used for incubation with Streptomyces Venezuelae

Soil description	Type soil	Soil code	µg kg^{-1} biosynthesised chloramphenicol
Subsoil Robin	subsoil	Soil-1	379
Soil Robin	soil	Soil-2	1015
Clay Haarweg	clay	Soil-3	1099
Soil meenthoeve	soil	Soil-4	1008
Sand Born	Sand	Soil-5	335
Peatland Venenkamp	Peatland	Soil-6	1871
Clay Afweg	clay	Soil-7	1480

Chloramphenicol-di-TMS derivatives were analysed using GC-IRMS. These measurements (Figures 3 and 4) showed a good reproducibility of the $^{12}C/^{13}C$ isotope ratios from triplicate injections of the extracts. The amount of chloramphenicol-di-TMS in soil samples was high enough to meet the LOD of the GC-IRMS (approximately 5 ng absolute on a GC column). In soil extracts, no interfering peaks that might disturb the signal for chloramphenicol-di-TMS derivatives were observed in the chromatograms. However, in straw and swine urine extracts interferences were present in the chromatogram disturbing the $^{12}C/^{13}C$ isotope ratio. A small difference in $^{12}C/^{13}C$ isotope ratio was observed between the chloramphenicol produced in soil (biosynthesis) and the solutions of reference standards (synthetic). Further research should indicate if this difference is sufficient to discriminate synthetic from naturally produced chloramphenicol.

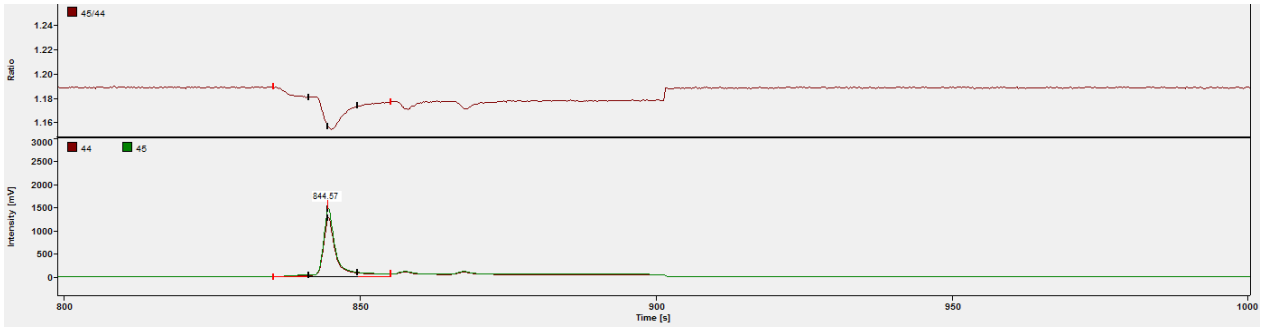

Figure 3. GC-IRMS chromatogram of a soil extract containing biosynthesised chloramphenicol-di-TMS derivative.

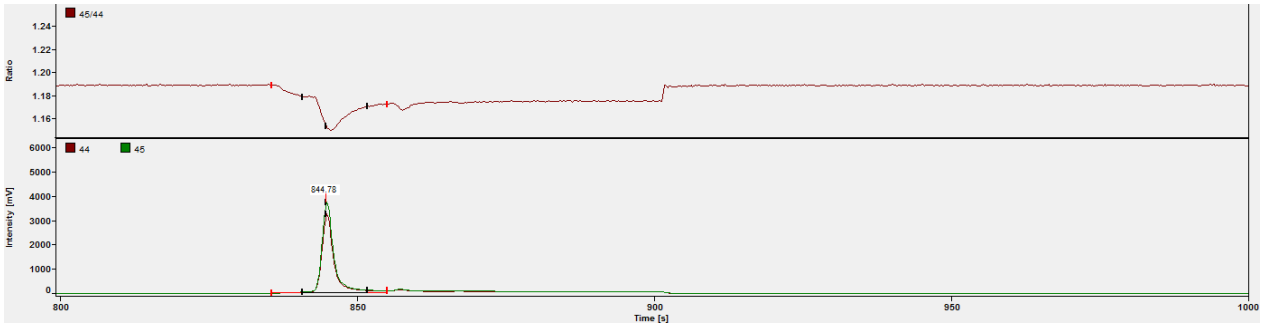

Figure 4. GC-IRMS chromatogram of a pure standard chemically synthesised chloramphenicol-di-TMS derivative.

Conclusions

During biosynthesis of chloramphenicol by *Streptomyces Venezuelae*, many by-products are produced in soil. A selection of 49 most abundant by-products was used to check chloramphenicol contaminated straw samples. 2 out of the 49 selected by-products were detected in 2 out of 4 straw samples. These by-products were identified based on their product ion spectra. Unfortunately, these by-products were present at levels much lower that chloramphenicol itself and therefore it is currently impossible to apply this approach for discriminating synthetic from naturally produced chloramphenicol.

The use of GC-IRMS for determination of the $^{13}C/^{12}C$ isotope ratios of biosynthesised chloramphenicol in soil is promising. First experiments showed that this technique is capable to determine $^{13}C/^{12}C$ isotope ratios of chloramphenicol in soil and a difference in the isotope ratio of synthetic versus naturally produced chloramphenicol was found, though very small. Final results from these experiments will be published elsewhere later.

Acknowledgements

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DEPOSITION AND DEPLETION OF MADURAMICIN AND MONENSIN RESIDUES IN EGGS RESULTING FROM MISUSE OF FEED FOR TARGET SPECIES

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Abstract

Coccidiostats are often used as additives in poultry breeding due to their prophylactic and therapeutic effects. According to Commission Regulation (EU) No 495/2011, monensin is authorised in feedstuffs at maximum doses of 125 mg kg^{-1}, and maduramicin at a maximum content of 5-6 mg kg^{-1} (EU No 388/2011). In the present study, laying hens, as the non-target species, were treated with maduramicin and monensin added to feedstuffs below and at the concentration authorised for target species. Hens were divided into groups and fed for 14 days with medicated feed. Two groups were treated with feed containing maduramicin at either 1 or 5 mg kg^{-1}, while two groups were treated with feed containing monensin at either 62.5 or 125 mg kg^{-1}. The control group received feed free of coccidiostats. Eggs were collected during the treatment period and 26 days after withdrawal of the medicated feed. Maduramicin residues were determined exclusively in egg yolk, while residues of monensin were determined in both egg white and yolk. The transfer factor was significantly higher for maduramicin than for monensin. Residue levels of maduramicin and monensin in eggs, after administration at the maximum concentration in feed, were below the maximum residue limits for eggs after 16.6 days and 6.0 days, respectively.

Introduction

Coccidiosis is a disease characterised by acute invasion and destruction of the intestinal mucosa of the infected animal by protozoa of the genera *Eimeria* or *Isospora*. Due to its high reproduction potential, ability of sporulated oocysts to survive in poultry litter and its varying pathogenicity, cocciodosis is the most significant disease of intensive poultry farming.

Coccidiostats are chemicals that inhibit or destroy the protozoan parasites causing coccidiosis in farmed animals. Coccidiosis has been controlled by adding substances to animal feed and therefore regulatory bodies have authorised coccidiostats as feed additives. According to Commission Regulation (EU) No 495/2011, monensin is authorised in feedstuffs at a maximum dose of 125 mg kg^{-1}, and maduramicin at a maximum content (MC) of 5–6 mg kg^{-1} (EU No 388/2011). Despite the prescribed procedures in the production of animal feed to minimise the risk of errors and cross-contamination, the likelihood of detecting anticoccidials in food for non-target groups of animals is very high. The most common causes of contamination are related to technological equipment and physico-chemical characteristics of the substances. Production of feed for different animals is maintained on the same production line and, if not properly cleaned from the previous batch, presents a high risk of contamination.

Maduramicin and monensin are not authorised for use in laying hens. The European Commission united the maximum residue levels (MRLs) of coccidiostats in food (Commission Regulation (EU) No 610/2012) that occur due to the unavoidable carry-over of coccidiostats to non-target feed. Concentration in feed materials should not exceed 0.05 mg kg^{-1} for maduramicin and 1.25 mg kg^{-1} for monensin (Commission Directive 2009/8/EC). This concentration represents cross-contamination at a level of 1% of the maximum authorised concentration, which resulted in maximum residue limits (MRL) set for the presence of coccidiostats in food resulting from unavoidable carry-over. The maximum residue limit in eggs was set at 12 µg kg^{-1} for maduramicin and 1.25 mg kg^{-1} for monensin (Commission Regulation No 610/2012).

Affinity of the drug to bind to plasma proteins, hydrophobic and hydrophilic qualities, and the ability of drug to transport through different types of tissues are important characteristics determining the distribution of the drug in the yolk or albumen of the egg (Martinez *et al*., 2006). Laying hens lay an egg every 24 hours, and each egg takes several days to develop *in vivo*, with some components of the egg present in the body months before the egg is completely developed and spawned. After treatment with veterinary drugs, it can take up to several weeks for an egg to contain no drug residues (Goetting *et al*., 2011). Egg yolk has the longest development period of all three main components of eggs. Rapid accumulation of veterinary medicines occurs during 10 days of intensive growth of egg yolk (Goetting *et al*., 2011).

Although an earlier model was developed to describe the transfer of veterinary drugs to different species (Vandenberg, 2012), it cannot explain the distribution of coccidiostats. The concentration limits that describe the permitted amount of coccidiostats in eggs derived from animal feed containing 1% of the maximum permitted level for target species are not often supported by elimination studies. Moreover, due to differences in the kinetics between coccidiostats, studies on retention and elimination must be carried out for each anticoccidial.

There are few data on the pharmacokinetics of non-target groups of animals, and laying hens are the most common non-target group to unintentionally receive coccidiostats via feed in low or high concentrations. Several papers have provided experimental data indicating that the administration of compliant contaminated feed may result in non-compliant samples of

eggs (Olejnik *et al*. 2014; Bodi *et al*. 2012). Mortier *et al*. (2005) concluded that a 5% carry-over of coccidiostats can result in residue persistence in eggs for 15 days. The tested coccidiostats were Diclazuril, halofuginone, robenidine, narasin and nicarbazin, and the conclusions showed that the maximal doses for broilers can result in a withdrawal period of 22, 19, 26, 18 and 23 days, respectively.

This study investigated the depletion time of maduramicin and monensin in eggs after administration via feed. The objective of this study was to compare the different chemical properties of maduramicin and monensin on the kinetics of elimination and their deposition rate in eggs following the treatment of laying hens. For the purpose of calculating the transfer factor of coccidiostats from the feed mixture to the egg matrix, the plateau value of elimination curve was calculated for each coccidiostat.

Materials and Methods

Animal treatment and sampling

This study was performed in accordance with the regulations of the Ethics Committee for research and animal experiments in Croatia, which are compliant with EU legislation. Laying hens (ISA Brown, n=30) were obtained from a breeding station and were set up at approximately 21 weeks of age. In cages, hens had free access to water and feed, and intake was observed over 2 weeks prior to treatment during the adaptation period. Birds consumed an average of 140 g feed per day. Hens were under daily surveillance by veterinarians and qualified personnel. Hens were randomly divided into five groups (n=10), one of which was the control group and four treatment groups, and each group was housed in a separate cage. In the treatment groups, Groups I and II were treated for 14 days with 100% and 20% of the maximum authorised maduramicin concentrations, respectively, while Groups III and IV received 50 % and 100 % of the maximum dose of monensin (MC) (Table 1). Duration of the treatment period was chosen according to the maximum period of the treatment for chickens for fattening.

Preparation of feed with the specified amount of the maduramicin or monensin premix was performed using a special feed mixer. Considering the daily intake of feed, groups that consisted of 10 laying hens required approximately 19.6 kg of medicated feed for the 14-day treatment period. Preparation started with weighing the calculated amount of the maduramicin ammonium and monensin sodium premix as shown in Table 1. Feed samples were prepared starting with the group with the lowest coccidiostat concentration.

Table 1. Experimental groups and preparation of the medicated feed with the addition of maduramicin.

	Control group	Group I	Group II	Group III	Group IV
Maduramicin conc. in feed (mg kg^{-1})	-	5.0	1.0	-	-
Monensin conc. in feed (mg kg^{-1})	-	-	-	62.5	125
Feed (kg)	20	20	20	20	20
1 % maduramicin ammonium premix (g)	-	0.204	0.102	-	-
10 % monensin sodium premix (g)	-	-	-	12.910	25.819

The 14-day treatment period was labelled with numbers of days from -14 to -1, where -14 was the first and -1 the last treatment day. Post-treatment days were labelled with numbers starting from 1 to 26.

Eggs were collected daily throughout the study period and labelled to indicate the day number, group and cage number, and stored at +4°C. Eggs collected prior to administration were used as the blank control. After receiving eggs in the laboratory, samples were separated into egg white and egg yolk, homogenised and stored at -20°C until analysis.

Chemicals, standards and apparatus

All reagents were of analytical, HPLC or LC-MS grade. LC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Ammonium formate (97%) and formic acid (≥ 96%) were purchased from Sigma Aldrich Chemie GmbH (Germany). Solid phase extraction (SPE) cartridges Strata X, 33 µ Polymeric RP (200 mg/ 6 mL) were supplied from Phenomenex Inc. (Torrance, USA). Nitrogen 5.0 and 5.5 were purchased from SOL spa (Monza, Italy). Ultra pure water was obtained by the Milli-Q system (Millipore, Bedford, USA). Maduramicin ammonium and monensin sodium salt hydrate standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). For the internal standard, nigericin was selected, as a structurally similar compound that is not in commercial use. Nigericin was obtained from Sigma-Aldrich (St. Louis, MO, USA). The maduramicin ammonium alpha premixture Cygro® 1% was purchased from Bayer AG, Germany. Monensin sodium premixture Mondolar

10% was purchased from Krka, Slovenia. Feed for laying hens was supplied from the local market and tested for the presence of maduramicin and monensin with the appropriate method for feed. Eggs used for the validation and matrix calibration curve were supplied from a local laying hen farm and previously tested for the presence of maduramicin and monensin.

The following equipment was used in sample preparation: Waring Commercial Blender 7011HS (Connecticut, USA), IKA® Vortex model MS2 Minishaker (Staufen, Germany), Iskra ultrasonic bath (Slovenia), Supelco vacuum manifold (Bellefonte, PA), centrifuge Rotanta 460R (Hettich Zentrifugen, Tuttlingen, Germany) and Nitrogen evaporation system N-EVAP® model 112 (Orgamonation Associates Inc., USA). Analysis by high performance liquid chromatography with tandem mass spectrometry was carried out with the LC-MS/MS system consisting of HPLC 1200 and Triple Quad LC/MS 6410 mass spectrometer (Agilent, USA).

Determination of maduramicin and monensin in eggs by the validated LC-MS/MS method

A total of 2 g homogenized egg white or egg yolk was weighed. The internal standard was added to all samples at a concentration of 8 µg kg^{-1} and samples were left in the dark for 10 min. Extraction was achieved by adding 2 mL acetonitrile, mixing on the vortex for a few seconds, followed by a further 15 min mixing. After centrifugation (3,000 x g, 5 min at 4°C) the supernatant was evaporated with a slow stream of nitrogen at 60°C. Residues were dissolved with 6 mL distilled water. SPE columns were activated with 6 mL methanol and 6 mL distilled water and the sample extract was applied to the column. Analytes were eluted with 10 mL methanol. Samples were further evaporated with nitrogen at 60°C followed by dissolution of residues in 250 µL methanol: water (50:50; v/v). Prior to injection in the LC-MS/MS, the final extract was filtered through 0.45 µm RC membrane filters. For quantification, eight blank samples were spiked for a 4-point matrix calibration curve (MCC). Egg samples used for MCC were previously tested for the presence of maduramicin.

Results and discussion

Using animal feed containing maduramicin and monensin at therapeutic doses, deposition and depletion time in eggs were investigated. The method used for determination of coccidiostats in eggs was validated according to Commission Decision 2002/657/EC. Low sensitivity of the method and thus compatibility for use in the depletion study is described with the decision limit (CCα) and detection capability (CCβ) (Table 2).

Table 2. Limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ) determined for eggs at the lowest validation level (C_0) and MRL (µg kg^{-1})

	LOD	LOQ	C_0	$CC\alpha_{C0}$	$CC\beta_{C0}$	MRL	$CC\alpha_{MRL}$	$CC\beta_{mrl}$
Maduramicin	0.13	0.51	0.5	0.58	0.68	12	13.9	17.0
Monensin	0.14	0.53	0.5	0.59	0.69	2	2.1	2.4

Recovery ranged from 97.7 to 104.3% for maduramicin and 97.3 to 103.5% for monensin. The coefficient of variation (CV) was highest for maduramicin with 11.3% at the lowest validation level. For each batch of the analysis, a matrix matched calibration (MCC) curve was prepared for eggs. All MCC were linear and coefficients of variation (R2) were higher than 0.98. The internal standard nigericin sodium was used to overcome the matrix suppression effect.

Eggs were sampled during and after treatment of hens with feed prepared with maduramicin at two different concentrations (5 and 1 mg kg^{-1}) and monensin (62.5 and 125 mg kg^{-1}). Eggs were divided into subsamples of egg yolk, egg white and whole egg. Results of the analysis of whole egg samples are not shown as samples were not homogenous. However, for the purpose of comparing the data with other studies, concentrations in whole egg were calculated on the basis of an egg yolk:white ratio of 34:7% as defined previously (Warren & Ball 1991). The depletion curve of maduramicin in egg yolk is shown in Figure 1. On the second day of treatment (day -13), the concentration of maduramicin in egg yolk was 13.0 µg kg^{-1} for Group I and 2.6 µg kg^{-1} for Group II, leading to a sudden increase of concentrations up to maximum values of 3,313 µg kg^{-1} and 1350 µg kg^{-1}, observed on the last day of the treatment (day -1) for Groups I and II, respectively. Ten days after treatment, the maximum concentration of maduramicin in egg white was 3.0 µg kg^{-1} for Group 1 and 0.7 µg kg^{-1} for Group II, and the highest concentration of maduramicin found in egg yolk from eggs sampled on the same day was 2,369 µg kg^{-1} from Group I. It can be concluded that even at high concentrations of maduramicin in poultry feed, residues of maduramicin in egg white could not be detected at significant values.

In the experiment with monensin (Groups III and IV), an equal distribution of monensin was observed between egg yolk and egg white. The depletion curve of monensin detected in egg yolk is shown in Figure 2. Maximal concentrations of monensin in egg yolk were observed on first day after treatment (day 1) at 29.0 µg kg^{-1} and 87.3 µg kg^{-1} for Groups III and IV, respectively. At the end of treatment, the concentration of monensin in egg white reached twice the concentration found in egg yolk (Figure 3). The maximum concentration observed in albumen was 174 µg kg^{-1}, while in egg yolk, the maximum concentration was 87.3 µg kg^{-1}. A higher accumulation of monensin in egg white can be explained by the high concentration of

monensin added to the animal feed. In a study conducted with halofuginone added to feed for laying hens, an equal distribution of halofuginone was found in egg and egg white (Yakkundi *et al*., 2002), while due to the lower polarity of molecules, similar to maduramicin, a higher deposition of dinitrocarbanilid (Cannavan *et al*., 2000) and lasalocid (Kennedy *et al*., 1996) was found in egg yolk. Non-polar molecules with high lipophilic activity will be absorbed at a lower rate, though if added to animal feed at higher concentrations, it could be also detected in albumen.

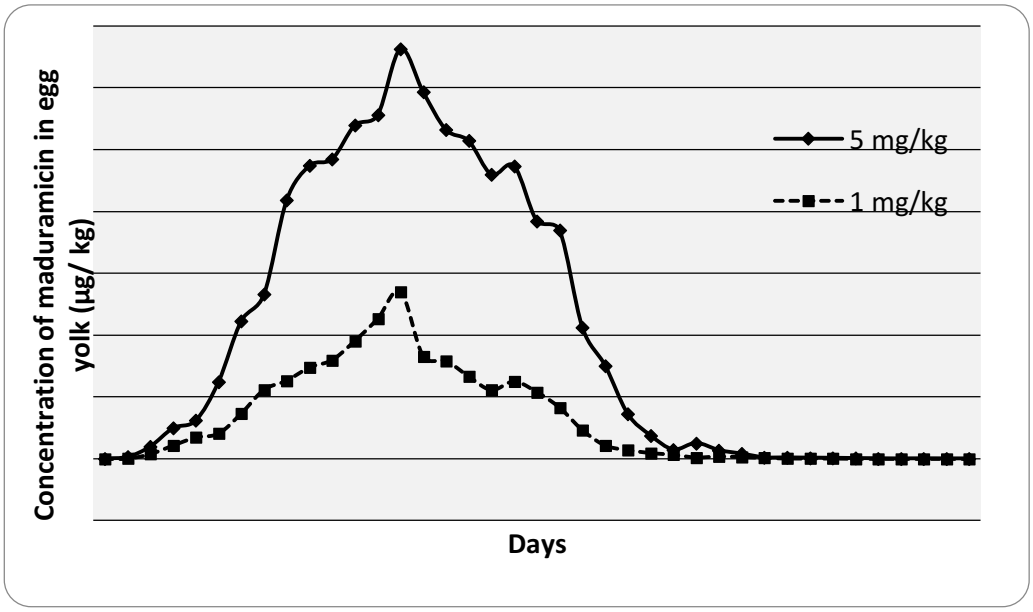

Figure 1. Accumulation and elimination of maduramicin from the yolks of Groups I and II.

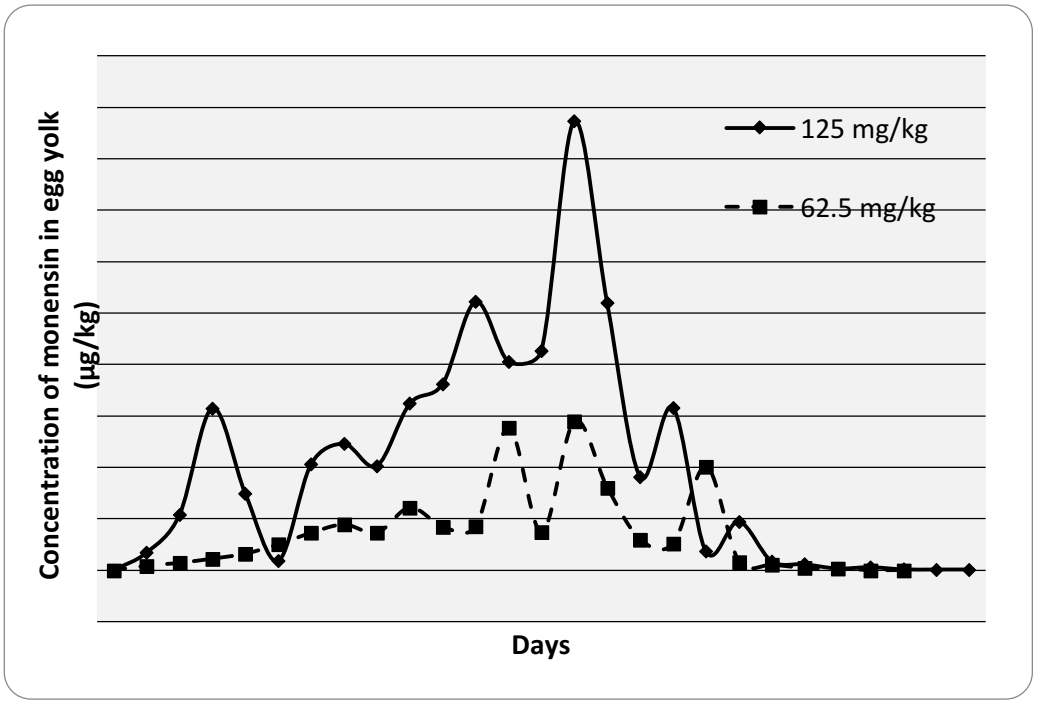

Figure 2. Accumulation and elimination of monensin from egg yolk in Groups III and IV.

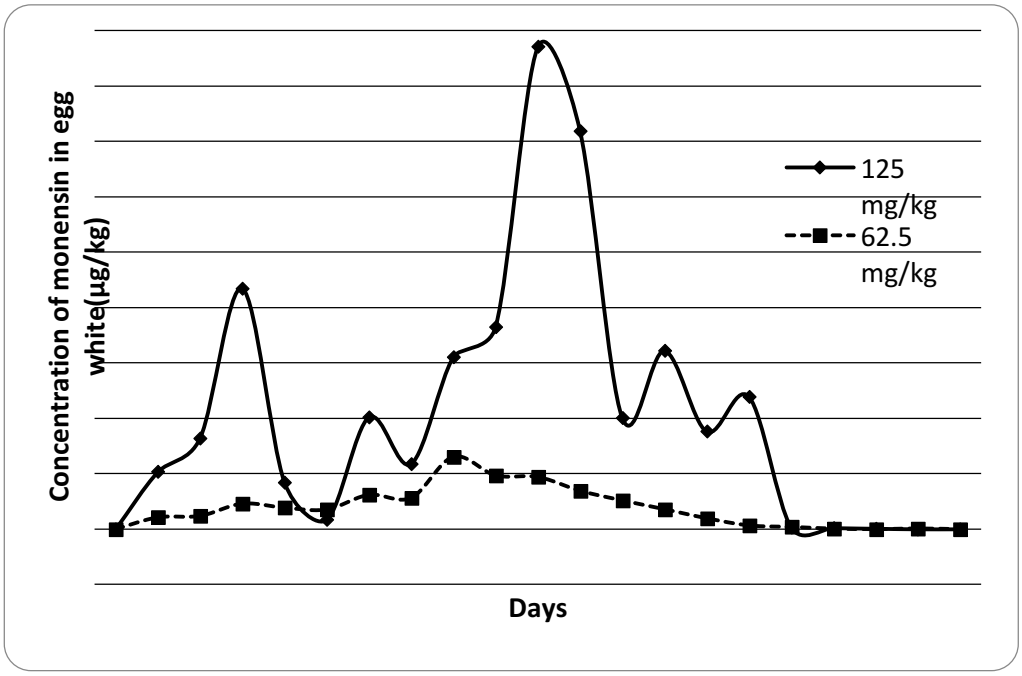

Figure 3. Accumulation and elimination of monensin from egg white of Groups III and IV.

In order to calculate the transfer factor (TF) of coccidiostats from the feed to matrix, for each of the elimination curves, average plateau values were calculated and divided by the concentration of coccidiostats in animal feed. The depletion curve for the maximum concentrations of coccidiostats in feed showed no plateau period. This is due to the non-homogeneous distribution of maduramicin and monensin in compound feed or different intakes of food by each laying hen. However, plateau values were calculated for the concentrations around the maximum achieved values. Transfer factors for different concentrations of coccidiostats in feed are given in Table 3. Transfer factors calculated for the two experimental groups with the addition of maduramicin were in the range from 0.19 to 0.36. In comparison to a previous study where lower concentrations of maduramicin were added to feed (0.05, 0.1 and 0.5 mg kg^{-1}), the transfer factors were consistent, i.e. in the range from 0.17 to 0.37 (Varenina *et al*. 2015). Experimental groups with monensin with markedly higher concentrations in feed (125 and 62.5 mg kg^{-1}) resulted in transfer factors in the range from 0.0002 to 0.0003. It can be concluded that higher concentrations of coccidiostats in feed do not affect deposition of the substance in eggs. In comparison to other deposition studies, the transfer of maduramicin is markedly higher than other investigated coccidiostats. For lasalocid, halofuginone and nicarbazin, TF values ranged from 0.051 to 0.077 (Kennedy *et al*. 1996; Yakkundi *et al*. 2002; Cannavan *et al*. 2000), while for salinomycin, monensin and narasin, they ranged from 0.0001 to 0.004 (Olejnik *et al*. 2014).

Table 3. Transfer factors (TF) from feed mixture into egg calculated for the four experimental groups (µg equivalents of coccidiostat in eggs / µg of coccidiostat in compound feed).

Coccidiostats	days	Concentration of coccidiostat in feed (mg kg^{-1})	Plateau concentration in egg yolk (µg kg^{-1})	Plateau concentration in egg white (µg kg^{-1})	Plateau concentration in whole egg (µg kg^{-1})	Transfer factor
Maduramicin	-3 to 2	5	2885	0	980	0.19
	-3 to 1	1	1066	0	362	0.36
Monensin	-2 to 2	62.5	20.0	21.5	14.3	$0.23\ 10^{-3}$
	-3 to 2	125	54.9	159	68.4	$0.55\ 10^{-3}$

Concentrations of maduramicin and monensin in eggs after treatment showed exponential decay (Figures 1 and 2), and the logarithmic function of maduramicin concentrations in eggs was linearly dependent with time. Using this linearity, the time required for the concentration of coccidiostats to decrease below the maximum permitted limits (MRLs) or below the limit of quantification (LOQ) was calculated (Figures 4 and 5).

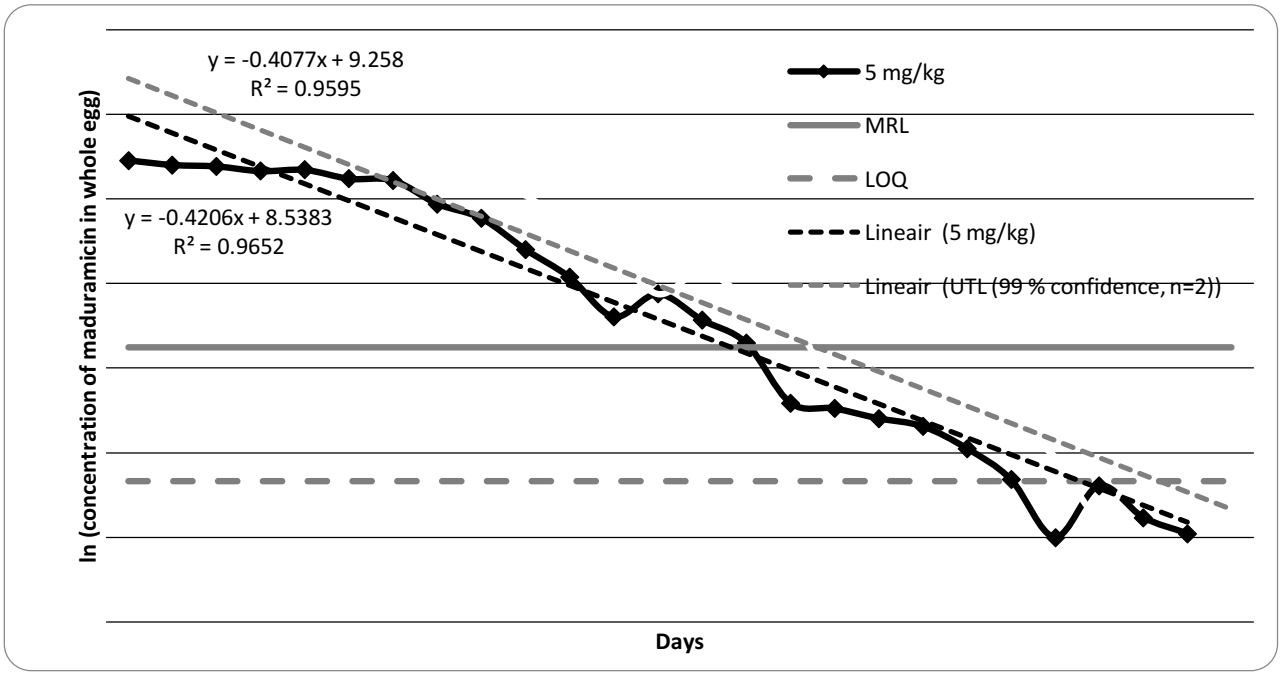

Figure 4. Depletion of maduramicin from eggs after the administration of feed containing maduramicin at 5 mg kg^{-1} (Group I).

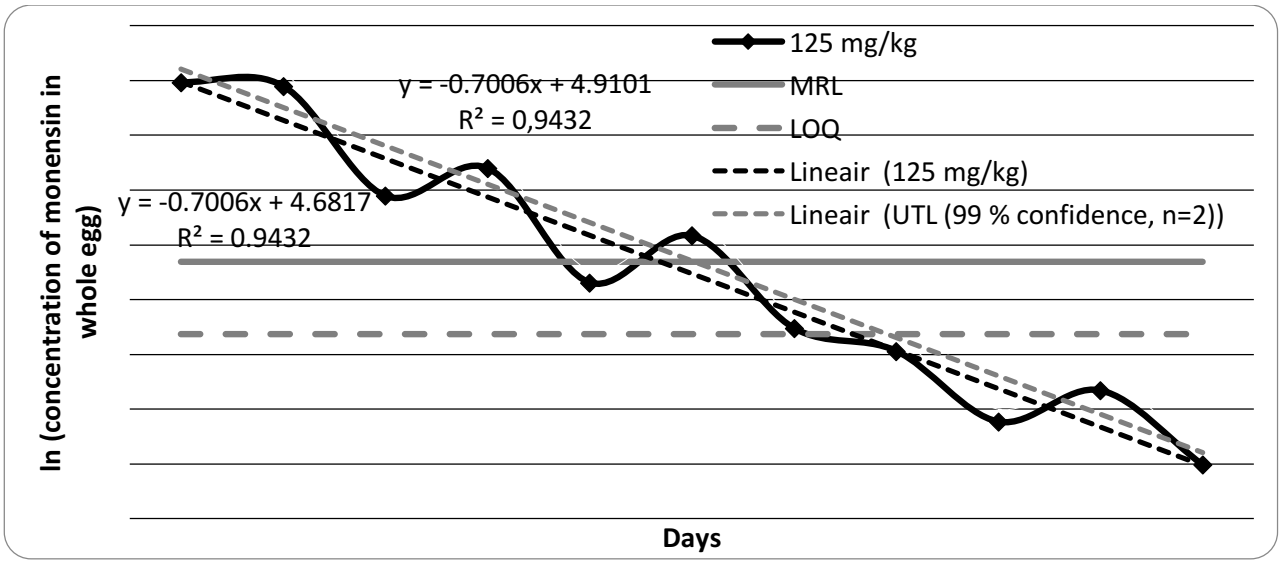

Figure 5. Depletion of monensin from eggs after the administration of feed containing monensin at 125 mg kg^{-1} (Group IV).

According to the reproducibility of the method, the linear regression of the upper tolerance limit was plotted, which served for determination of the depletion time. The t-value was determined by the probability (0.01 for a 99% confidence interval) and the degrees of freedom (n-1). After administration of maduramicin to laying hens at concentrations of 5 mg kg^{-1} and 1 mg kg^{-1} (Groups I and II), the concentration of the given coccidiostat were below MRL values for eggs 16.6 and 13.8 days after treatment, respectively (Figure 4). After the addition of monensin to feed at concentrations of 62.5 mg kg^{-1} and 125 mg kg^{-1} (Groups III and IV), concentrations of residues were below the MRL after 4.7 and 6.0 days, respectively. Concentrations of residues were below the LOQ values 24.4 and 22 days after treatment of maduramicin at concentrations of 5 mg kg^{-1} and 1 mg kg^{-1} in feed, respectively. Monensin in animal feed at concentrations 25 times higher than in experiment with maduramicin resulted in the detection of monensin residues in eggs for 6.9 and 7.9 days after the end of treatment.

Conclusions

In this experiment, maximum doses of maduramicin and monensin were added to feed for laying hens and the deposition of coccidiostats in egg yolk and egg white was examined. It was found that if the substances dominate in egg yolk, then elimination time may take more than 10 days. This can be explained by the fact that the development process of egg yolk begins in

the liver, where the first precursors of egg yolks are generated several days prior to the laying of the egg. In comparison, egg white develops only hours before laying eggs.

The absorption of chemicals from the gastrointestinal tract depends on the physico-chemical properties of the compound, such as its lipophilicity and dissociation. The assumption that lipophilicity increases the absorption of chemicals does not apply to substances with extremely high lipophilicity, which can have low bioavailability. The reason is that highly lipophilic molecules may remain in the lipid structure of the plasma membrane, and therefore are poorly soluble in gastrointestinal fluids, thus preventing contact with the mucous membrane and absorption. Low bioavailability is the main reason why the administration of monensin in high concentrations results in a short retention period in tissues and eggs. This is also supported by previous findings, where non-compliant samples of animal feed were mostly related to monensin, while the most common causes of positive samples of eggs were high concentrations of lasalocid and nicarbazin (Canavan *et al*., 2000; O'Keeffe *et al*., 2007), coccidiostats with high transfer factors. Due to the long depletion time, the incidence of non-conforming samples for lasalocid and nicarbazin in eggs is higher than for other coccidiostats. Therefore, the occurrence of residues in non-target tissues is not only affected by the degree of contamination of feed mixtures for non-target groups, but also by the pharmacokinetic properties and accumulation capability of each coccidiostat.

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PRODUCTION OF SECONDARY ANTIBODY FOR THE DEVELOPMENT OF A SCREENING METHOD FOR THE DETERMINATION OF TETRACYCLINES RESIDUES IN MILK

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Abstract

Tetracyclines (TCs) are the most commonly used antibiotics for the treatment of bacterial infection. The use of these antibiotics in animal healthcare has raised concerns as the presence of residues in food may lead to increase microbial resistance. To protect consumers, many countries have set acceptable tolerance levels for these drugs. The maximum residue level (MRL) established by European Union for tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC) in milk is 100 μg kg^{-1}. In Russia the MRL for these compounds is 10 μg kg^{-1}. Therefore, it is necessary to develop a suitable analytical technique with specificity, sensitivity and simplicity. In this study, an indirect ELISA technique was developed for the detection of TC and CTC in milk. To get immunogens there have been synthesized conjugates of TC, CTC and OTC with *bovine* serum albumin (BSA) by modified Mannich reaction. Polyclonal specific rabbit sera have been received by immunization of rabbits. Secondary diagnostic antibodies were obtained by immunization of sheep with anti-CTC, anti-OTC and anti-TC antibodies. The method was validated, in line with EU requirements (Commission Decision 2002/657/EC) concerning screening methods. The limit of detection was 0,3 μg L^{-1}.

Introduction

Tetracyclines are a broad spectrum antibiotic, and have a good activity against a variety of both Gram-positive and Gram-negative microorganisms (Cinquina *et al*. 2003). They are widely used for treatment and prevention of diseases in food-producing animals. The use of these antibiotics in animal healthcare has raised concerns as the presence of residues in food may lead to increase microbial resistance. And it causes harmful effects, such as allergic reactions, liver damage, yellowing of teeth and gastrointestinal disturbance. To protect consumers, many countries have set acceptable tolerance levels for these drugs. The maximum residue level (MRL) established by European Union for tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC) in milk is 100 μg kg^{-1} (Commission Regulation (EU) No 37/2010). In Russia the MRL for these compounds is 10 μg kg^{-1} (Decision of the Customs Union Commission No. 299). Therefore, it is necessary to develop a suitable analytical technique with specificity, sensitivity and simplicity. There are several methods for the determination of TCs in foodstuff, mainly chromatography including HPLC - MS, LC-MS. These instrumental analyses have the advantage of determining individual tetracycline analogues at highly sensitivity, but needs time-consuming sample clean-up and expensive equipment. Enzyme-Linked Immunosorbent Assay (ELISA) is an immunological technique with simplicity, sensitivity, specificity that uses an enzyme to detect the presence of an antibody or an antigen in the examined sample. This method is suitable for screening purposes. In this study, an indirect ELISA technique was developed for the detection of TC and CTC in milk. This technique will provide optimal limit of detection (LOD) and reproducibility in a complex milk matrix.

Materials and Methods

Reagents and materials

TC, CTC, OTC, doxycycline (DC), epi-OTC, epi-TC, *bovine* serum albumin (BSA), 3,3',5,5'-tetramethylbenzidine (TMB), horseradish peroxidase (HRP), sodium periodate, sodium borohydride, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade. The ELISA reader (Sunrise) was from Tecan (Austria). The 96-well polystyrene microtitre plates were from Greiner Bio One (Frickenhausen, Germany).

The following buffers and solutions were used: (1) coating buffer: 0.05 mol L^{-1} carbonate buffer (pH 9.6) containing 10 mmol l^{-1} Na_2CO_3 and 40 mmol L^{-1} $NaHCO_3$; (2) assay buffer: 5 mmol L^{-1} TRIS-buffer (TRIS, pH 7.0) containing 5 mmol TRIS-HCl, 1% BSA; (3) washing buffer (PBST): 0.01 mol L^{-1} phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween-20; (4) 0.02 M HCl; (5) substrate solution: 1.000 mL of 24 mg mL^{-1} TMB dissolved in the mixture of dimethyl formamide (DMF):water (7:3 v/v), 3.5 μL of 30% H_2O_2 and 10 mL citrate buffer; (6) stop solution: 1 mol L^{-1} sulphuric acid; (7) TCs standard solutions at concentrations of 0.2, 0.6, 1.8, 5.4, 16.2 μg L^{-1} were prepared by diluting the stock solution (1.00 mg mL^{-1}, by dissolving TC powder in methanol) with assay buffer.

Synthesis of immunogen

TC, CTC and OTC were directly linked to BSA via the Mannich reaction (Pastor-Navarro *et al*. 2007) giving the immunogens and the coating conjugates, respectively. Briefly, the corresponding tetracycline (0.1125 mmol) was dissolved in water (2 mL)

and added to a solution containing BSA (2.25 µmol) in 1.5mL water. To this solution 3 mol L^{-1} sodium acetate (1 mL, pH 5.5) and 37% (w/v) formaldehyde (0.4 mL) were added. The reaction mixture (pH 6–7) was stirred for 2 h at room temperature. The mixture was centrifuged at 10,000 rpm for 12 min and the supernatant was purified by dialysis against PBS. The immunogens were stored at −20°C.

Immunization of rabbit

Polyclonal specific rabbit sera were obtained by immunization of rabbits. Three groups of male Chinchilla rabbits were immunized intracutaneously with 300 µg of TC-BSA, OTC-BSA and CTC - BSA in 9 mL of mixed solution of physiological saline and complete Freund's adjuvant. The booster injections were carried out at 4 week intervals with immunogens in physiological saline. The concentration of immunogen was decreased with future injections up to 20 µg per rabbit. Blood samples were obtained seven days after each immunization and were assayed using the enzyme-linked immunosorbent assay (ELISA) procedure.

Production of secondary diagnostic antibodies

Secondary diagnostic antibodies were obtained by immunization of sheep with anti-CTC, anti-OTC and anti-TC antibodies. The booster injections were carried out at 4-6 weeks intervals. Blood samples were obtained seven days after each immunization and were assayed using the enzyme-linked immunosorbent assay (ELISA) procedure. The scheme of immunization is presented in Table 1.

Table 1. Scheme of sheep immunization.

Number of immunization	Immunogen	Adjuvant	Sheep anti-rabbit IgG number
1	∑Ig anti-CTC-BSA	complete Freund's adjuvant	01
2		incomplete Freund's adjuvant	02
3		incomplete Freund's adjuvant	03
4		incomplete Freund's adjuvant	04
5		incomplete Freund's adjuvant	05
6		incomplete Freund's adjuvant	06
7	Anti-sera anti-CTC-BSA, anti-TC-BSA, anti-OTC-BSA	incomplete Freund's adjuvant	07
8	∑Ig anti-CTC-BSA, anti-TC-BSA, anti-OTC-BSA	incomplete Freund's adjuvant	08

Synthesis of secondary diagnostic antibodies peroxidase conjugate (anti –rabbit IgG-HRP)

An amount of 8 mg horseradish peroxidase (HRP) was dissolved in 2 mL deionized water. Then 0.4 mL 0.1 M sodium periodate was added and the mixture was stirred for 20 min at room temperature. HRP was separated on a column of PD-10 in carbonate buffer. A portion of 16 mg isolated immunoglobulins (anti rabbit IgG) in 2 mL carbonate buffer was added. The mixture was stirred for 2 h at room temperature. A volume of 2 mL 4 mg mL^{-1} sodium borohydride in deionized water was added and the mixture was stirred again for 2 h at 4 °C. Then the conjugate (anti –rabbit IgG-HRP) was purified by dialysis against PBS.

ELISA procedure

An indirect competitive ELISA format was adopted for analysing TCs. The ELISA procedure was as follows. Coating antigen stock solution (1 mg mL^{-1}) was diluted with TRIS - buffer at the required concentration and aliquots of 150 µL per well were added to a 96-well microtitre plate. After incubation during 2.5 h at 37°C the plate was washed with PBS. Standard solutions or samples (50 µL per well) and diluted antiserum (50 µL per well) were then added and incubated for 1 h at 37°C. After washing, anti-IgG-HRP (150 µL per well) was added and the plate was incubated for 1 h at 37°C. Then the plate was washed and substrate solution (100 µL per well) was added. After incubation with shaking for about 10 min, 0,5 M sulfuric acid (100 µL per well) was added and the absorbance was measured at 450 nm using a microplate reader. A calibration curve was constructed in the form $(B/B_0)\times100$ % *vs* log*C*, where *B* and B_0 are the absorbance of the analyte at the standard point and at zero concentration of analyte respectively. The concentrations of TCs in the samples could be calculated from the standard curve run in the same microtitre plate.

Preparation of milk samples

Milk samples were purchased from a local market. Semi-skimmed and full fat milk samples were centrifuged at 4,000 g for 10 min at 4°C. The lower layer was used directly in ELISA.

Cross-reactivity study

A cross-reactivity study was performed using DC, OTC, CTC and epi-form of TCs which are structurally similar to TC. Study was accomplished in the same manner as the studies performed for TC assay.

Results and discussion

Since TCs are low molecular weight molecules, containing only one antigen determinant, a competitive indirect immunoassay format was performed. In the competitive scheme employed in this work, TCs antibiotics competed with TC-BSA immobilized on microtitre plates for a limited number of the antigen binding sites of the capture antibodies. Secondary diagnostic antibody conjugated with HPR was applied for the determination of bounded antibody. The main aim of this work was to produce secondary diagnostic antibody.

In this work, anti-CTC, anti-TC and anti-OTC antisera were used for the production of a secondary antibody coupled to HRP. Rabbit antisera or rabbit IgG injected during an eight cycle of immunization every 4-6 weeks (Table 1). Antisera samples were obtained 14 days after each immunization. Then IgG fraction was isolated and conjugated with HRP. Then every anti-rabbit-IgG-HRP conjugate was tested using the enzyme-linked immunosorbent assay (ELISA) procedure.

The IC_{50} for TC and CTC depended on cycle of immunization and specificity of immunogen (Table 2). Anti-rabbit-IgG-HRP conjugates number 01-06 were obtained with anti-CTC-BSA IgG. The assay showed strong specificity for CTC. The IC_{50}s ranged from 100-1,000 μg L^{-1}. The anti-rabbit-IgG-HRP conjugate 07 was produced with sum of anti-sera anti-CTC-BSA, anti-TC-BSA, anti-OTC-BSA. The sensitivity was increased and IC_{50}s for TC and CTC were 20 and 5 μg L^{-1}, respectively.

Table 2. The scheme of sheep immunization

Number of sheep anti-rabbit IgG -HRP	IC_{50}, μg L^{-1}	
	TC	CTC
06	200	20
07	20	5
08	3.9	3.7

The anti-rabbit-IgG-HRP conjugate 08 showed the highest sensitivity and a broad specificity for TCs. The limit of detection was 0.3 μg L^{-1}. The specificity of the assay was evaluated via the cross-reactivity (CR) of the anti-TC antibody with structurally related TC (CTC, DC, OTC, epi-TC, epi-OTC). The coefficients of cross-reactivity were presented in Table 3. Typical calibration curve for TC constructed with the anti-rabbit-IgG-HRP 08 was presented in Figure 1.

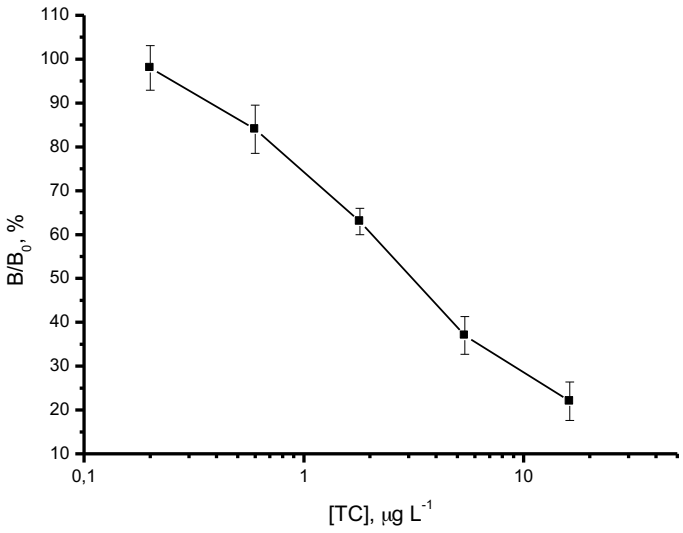

Figure 1. Typical calibration curve for TC constructed with the anti-rabbit-IgG-HRP 08.

The optimized ELISA was applied for milk analysis. Firstly, milk samples were defatted and then skimmed milk directly used in analysis. For the evaluation of analysis, five milk samples were fortified at three concentrations, namely at 1, 5 and 10 μg·L^{-1}. The recoveries ranged from 90 to 130%, 82 to 97% and 84 to 125%, respectively. The method was validated, in line with EU requirements (Commission Decision 2002/657/EC) concerning screening methods. The CCβ was 0.5 μg L^{-1}.

Table 3. Cross-reactivity.

TCs	CR (%)
tetracycline	100
chlortetracycline	125
epi-oxytetracycline	29
doxytetracycline	15
oxytetracycline	5
epi-tetracyline	<1

Conclusions

In this study the indirect competitive ELISA was developed and applied for the determination of TCs in milk. The Limit of detection was 0.3 $\mu g \cdot L^{-1}$ which is less than set by Russian Federation for the detection of a sum of TCs.

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VETERINARY DRUG ANALYSIS IN ANIMAL ORIGIN FOOD AND FEED AND THEIR RELEVANT PRODUCTS: A MODERN MULTI-CLASS, MULTI-RESIDUE METHOD USING UHPLC-MS/MS

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Abstract

A multi-class, multi-residue method based on UHPLC-MS/MS has been developed for the analysis of around 150 compounds belonging to a variety of veterinary drug classes: anthelmintics, antibiotics (aminoglycoside, amphenicols, β-lactams -penicillins and cephalosporins, lincosamides, macrolides, quinolones, sulfonamides, tetracyclines, and others), antimicrobial growth-promoters, anti-protozoals, β-agonists, coccidiostats, dyes, other-no class, pesticides, and tranquilizers. The method development and optimization was divided into five main phases: (*i*) MS/MS conditions for individual compounds; (*ii*) LC conditions; (*iii*) final LC-MS/MS method; (*iv*) sample preparation procedure; and (*v*) method validation, data acceptance criteria, and method implementation. Particular attention was devoted to mobile phase composition optimization and to comparison of different sample preparation approaches. Different concentrations of formic acid in the aqueous mobile phase or different ratios of acetonitrile and methanol in the organic mobile phase were evaluated to achieve a well-distributed elution profile and minimum analyte interferences. The sample preparation optimization was divided into three stages: (*i*) extraction procedure; (*ii*) different clean-up options (such as hexane defatting, dispersive SPE clean-up, or supported liquid extraction); and (*iii*) establishment of the sample extract dilution scheme.

The final method consists of dispersing the sample in water with an EDTA buffer, followed by extraction with 0.1% formic acid in acetonitrile, drying down an aliquot of the extract, and reconstituting it in a mixture of water and acetonitrile. The analytical determination is performed by UHPLC-MS/MS using positive electrospray ionization mode. Depending on the purpose (screening or accurate quantitation) of the analysis, the analyte concentrations in the samples can be reported without or with compensation for any potential matrix effects and recovery losses. In the former case, the analyte concentrations are determined using an external calibration based on standards prepared in solvent. In the latter case, when compensation for matrix effects and recovery losses is required for highly accurate quantification, the method of standard addition or extracted matrix-matched calibration is employed. The method was validated, and the results showed that the method was sensitive, selective and reliable for multi-class, multi-residue veterinary drug analysis.

Introduction

Veterinary drugs are a complex group of different chemical classes and therapeutic agents. They are used within animal husbandry to treat and prevent disease and ensure animal health and growth. Residues of such drugs in animal edible tissues are not desirable because they could pose a potential threat to consumer health and promote antibiotic resistant bacteria strains. Therefore, these substances are strictly regulated and monitored in food products to ensure food safety and prevent the unnecessary exposure of consumers to veterinary drugs. For that purpose, multi-class, multi-residue methods are becoming increasingly popular in regulatory monitoring programs globally owing to their extended analytical scope and laboratory efficiency [1-2].

Development of any large multi-class, multi-residue detection method poses significant challenges, including a large number of analytes; co-existence of parent drugs and metabolites; different physical/chemical properties ranging from hydrophilic to hydrophobic and acidic, neutral to basic; analyte stability and interaction with matrix components; compromise between analytical scope and performance characteristics; matrix effects and potential interference from co-extractives. In this study, a modern large scale method based on ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) was developed and validated to provide screening, identification and quantification of approximately 150 compounds from 10 veterinary drug classes, including 9 antibiotic sub-classes. The method provides the benefits of cost- and time- effective analysis of a large number of important veterinary drugs, selective detection of individual analytes, improved sensitivity for low limit of detections (LODs)/limit of quantifications (LOQs), and high level identification/confirmation confidence.

Materials and Methods

Reagents and Standards

Acetonitrile, methanol, water and formic acid (99%) were LC-MS grade and obtained from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Ethylendiaminetetraacetic acid (EDTA) disodium salt was ACS grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

The veterinary drug reference standards were acquired from various vendors and were of the highest available purity. The individual analyte and internal standard stock solutions were made at concentrations of 1,000 to 2,000 µg mL^{-1}. The analytes

were divided based on their classes into nine different groups (A to I) listed in Table 1. The analyte group composite stock solutions were prepared at 40 to 100 µg mL^{-1}. The internal standard composite stock solution, including ten isotopically labelled compounds representing different veterinary drug classes, was made at 1 to 20 µg mL^{-1}. All stock solutions were stored at -20°C. The analyte spiking solutions were prepared at different concentration levels by mixing appropriate volume of each of the 40 to100 µg mL^{-1} composite stock solutions with 75: 25 water-acetonitrile (*v*/*v*). The internal standard spiking solution was made by diluting the internal standard composite stock solution with 75: 25 water-acetonitrile (*v*/*v*). In this study, three sets of standards (extracted matrix-matched standards, post-extracted matrix matched standards, and solvent working standards) were prepared fresh daily. All three types of standards were made with the same range of analyte concentrations as matrix equivalence (0.5, 1, 5, 10, 50 and 100 ng g^{-1}).

Table 1. Analyte groups.

Group (# of analytes)	Veterinary drug classes
Mix A (22)	Anthelmintics
Mix B (20)	Antibiotics – β-lactams (cephalosporins and penicillins)
Mix C (13	Antibiotics - Macrolides and lincosamides
Mix D (23)	Antibiotics - Quinolones and others
Mix E (24)	Antibiotics - Sulfonamides
Mix F (9)	Antibiotics - Tetracyclines
Mix G (22)	β-agonists, coccidiostat, and antimicrobial growth promoter
Mix H (12)	
Mix I (4)	Tranquilizers, dyes and pesticides
	Antibiotics - Amphenicol, aminoglycoside, and other

LC-MS/MS Analysis

The UHPLC-MS/MS analysis was performed with an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity binary solvent delivery system and autosampler. The UHPLC system was coupled with an Agilent QQQ 6495 triple quadrupole mass spectrometer equipped with an Agilent Jet Stream electrospray ionization source and iFunnel technology. The instrument control, data acquisition and analysis were performed with Agilent MassHunter software.

Chromatographic separation was performed using an Agilent ZORBAX RRHD Eclipse Plus C18 column (100 x 2.1 mm, 1.8 µm particle size) with an Agilent Eclipse Plus C18 guard column (5 x 2.1 mm, 1.8 µm particle size). Mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in methanol, respectively. The following gradient elution program (mobile phase A and B) was used: 0-0.75 min: 2% B; 0.75-7 min: 2-40% B; 7-11 min: 40-100% B; 11-14.5 min: 100% B; 14.6-17.5 min: 2% B. Flow rate was 0.5 mL min^{-1}. The column was maintained at 40°C, and the autosampler was at 5°C. The injection volume was 5 µL.

The dynamic MS/MS acquisition was carried out using ESI in positive mode. The MS parameters were optimized and set as follows: drying gas - N_2 (250 °C, 12 L min^{-1}); nebulizer gas - N_2 (60 psi); sheath gas - N_2 (350°C, 10 L min^{-1}); capillary voltage – 4,000 V; nozzle voltage – 500 V; positive high pressure RF - 75 V; positive low pressure RF - 60 V. Three MS/MS (multiple reaction monitoring, MRM) transitions of each analyte were chosen with optimized collision energy (CE) and cell accelerator voltage (CAV) parameters.

Sample Preparation

Sample (1 g) was weighed into a 50-mL disposable centrifuge tube. To prepare fortified samples and extracted matrix-matched standards, blank samples were fortified with 25 µL of appropriate analyte spiking solutions and 25 µL of the internal standard spiking solution and incubated for 15 min. Extraction solvent A (10 mL 0.05M EDTA in water) was added and vortexed briefly until the sample was homogeneous. Extraction solvent B (10 mL 0.1% formic acid in acetonitrile) was added and vortexed and then shaken for 15 min. Sample was centrifuged at 2,000 rcf for 5 min. A 2-mL aliquot of the supernatant was transferred to a 15-mL centrifuge tube for evaporation. The supernatant was evaporated to dryness at 40°C under a flow of N_2. The extract was then reconstituted with 1 mL of 75:25 water-acetonitrile (*v*/*v*). At this point, post-extraction matrix-matched standards were fortified with 25 µL of appropriate analyte spiking solutions and 25 µL of the internal standard spiking solution plus 950 µL of the dilution solution. The sample was mixed thoroughly and then transferred to a micro-centrifuge tube, centrifuged at 15,000 rcf for 5 min and filtered (0.2 µm PTFE) into an autosampler vial for LC-MS/MS analysis.

Method Validation

Infant formula powder was selected as a representative matrix for the initial method validation due to its high complexity. Method performance was evaluated by analysing a representative infant formula powder sample in duplicate (as a matrix

blank) together with five replicates of spikes at 0.5, 1, 5, and 10 ng g^{-1} on Day 1 and with five replicates of spikes at 1, 5, 10, 50, and 100 ng g^{-1} on Day 2. The quantitation was performed both using extracted matrix calibration curve (prepared pre-extraction by spiking standards into the blank sample matrix) and matrix-matched calibration curve (prepared post-extraction). The analysis was conducted by two different analysts on Day 1 and Day 2.

Results and Discussion

LC-MS/MS Analysis

Agilent MassHunter Optimizer was used for MRM optimization, which include generation of product ion scans, selection of MRMs (up to 10 for each compound); and selection of the optimum collision energy for each MRM. The dynamic MRM (dMRM) acquisition method was further optimized including ESI source conditions, cell accelerator voltage, and evaluating the MRMs for sensitivity and selectivity to choose the best three MRMs for the method. The selectivity of the MRMs was verified in infant formula powder using the final sample preparation procedure to make sure that the selected MRMs do not have any matrix interferences from closely eluting matrix components, which would affect accurate quantitation and/or analyte identification.

Mobile phase composition and gradient are highly important to achieve good ionization efficiency and high sensitivity, reduce potential interferences between compounds, and separate compounds that share the same precursor and even product ions (critical pairs/groups of analytes). In this study, we evaluated different organic mobile phase compositions, including comparison of 0.1% formic acid in acetonitrile, 0.1% formic acid in methanol and 0.1% formic acid in 1:1 acetonitrile-methanol (*v*/*v*) as organic mobile phases (mobile phase B). The best overall sensitivity and separation of critical pairs/groups of analytes was achieved using 0.1% formic acid in methanol. In addition, we also tested different buffers, such as 0.1% formic acid, 0.3% formic acid or 10 mM ammonium formate in both mobile phase A and B. The use of 0.1% formic acid provided the best sensitivity and separation selectivity results. Furthermore, the mobile phase gradient was optimized to achieve optimum chromatographic separation and peak shape. Figure 1 shows a typical extracted ion chromatogram of an infant formula powder sample spiked at 100 ng g^{-1} with all the analytes.

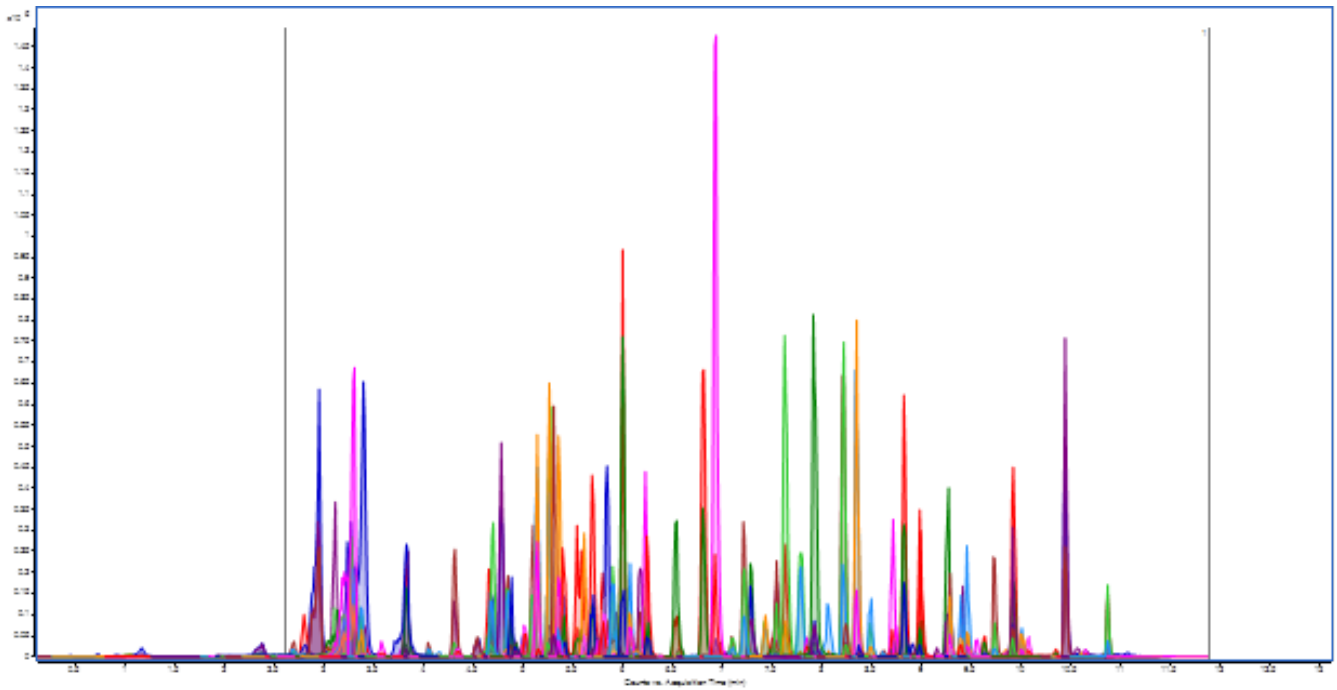

Figure 1. An LC-MS/MS extracted ion chromatogram of an infant formula powder sample spiked at 100 ng g^{-1} with all analytes (equivalent to 10 ng mL^{-1} in the final extract).

Sample Preparation

Infant formula powder was chosen as the initial matrix in the method development process due to its high complexity (high protein, fat, and carbohydrate content, and many additives, including metals) and strict requirements for LOQ. Two major sample preparation steps were evaluated: sample extraction and sample clean up.

Acetonitrile and water mixtures (75:25 and 50:50 acetonitrile-water, *v*/*v*) were investigated in detail because this solvent combination is frequently used in the extraction step in veterinary drug residue methods. Acetonitrile has the advantage of

precipitating unwanted proteins. Different ratio of acetonitrile and water yielded different absolute recoveries for some compounds. The 75:25 acetonitrile-water (*v*/*v*) aqueous extraction solution was capable of precipitating a larger fraction of protein and improved the recoveries for some compounds but rendered lower recoveries (<50%) for β-lactams, which is a highly important antibiotic class. Increasing the water content to 50:50 acetonitrile-water (*v*/*v*) was necessary to improve the recoveries of β-lactams. Furthermore, increasing the water ratio in the extraction solvent prevents extensive co-extraction of lipids and phospholipids. The 50:50 acetonitrile-water (*v*/*v*) mixture was selected as the extraction solvent. For infant formula powder, the sample is first homogenized with water, followed by the addition of acetonitrile. We added 0.1% formic acid to the acetonitrile extraction solvent to further improve recoveries of certain analytes. Also, to prevent chelation of tetracyclines and quinolones with metals, we added EDTA (0.05 M) to the water used for the initial sample homogenization, which resulted in significantly increased recoveries of these two analyte classes.

Several clean-up procedures were assessed because co-extracted matrix components could reduce column lifetime, cause more frequent instrument maintenance, or potentially interfere with qualitative and quantitative analysis. To evaluate the clean-up efficiency, the sample extract was divided into aliquots and then subjected to various procedures, including no clean-up, Enhanced Matrix Removal (EMR sorbent for lipid removal in dispersive SPE format), supported liquid extraction (SLE) for removal of proteins, phospholipids and salts , C18 in a SPE cartridge format for removal of lipids and other less polar compounds, PLD+ (96 well plate format for phospholipid removal) and hexane defatting plus C18 cartridge SPE for removal of lipids and other less polar compounds. Three factors were utilized to evaluate the clean-up efficiency: recovery and precision; matrix co-extractive removal efficiency by a gravimetric test; matrix suppression/enhancement evaluation using post-column infusion of veterinary drug standards into the final extract with and without applying the clean up procedures. From gravimetric test and post-column infusion, EMR and SLE provided the best co-extractive removal efficiency. The comparison of recoveries and precisions from sample preparation procedure without clean up and with clean up is provided in Table 2. Considering the lower recoveries observed for some critical compounds after applying the various clean-up procedures, these steps were omitted from the final method.

Table 2. Sample clean up option comparison on recovery (at spiked level of 100 ng g^{-1} in sample) and CV% (n =3).

Procedure	Recovery for critical compounds (*e.g.* tetracyclines, β-lactams)	CV%
No clean up	As control	All within 20%
EMR (dSPE): enhanced matrix removal-lipid	Negatively impacted	30% analytes having CV% > 20%
SLE: supported liquid extraction for proteins, phospholipids and salts removal	Negatively impacted	40% analytes having CV% > 20%
C18 SPE cartridge format: removal of lipids and other less polar compounds	Negatively impacted on several critical compounds and dyes	10% analytes having CV% > 20%
PLD+: 96 well plate format for phospholipid removal	Negatively impacted on several critical compounds and dyes	10% analytes having CV% > 20%
Hexane defat + C18 cartridge SPE: removal of lipids and other less polar compounds	Negatively impacted on several critical compounds and dyes	15% analytes having CV% > 20%

Validation

Specificity was demonstrated by monitoring multiple MS/MS transitions along with the evaluation of their signal ratios, which allows distinguishing of the target analyte from potential interferences.

To test the linearity of the method and compare quantification results, three sets of standards (extracted matrix matched standards, post-extracted matrix matched standards, and standards in solvent) were prepared [3]. All three types of standards were made with the same range of analyte concentrations equivalent to 0.5, 1, 5, 10, 50 and 100 ng g^{-1} in the infant formula powder sample. Solvent-based calibration standards were used to monitor matrix effects. Post-extraction matrix-matched calibration was employed to determine absolute analyte recoveries. The extracted matrix curve was used to mimic the standard addition procedure that will be employed for routine quantitation of potential veterinary drug residues in the samples. The coefficient of determination (r^2) values and linear range (LR) for the extracted matrix curves from the two days of analysis were determined by using a linear calibration with 1/x weighting factor. The r^2 values were >0.99 for the majority of analytes ranging from 1 (or 5 or 10) to 100 ng g^{-1}.

The accuracy, precision and intermediate precision results were obtained by two different analysts on two different days analysis. The accuracy results include absolute spike recoveries calculated using post-extraction matrix-matched calibration curves and corrected spike recoveries calculated using matrix extracted calibration curves. The latter calibration approach is frequently employed in the veterinary drug analysis field. It mimics the standard addition procedure and provides highly ac-

curate results by compensating for both matrix effects and potential recovery losses. Ten labelled internal standards, representing different veterinary drug groups/classes, are used in the method to monitor routine performance but are not used for response normalization. The precision was evaluated by analysis of six fortification levels of infant formula in five replicates. The intermediate precision of the method was investigated at three fortification levels of 1 ng g^{-1}, 5 ng g^{-1} and 10 ng g^{-1} in five replicates on two different days. Acceptable analyte recoveries (within the 70-130% range) and CVs (≤30%) were obtained for all analytes at and above their LOQs, except for 4-epidemeclocycline, decoquinate, diminazine, and colistin A and B, which were excluded from the final method used for the routine analysis.

The method validated LOQ (the reporting limit) was determined for each analyte as the lowest spiking level that met the validation criteria for recoveries and CVs. The typical analyte reporting limits in infant formula powder are between 1 and 10 ng g^{-1}.

Conclusions

A modern multi-class, multi-residue method for detection, identification and quantification of veterinary drugs using LC-MS/MS was developed and validated in infant formula powder. The method currently covers approximately 150 analytes belonging to a variety of veterinary drug classes. Ten MRMs were selected and optimized for each individual analyte. Collision energy and cell accelerator voltage were optimized for each MRM of each analyte. Three MRMs from each analyte with the highest intensity and the best selectivity were chosen to establish the dynamic MRM acquisition method. LC mobile phase composition and gradient program were optimized to achieve a well-distributed elution profile and minimum analyte interferences in the sample matrix. Sample extraction conditions and clean-up options were evaluated to determine the best approach providing a reasonable balance between the efficient sample preparation and acceptable recovery and precision. Lower recoveries were observed for certain compounds after applying various clean-up procedures, including EMR, SLE, C18 SPE (Quick QuEChERS cartridge format) and PLD+. Therefore, the clean-up step was omitted from final method. The proposed method was validated in infant formula powder and quantified with extracted matrix-matched standards, showing satisfactory validation results, including identification, selectivity, matrix effects, linearity, LOQs, accuracy and precision. Moreover, this method can be used in routine analysis for the simultaneous detection and quantification of a large number of veterinary drug residues in infant formula. In the future, the method will also be validated for screening/quantification of veterinary drugs in other relevant matrices, such as dairy products, seafood, meat, eggs, honey, pet food and animal feed.

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DEVELOPMENT AND VALIDATION OF A CONFIRMATORY METHOD FOR THE DETERMINATION OF CORTICOSTEROIDS IN MEAT, MILK AND LIVER

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Abstract

In accordance with the Russian Federation regulations the residues of corticosteroids are not allowed in food products. Therefore, the monitoring of these residues is necessary to ensure that there is no abuse of hormonally active substances. The aim of this study was to develop a rapid and sensitive method for the analysis of dexamethasone, prednisolone, methylprednisolone, and triamcinolone acetonide in meat, milk and liver using LC-MS/MS. A binary solvent delivery system (Eksigent UltraLC-100, Eksigent, USA) and a triple quadrupole mass spectrometer (QTRAP 5500, Sciex, Canada) were used. Separation of analytes was carried out on a Pursuit 3 C18 column (150 mm× 2.1 mm, Agilent). The sample preparation includes liquid-liquid extraction with TBME and defatting with hexane. The method was validated in the range of 0.1 – 30 µg kg^{-1} for all analytes. The recovery lies in the range of 75 – 110%, the combined uncertainty lies below 40% for all analytes. Thus, a rapid LC-ESI-MS/MS method for the determination of four corticosteroids in meat, milk and liver has been developed and validated.

Introduction

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex of vertebrates, as well as the synthetic analogues of these hormones. Corticosteroids are involved in a wide range of physiological processes, including stress response and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behaviour. Because of their anti-inflammatory properties, chemical synthesis of many corticosteroids was investigated with applications in human and veterinary medicine. Besides their therapeutic use, these compounds are capable to increase weight gain and reduce feed conversion ratio, and they have a synergetic effect when combined with other molecules like ß-agonists or anabolic steroids. Thus, corticosteroids could be illegally used as growth promoters in cattle, administered through livestock food or by injection. Corticosteroid residues can cause metabolic disorders and vision disturbances, diseases of neural and blood systems. Some corticosteroids are allowed for usage in EU, and their MRL's are established. But in accordance with the Russian Federation regulations the residues of corticosteroids are not allowed in food products at all. Consequently, control is demanded to survey the misuse of corticosteroids and to assure the food safety.

Materials and Methods

Standards

Dexamethasone, prednisolone, methylprednisolone, and triamcinolone acetonide were purchased from Sigma (MO, USA). Triamcinolone acetonide-d6 was obtained from TRC (Canada).

Instrumentation

The HPLC system was Eksigent ekspert ultraLC-100 System (Sciex, Canada), consisted of a binary pump, column compartment and autosampler. Separation of analytes was carried out on a Pursuit 3 C18 column (150 mm×2.1 mm, Agilent). The hybrid triple quadrupole\linear ion trap mass spectrometer 5500 QTRAP (Sciex, Canada) was coupled to HPLC using an electrospray ionization interface in negative ionization mode (ESI-). Data acquisition was conducted with help of Analyst 1.6.1 software (Sciex, Canada). Parameters for multiple reaction monitoring (MRM) transitions are presented in Table 1.

The injection volume was 20 µL and the analysis was carried out with gradient elution using solvent A (deionised water) and solvent B (methanol) at a flow rate 0.15 mL min^{-1}. The gradient was 80% of solvent A at start with linear decrease to 30% of A at 20th min of analysis. Then the portion of solvent A was reduced to 22% in the next 10 min. At 30.1 min solvent A was increased to 80% and was not changed during the last 10 min. Total run time was 40 min. The ESI/MS/MS conditions were such as: Ionspray Voltage (IS) -4,500 V and collision gas (CAD) 6 psi, GAS1 50 psi, GAS2 30 psi. The heater gas temperature (TEM) was set to 500°C. MS/MS product ions were produced by collisionally activated dissociation (CAD) of selected precursor ions in the collision cell of mass spectrometer. MRM experiments were performed using a dwell time 50 ms and compound-dependent collision energies (shown in Table 1). Two transitions were used for the identification of each analyte but only most intense one was used for quantification.

Table 1. MRM transition parameters

№	Analyte	Precursor ion (*m/z*)	Transition products (*m/z*)	Retention time (min).	Collision energy (V)/ Collision cell exit potential (V)
1	Prednisolone	405.2 (-)	295.1\280.1	18.0	-45/-20, -50/-20
2	Methylprednisolone	419.2 (-)	343.3\309.2	20.5	-23/-20, -43/-20
3	Dexamethasone	437.1 (-)	361.2\345.2	20.1	-24/-20, -34/-20
4	Triamcinolone acetonide	479.2 (-)	413.2\337.2	20.4	-26/-20, -32/-20
5	Triamcinolone acetonide-D6	485.3 (-)	337.2	20.5	-65/-20

Sample preparation

Samples (meat, milk, liver) were homogenized and 5 g of each sample was weighed into 50 mL plastic tube. Samples were fortified by addition of 50 uL of internal standard (triamcinolone acetonide-d6, concentration 1 µg mL^{-1}). Tubes were vortexed during 1-2 min and left for 10 min in a dark place at room temperature. Then 10 mL of acetic buffer pH 5.2 were added and sample was shaken for 10 min. A volume of 15 mL of tert-methylbutyric ether was added into sample tube. Sample was shaken for 20 min and then centrifuged for 20 min at 15,000 rpm at 4°C. Upper layer was transferred into 50 mL plastic tube and the liquid evaporated on a Pierce Reacti-Vap 3 System at a temperature below 40°C. Then the sample was reconstituted in 1 mL of a mixture of water-methanol in a ratio 1:4. The tube was placed on a ultrasonic bath for 1 min and shaken on vortex-type shaker for 1 min. A volume of 3 mL of hexane was added to the sample followed by vortex-mixing. Then it was centrifuged again for 20 min at 15,000 rpm at 4°C. The hexane layer was discarded and the rest of the sample was filtered through a membrane filter and transferred into a 2-mL glass vial for LC-MS/MS analysis.

Validation

The method was validated for beef according to RMG 61-2010 "State system for ensuring the uniformity of measurements. Accuracy, trueness and precision measures of the procedures for quantitative chemical analysis. Methods of evaluation". The whole validation study consisted of four individual experiments with two factors: operator and storage time before LC-MS/MS analysis. The same sample was used for all experiments. The validation levels were 0.1, 0.5, 2, 10, 30 ng mL^{-1} for dexamethasone, prednisolone, methylprednisolone and 2, 6, 12, 20, 30 ng mL^{-1} for triamcinolone acetonide. Quantification was performed using individual matrix calibration curve for each series with same concentration levels for each analyte. In order to prove the specificity and the lack of susceptibility to matrix interferences, several blank samples fortified with internal standard were additionally analysed in each series. The in-house validation study was performed within 4 weeks.

The specificity of the method was demonstrated as no interfering peaks were observed at the retention time of dexamethasone, prednisolone, methylprednisolone, and triamcinolone acetonide in a variety of blanks and by analysing structurally related compounds like the labelled internal standard which can be separated from the analytes. The recovery, corrected by matrix calibration and the use of internal standards, lies in the range of 75 – 110% for all analytes in the validated concentration range and are given in Table 2. Measurement range and expanded uncertainty are given in Table 3.

Table 2. Recoveries for corticosteroids.

Analyte	Recovery (%)
Prednisolone	78
Methylprednisolone	75
Dexamethasone	81
Triamcinolone acetonide	87

Table 3. Validation parameters.

Analyte	Measurement range (ng mL^{-1})	Expanded uncertainty (%)
Prednisolone	0.5-30	65
Methylprednisolone	0.5-8; 8-30	46; 54
Dexamethasone	0.5-30	27
Triamcinolone acetonide	2-30	21

Conclusion

A fast and reliable LC-ESI-MS/MS multi-residue method allowing simultaneous determination of four corticosteroids in meat was developed and validated. The validation was carried out according to national guide RMG 61-2010.

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INVESTIGATION OF CORTICOSTEROIDS PROFILES IN *BOVINE* URINE: PART A. DEVELOPMENT OF A METHOD FOR CORTISOL, PREDNISOLONE AND THEIR METABOLITES DETERMINATION

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Abstract
Prednisolone is a corticosteroid widely used for therapeutic purposes both in human and veterinary medicine. Corticosteroids are often fraudulently administered to meat-producing animals because of their grow-promoting effects. Their use outside therapeutic prescription is banned by the European legislation. Until a few years ago, prednisolone was considered exclusively a synthetic molecule, but nowadays many studies have shown that it may be endogenous too. Several independent research groups have obtained experimental evidence confirming the hypothesis of a possible biosynthesis of prednisolone in *bovine*, under specific stressful conditions. A debate on the possibility to distinguish endogenous from therapeutic prednisolone administration is underway. In order to try to solve this issue, we developed an accurate and sensitive method to map 22 molecules correlated to prednisolone and cortisol metabolism in *bovine* urine in this study (Part A). Samples (5 mL) were hydrolysed with glucuronidase/arylsulfatase and purified using solid phase extraction (SPE). The instrumental determination was performed by LC-MS/MS, using electrospray ionization in negative mode (ESI-). Our following goal will be to investigate through the developed method, using multivariate statistics, the alteration of endogenous pattern in case of stress or pharmacological treatment and therefore to obtain a powerful instrument to detect illicit operations.

Introduction
Prednisolone is a synthetic corticosteroid with anti-inflammatory and gluconeogenetic activities. Corticosteroids are used for the treatment of inflammatory diseases. For this reason, Commission Regulation 2010/37/EC established maximum residue levels (MRLs) for corticosteroids in liver and muscle. In urine no MRLs have been set and therefore their presence at any concentration is not allowed. On the other hand, corticosteroids are known to increase weight gain, to reduce the feed conversion ratio and to have a synergic effect with other anabolic agents like sex steroids and β-agonists. In EU legislation, their use as growth-promoters in livestock breeding is forbidden.

In recent years, prednisolone was frequently observed at concentrations < 5 μg L^{-1} in *bovine* urine, in particular in samples collected at the slaughterhouse, but also at farms. In all cases, no therapeutic use was declared in advance by the responsible veterinarian (Sterk *et al*. 2012; Leporati *et al*. 2012, Dusi *et al*. 2011).

Recent studies have confirmed the hypothesis that prednisolone can be formed *in vivo* from endogenous cortisol in animals subjected to conditions of acute (*e.g*. transport and slaughter), or chronic (*e.g*. chronic diseases, inflammation, ill-treatment) stress (Arioli *et al*. 2010; Sterk *et al*. 2012; Dusi *et al*. 2012). In 2012, the Italian Ministry of Health has set the limit value for not compliant results at 5 μg L^{-1}. This provision should be temporary, pending on the identification of biomarkers reliable of exogenous origin of prednisolone. Thus, the interest for pattern recognition of corticosteroid metabolites profiles increased. A study on the metabolites could be a promising approach. It can be assist to distinguish treated from non-treated animals and thus abuse of these substances.

In literature, no studies on the metabolism of prednisolone in cattle were found. However, there are several studies in human medicine, which have led to the isolation of 20(S)-dihydroprednisolone, 20(R)-dihydroprednisolone, 6β-hydroxyprednisolone and 20β-hydroxyprednisone in urine. A recent study carried out on urine of cows treated with low-dose prednisolone, showed the absence of the drug and the presence of the metabolite 20β-hydroxyprednisolone.

The aim of this study was the development of an accurate and robust LC-MS/MS procedure for simultaneous quantitative determination of 22 molecules correlated to prednisolone and cortisol metabolism in *bovine* urine. This method will be used to map the metabolite profile and investigate the alteration of endogenous pattern in case of stress or pharmacological treatment and therefore to obtain a powerful instrument to detect illicit operations.

Materials and Methods

Standards and reagents

All reagents used were of analytical grade (Sigma Aldrich, St. Louis, MO, USA). Samples were cleaned up on polymeric RP SPE cartridge 60 mg/ 3 mL (Oasis HLB Waters, Milford, MA, USA or STRATA X Phenomenex, Torrance, CA, USA). *Helix pomatia* glucuronidase/arylsulfatase (Sigma Aldrich, St. Louis, MO, USA) was used for hydrolysis of corticosteroid conjugates.

The standards were purchased from different manufacturers: Sigma Aldrich (St. Louis, MO, USA), Steraloids (Newport, RI, USA), CDN Isotopes (Pointe-Claire, Quebec, Canada), TRC (North York, Ontario, Canada). To each substance, a fast identification number (ID) was associated (Table 1). Corticosteroids and their ISTDs stock solutions at the concentration of 0.2 or 0.1 mg mL^{-1} in methanol were stored at -20°C. Suitable working standard solutions were obtained by appropriate dilution of the corresponding stock solutions and stored at -20°C.

Table 1. Corticosteroids of interest in this study.

ID	ANALYTE	ID	ANALYTE	ID	ANALYTE
1	cortisol	2	cortisone	3	prednisolone
5	6β-hydroxycortisol	6	6β-hydroxycortisone	4	prednisone
9	5β-dhydrocortisol	17	5β-dihydroocortisone	7	6β-hydroxyprednisolone
10	3α, 5α-tetrahydrocortisol (allo-tetrahydrocortisol)	18	3α, 5α-tetrahydrocortisone (allo-tetrahydrocortisone)	26	20(S)-dihydroprednisolone (20α-hydroxyprednisolone)
11	3α, 5β-tetrahydrocortisol	19	3α, 5β-tetrahydrocortisone	27	20(R)-dihydroprednisolone (20β-hydroxyprednisolone)
12	20(R)-dihydrocortisol (20β-hydroxycortisol)	20	20(S)-dihydrocortisone (20α-hydroxycortisone)	33	20β-hydroxyprednisone
13	20(S)-dihydrocortisol (20α-hydroxycortisol)	21	20(R)-dihydrocortisone (20β-hydroxycortisone)	38	prednisolone-d6
22	α-cortoloneco			40	cortisone-d2
23	β-cortolone			49	6β-hydroxycortisol-d4

Sample preparation

Urine sample (5 mL) spiked with 100 µL 100 ng mL^{-1} internal standard solution (prednisolone-d6, cortisone-d2 and 6β-hydroxycortisol-d4), was buffered with 10 mL 0.15 M ammonium acetate buffer (pH 4.8). Enzymatic hydrolysis was then carried out by incubation for 2 h at 50°C using 50 µL *Helix pomatia* glucuronidase/arylsulfatase.

After cooling and centrifugation, 12 mL of the hydrolysed sample was loaded onto a polymeric RP SPE cartridge (60 mg, 3 mL) conditioned with 3 mL methanol and 3 mL 0.15 M ammonium acetate buffer (pH 4.8) prior to use. The cartridge was washed in sequence with 4 mL water, 8 mL methanol-sodium hydroxide 0.02 M 25/75 (v/v), 5 mL water and finally vacuum-dried. The analytes were eluted with 5 mL ethyl acetate. The solvent was evaporated to dryness under a stream of nitrogen at 40°C and the residue dissolved with 400 µL of a 50:50 (v/v) methanol-water mixture; after defatting with 2 x 1 mL of petroleum ether (40-60°C), twenty µL were injected in the LC-MS/MS apparatus.

LC-MS/MS conditions

LC-MS/MS analysis was carried out by an HPLC Accela TM system coupled to a triple quadrupole mass-spectrometer TSQ Vantage EMR (Thermo Fisher Scientific, San José, CA, USA), equipped with an H-ESI II operating in negative multiple reaction monitoring (MRM) mode. Chromatographic separation was performed on a Waters Xbridge BEH Phenyl® (150 x 3.0 mm i.d., 2.5 µm) analytical column equipped with a guard column. The LC eluents were: acetonitrile (A) and acetic acid 0,1% (B). The injection volume was 20 µL. The separation was obtained at a flow rate of 0.2 mL min^{-1} and an overall run time of 40 min with a gradient program summarised in Table 2.

Column and tray temperatures were respectively set at 40°C and 15°C. The electrospray ionization was used to obtain the precursor ions $[M+CH_3COO]^-$ and at least three product ions were monitored for each analyte (Table 3). The optimized parameters were: spray voltage (3,000 V), sheat gas pressure (40 arbitrary unit), auxiliary gas pressure (15 arbitrary unit), sweep gas pressure (0 arbitrary unit), ion transfer capillary temperature (270°C), collision gas pressure (argon at 1.0 mTorr) and peak resolution on Q1 and Q3 (0.7 FWHM). Scan width (0.2 *m/z*) and scan time (0.05 s for 6β-hydroxycortisolo, 6β-hydroxycortisone, 6β-hydroxyprednisolone, 6β-hydroxycortisol-d4 and 0.01 s for all other analytes) for each monitored transition were optimized to obtain a sufficient number of points for peak and a good shape. The four ions monitored (one precursor ion and three product ions allowed to achieve 5.5 identification points for each compound, calculated as described in the EU Decision 2002/657/EC; the multiple reaction monitoring (MRM) conditions for the analytes are shown in Table 3.

Table 2. Gradient elution program

Time (min)	%A	%B
0.0	10	90
24.0	53	47
24.5	70	30
25.0	80	20
30.0	80	20
35.0	10	90
40.0	10	90

Table 3. MSMS conditions

ID	Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)
7	6β-hydroxyprednisolone	435,2	345.2 - 296.0 - 203.0
5	6β-hydroxycortisol	437	298.3 - 313.3 - 347.2
49	6β-hydroxycortisol-d4	441	317.4
6	6β-hydroxycortisone	435,2	151.2 - 219.2 - 345.2
26	20(S)-dihydroprednisolone	421,2	313.3 - 316.2 - 331.2 - 343.2
13	20(S)-dihydrocortisol	423,2	333.3 - 273.3 - 258.1
27	20(R)-dihydroprednisolone	421,2	313.3 - 316.2 - 331.2 - 343.2
21	20(R)-dihydrocortisone	421,2	271.0 - 316.2 - 331.2
12	20(R)-dihydrocortisol	423,2	333.3 - 345.4 - 315.3
33	20β-hydroxyprednisone	419,2	329.2 - 314.3 - 243.2
20	20(S)-dihydrocortisone	421,2	331.2 - 316.2 - 271
38	prednisolone-d6	425,2	333.2 - 284.1 - 299
22	α-cortolone	425,2	335.3 - 275.3 - 307.3
3	prednisolone	419,2	329.2 - 295.1 - 280.1
4	Prednisone	417,1	327.2 - 299.2 - 285.2
23	β-cortolone	425,2	335.3 - 275.3 - 299.0 - 247.1
1	cortisol	421,2	331.2 - 282.0 - 297.0
2	cortisone	419,2	329.2 - 311.0 - 301.0
40	cortisone-d2	421,2	331.2 - 303.4
10	3α, 5α-tetrahydrocortisol	425,2	301.2 - 319.2 - 335.3
11	3α, 5β-tetrahydrocortisol	425,2	335.3 - 319.2 - 301.2
18	3α, 5α-tetrahydrocortisone	423,2	333.3 - 305.3
19	3α, 5β-tetrahydrocortisone	423,2	333.3 - 305.3 - 261.0
9	5β-dhydrocortisol	423,2	333.3 - 299.2 - 317.2
17	5β-dihydroocortisone	421,2	331.2 - 303.4

Results and Discussion

A meta-analysis of more than 70 scientific publications has shown the metabolic routes of corticosteroids cortisol, cortisone, prednisolone and prednisone. Natural hormones undergo an excessive phase I metabolism and are converted to more polar compounds. Major metabolic pathways are: oxidation at C11 (C-OH→C=O) catalysed by 11β-HSD, dehydrogenation at 1-2 position catalysed by Δ1-HSD, oxidation at 6β-hydroxy derivative catalysed by cytochrome P450, reduction at 20(R) and 20(S)-dihydroxy derivatives (C=O→C-OH) catalysed by 20-HSD, reduction at the 5β-and 5α-position catalysed by 5β-reductase and 5α-reductase, respectively.

A number of 19 out of 33 metabolites described in literature were available commercially. They could provide a reliable pattern of metabolites for each corticosteroid of interest: eight for cortisol, six for cortisone and five for prednisolone; both C-6 oxidative and C-3, C-5 and C-20 reductive ways were guaranteed. As ISTDs were used prednisolone-d6, cortisone-d2 and 6β-hydroxycortisol-d4. The chromatographic separation was a challenge, and a lot of different HPLC columns were tested (Table 4). Best results were obtained with a Waters Xbridge BEH Phenyl® (150 x 3.0 mm i.d., 2.5 µm) column, which allowed a good separation of compounds 26-27-21-20 (all with parent ion at 421.2 *m/z*) and between epimers 10-11 (parent ion at 425.2 *m/z*). The LC-MS/MS analytical method allowed detection and quantification of all the analytes above 0.5 µg L^{-1} in spiked urine samples (Figure 1); the instrumental response was stable during long sequences and the S/N of analytes was good.

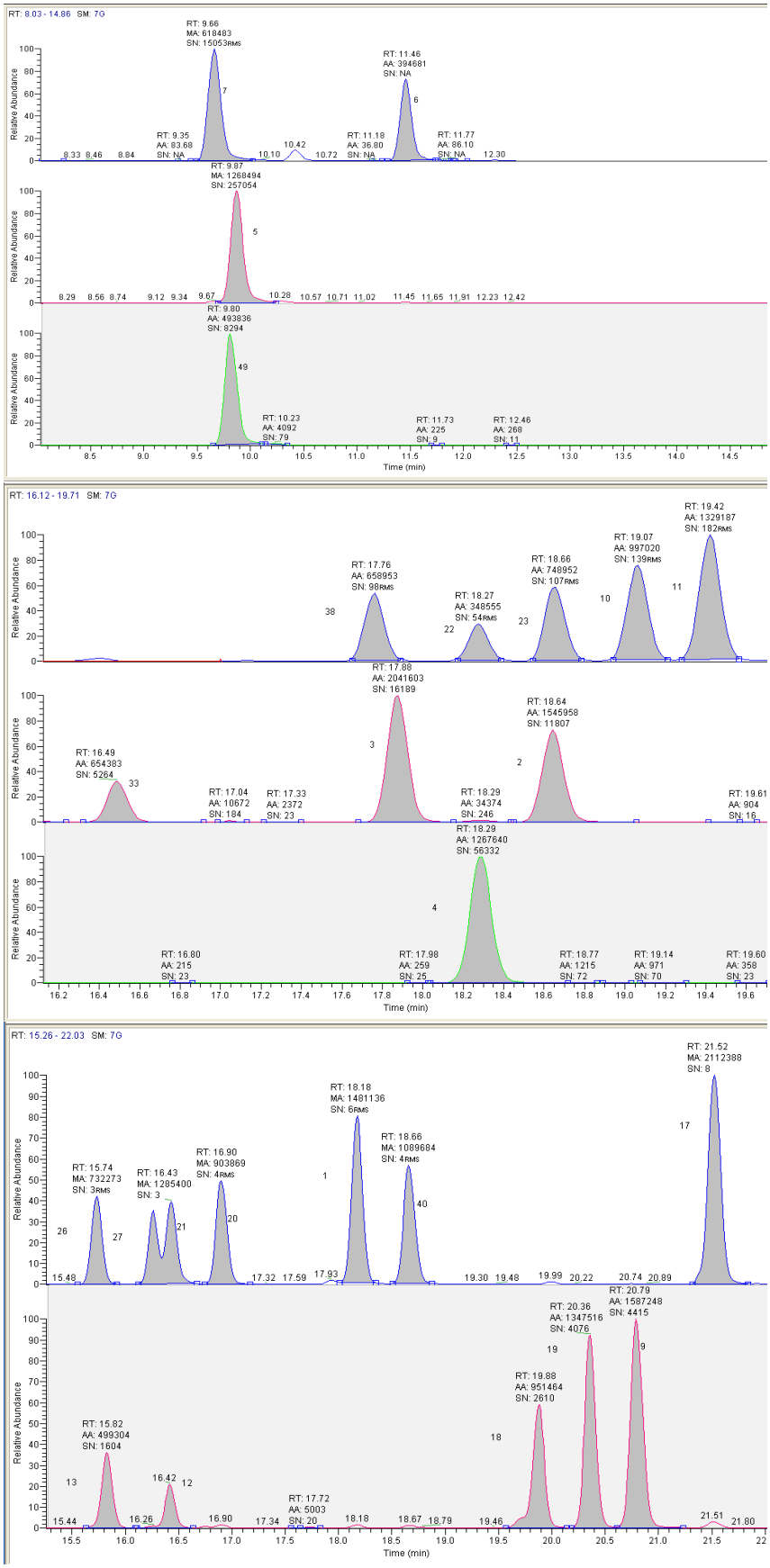

Figure 1. LC-MS/MS chromatograms of a urine spiked at 2 µg L^{-1} with all the analytes and ISTDs.

Table 4. HPLC columns

Manufactures	Description	Dimensions
Waters	Xselect HSS T3®	(150 x 3 mm, 2.5 μm)
Waters	CORTECS C18®	(150 x 3 mm, 2.5 μm)
Waters	CORTECS C18+®	(150 x 3 mm, 2.5 μm)
Waters	Xselect CSH Phenyl-Hexyl®	(150 x 3 mm, 2.5 μm)
Phenomenex	Synergi Fusion® RP 80 A	(150 x 2.0 mm, 4 μm)
Phenomenex	Synergi Polar® RP 80 A	(150 x 2.0 mm, 4 μm)
Phenomenex	Luna C8 PFP®	(100 x 3.0 mm, 3.5 μm)
Waters	SunFire C18®	(100 x 3.0 mm, 3.5 μm)
Waters	XBridge C18®	(100 x 3.0 mm, 3.5 μm)
Waters	XBridge-BEH Phenyl®	(100 x 3.0 mm, 3.5 μm)
Waters	Xbridge BEH Phenyl®	(150 x 3.0 mm, 2.5 μm)

Conclusions

This method showed good fitness for the identification and quantification of corticosteroids cortisol, cortisone, prednisolone, prednisone and their major metabolites in *bovine* urine. Therefore, it could be a potential way to solve the question on the endogenous versus exogenous origin of prednisolone in positive animals. Further studies are needed to investigate the alteration of endogenous pattern in case of stress or pharmacological treatment with the developed method.

Multivariate statistics can be very helpful to unravel the complex corticosteroid metabolism and residue patterns. It may help to identify certain metabolites or the ratios between metabolites in urine as useful tools to discriminate between endogenous and administered prednisolone. This work is in progress.

Acknowledgements

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RESULTS OF PROFICIENCY TESTING FOR THE ANALYSIS OF (FLUORO) QUINOLONE RESIDUES IN TROUT FISH (*ONCORHYNCHUS MYKISS* SPECIES)

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Abstract
The organization of proficiency tests to the attention of the National Reference Laboratories (NRLs) is one of the duties of the European Union Reference Laboratory (EU-RL) according to the Directive (EC) No 96/23 in its Annex V Chapter2 paragraph g. The participation to proficiency testing schemes allows laboratories to assess their competence and to prove the reliability of their results. The aim of this study was to assess the ability of the participants to detect, identify and quantify quinolone residues in trout fish flesh. The participants were asked to implement their routine method in order to detect and quantify each sample for their possible content in quinolone residues. The organization and statistical analysis of this study were performed within a quality assurance system according to internationally recognized guidelines and internal procedures. Quinolones are antibacterial agents used in the treatment of infections in both humans and food-producing animals. The Maximum Residue Limit (MRL) established for the different quinolones are stated in the Commission Regulation (EU) No 37/2010 of 22 December 2009. Forty one National Reference Laboratories (NRL) agreed to participate and received samples prepared at the EU-RL facilities from mixed trout fish muscle spiked with various quantities of the four quinolones, sarafloxacin, flumequine, oxolinic acid and marbofloxacin.

Introduction
Early quinolones discovery occurred in the 1960s. They are antibacterial agents used in the treatment of infections in both humans and animals. Since the discovery of nalidixic acid in 1962, several structural modifications have enhanced their biological and pharmaceutical activities. The introduction of a fluorine at position 6, from which originates the fluoroquinolone sub-family, has enlarged the spectrum of activity against both gram-negative and gram-positive pathogens. Fluoroquinolones are widely used against various important diseases of farmed fish and consequently residues of these antimicrobials have to be monitored in this matrix.

The Maximum Residue Limit (MRL) established for the different quinolones included in this proficiency test are listed below (Commission Regulation (EU) No 37/2010 of 22 December 2009).

The EU-RL proposed to each NRL of the network in the 28 EU-Member States in charge of the analysis of antimicrobial residues and including quinolones, to participate to a proficiency testing study concerning these residues. A total of 41 laboratories including 23 Reference Laboratories of the European Union, 7 laboratories outside the EU and 11 official French laboratories agreed to participate. The three different test materials consisted in spiked trout fish flesh. The samples had to be analysed for their possible content in quinolone residues. Detected compounds had to be quantified and confirmed according to European Decision (EC) No 657/2002. The organization and the statistical analysis of this PT were performed under our COFRAC accreditation within an in-house quality management system and according to ISO/IEC 17043, to national document LABCIL ref 02 rev2. Each participant's results were evaluated by means of Z-scores derived from the consensus assigned value calculated with all participants' results.

Materials and Methods

Preparation of the test materials
Rainbow trout from local fish farming were collected; fillets were taken and were checked with our validated HPLC-Fluorescence detection procedure LMV/00/02-version 3 for the absence of quinolones. Fillets were minced and then the suitable spiking solutions were added to obtain the materials described below. Homogenized during 30 min, each of the materials were sampled (about 20 g each). The hree test materials are presented in Table 1.

Table 1: test materials

	Material 1	Material 2		Material 3
Molecules	Sarafloxacin	Flumequine	Oxolinic acid	Marbofloxacine
Targeted spiked concentration ($\mu g\ kg^{-1}$)	45	400	200	100
MRL ($\mu g\ kg^{-1}$)	30	600	100	/

Homogeneity

The analysis of the materials' homogeneity was carried out for each material by calculation of the inter-sample standard deviation (Ss) according to the Annex B1-3 of the International Standard ISO 13528. For each material, the procedure was carried out as follows:

ten containers were randomly selected among the containers stored at –20°C and thawed, the content of each of the ten containers was separately homogenized and two test portions were taken from each. The analysis of the 2 x 10 portions was carried out during the same day by using our validated HPLC-Fluo procedure LMV/00/02. The statistical test for homogeneity is as follows: the material was considered homogeneous when the calculated standard deviation Ss was lower than 0.3* σ with Sigma (σ) derived from the Horwitz equation modified by Thompson.

All materials were found sufficiently homogeneous for their residue content.

Stability

The stability of the three materials was studied according to the Annex B4-5 of the Standard ISO 13528.

After the deadline set for samples analysis by the participants, 3 containers were randomly selected among the remaining containers stored at -18°C. The content of each of the three containers was then separately homogenised and two test portions were taken from each, the analysis of the 2 x 3 portions was carried out during one day by using our HPLC-Fluo procedure LMV/00/02.

The difference between the final mean value and the initial mean value (initial mean value being determined during the homogeneity study) shall be found lower than 0.3*σ with Sigma (σ) derived from the Horwitz equation modified by Thompson.

The materials were stable during the period of analysis agreed upon with the participants.

Results

Qualitative

No preliminary indications were given to the participants about the exact identity of the quinolone residues to detect. Participants used either LC-MS/MS or LC/FLUO techniques, both methods being suitable for the confirmation of the Group B1 authorized substances. They all reported their values for the CCα and the CCβ limits.

Material 1 containing sarafloxacin. Among the 41 participants 17 did not confirm the presence of sarafloxacin in this material. This result includes the laboratories that claimed not monitoring this particular compound but also laboratories that obtained concentration below their calculated CCα. It must be added that some announced CCα were not well described/established. CCα for substances having a MRL shall be calculated above the MRL concentration and not calculated as for a banned substance according to European Decision (EC) No 657/2002 and ISO 118843-2.

Material 2 containing flumequine and oxolinic acid. Four participants unsatisfactorily obtained false non-compliant results. One laboratory falsely detected norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin and difloxacin as well as the correct presence of flumequine and oxolinic acid. The three other laboratories confirmed flumequine at a level above their calculated CCα

This fact also includes laboratories that claimed not looking for oxolinic acid compound but also laboratories that obtained oxolinic acid concentration below their calculated CCα.

Material 3 containing marbofloxacin. Among the 41 participants 12 did not confirm the presence of marbofloxacin. This result includes laboratories that claimed not looking for this specific compound but also participants that obtained marbofloxacin concentration below their calculated CCα. One laboratory obtained a false non-compliant result and detected norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin and difloxacin but no marbofloxacin in this material.

For this material containing a compound for which no MRL has been set in farmed fish (non-authorized compound), we can assume that the most important fact is then to detect first and to identify the residue according to the identification criteria. It would be enough information to condemn the consignment. The quantitative analysis is of second level.

Quantitative results

The assigned value (X) was determined as the consensus of the results of all of the participants in this proficiency test using robust statistics. The assigned value for each material was calculated as being the robust average of the results reported by all the participants as proposed in the ISO 13528 document. The uncertainty u_x of the assigned value (calculated as the robust mean using Algorithm A of the Annex C of ISO 13528:2005) is calculated from: $u_X = 1.25 s^* / \sqrt{p}$

where s* = robust standard deviation (*calculated using Algorithm A of Annex C ISO 13528:2005*)
p = number of participants.

The value of the standard deviation for the proficiency test σ, could be derived from predictive models appropriate to calculate the reproducibility of an analytical method, as the Horwitz equation or the complementary model from Thompson (Thompson, 2000). The values are presented in Table 2.

Table 2: Summary of the results.

	Material 1	Material 2		Material 3
Molecules	Sarafloxacin	Flumequine	Oxolinic acid	Marbofloxacin
Spiked concentration (µg kg^{-1})	45.0	400	200	100
Number of participants for calculation	34	39	38	32
Minimum value (µg kg^{-1})	19.8	214	93.9	67.0
Maximum value (µg kg^{-1})	80.0	1677	401	128
Assigned value X (µg kg^{-1})	**40.04**	**343.3**	**186.5**	**88.24**
Robust standard deviation (µg kg^{-1}) s*	10.18	73.4	33.3	12.64
Standard uncertainty of the assigned value u_X (µg kg^{-1})	2.18	14.7	6.8	2.79
σ target value for standard deviation (µg kg^{-1})	8.81	64.5	38.4	19.41
u_X / σ	0.25	0.23	0.18	0.14

According to Standard ISO 13528, the uncertainty of the assigned value is negligible and does not have to be included in the interpretation of the results of the proficiency test if: $u_x \leq 0.3\,\sigma$ (or $u_x / \sigma \leq 0.3$)
In this proficiency test, this equation is fulfilled for all the three materials.

Z-scores. Calculation of z-scores is used to assess the accuracy of the results in a proficiency test. Z-score is calculated as:

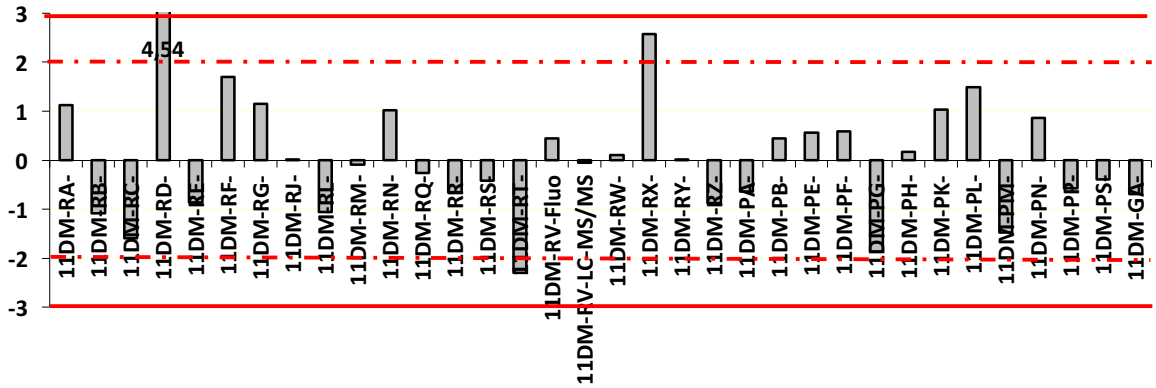
$$Z = \frac{x - X}{\sigma}$$

where Z = z-score ; *x* = Mean result of the laboratory ; *X*= assigned value
and σ = target value for standard deviation for the proficiency test.

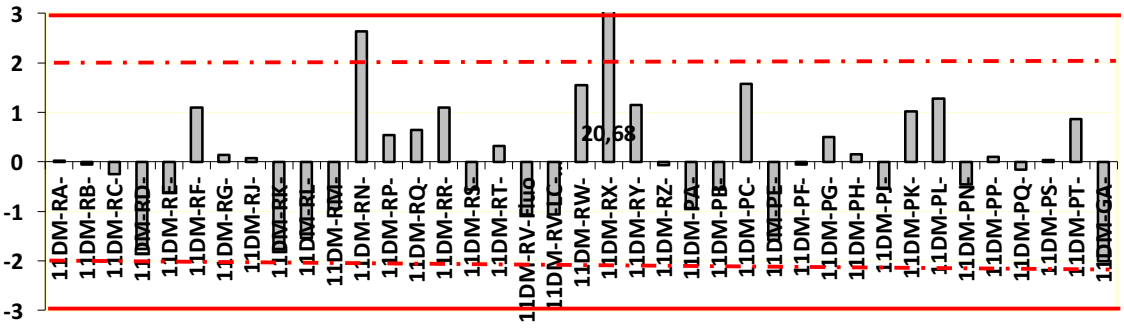
Interpretation of the Z-scores. The advantage of the z-score determination is to provide a standardized value suitable to compare the results. In a "well-behaved "analytical system, the probability that z-scores fall outside the range -2 < z < 2 is about 5 %. So the absolute value of z at 2 was established as a quality limit for the z-score. According to the 'International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories", it is possible to classify scores as follows:

$|Z| \leq 2$ Satisfactory
$2 < |Z| < 3$ Questionable
$|Z| \geq 3$ Unsatisfactory

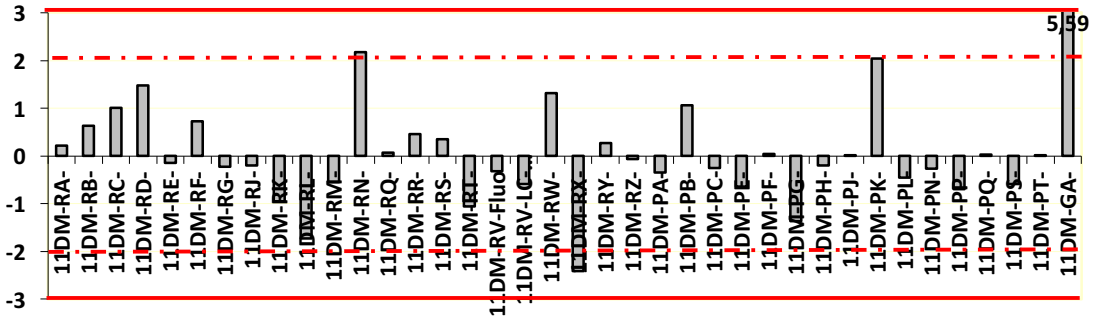
Z-scores - Material 1 containing Sarafloxacin
X = 40.04 µg/kg
σ = 8.81 µg/kg

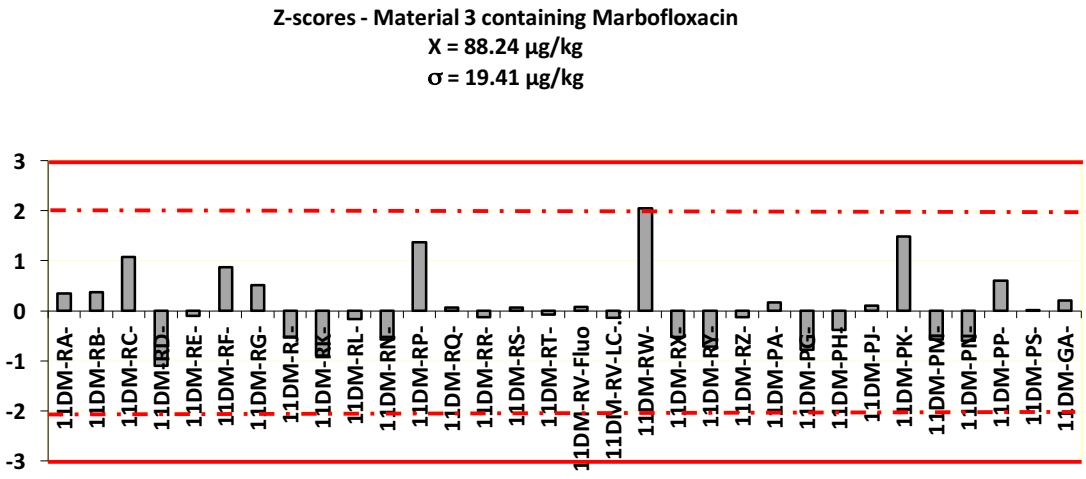


Z-scores - Material 2 containing Flumequine
X = 343.3 µg/kg
σ = 64.5 µg/kg



Z-scores - Material 2 containing Oxolinic acid
X = 186.5 µg/kg
σ = 38.4 µg/kg





Figures 1 to 4. Overview of the Z-scores obtained for the three PT materials, the lines indicate the Z-score tolerance limit of 2 or 3.

Discussion and Conclusions

Regarding the number of false negative results, it can be noticed that some improvements would have been appreciated to include at least the sarafloxacin (substance holding an MRL in fish) and possibly marbofloxacin in the analytical methods. In addition, cautions should be taken for the calculation of CCα for authorized substances in order to avoid taking a wrong decision. One of the laboratories did not detect sarafloxacin at 40.0 µg kg^{-1} in the material 1 even though its CCα was claimed at 24 µg kg^{-1}.

A quantitative evaluation of the results obtained in material 3 (containing a non-authorized substance in fish: marbofloxacin) was performed to inform participants and to allow them to assess the trueness of their results even if the detection and identification of marbofloxacin were the most important facts.

Results obtained by the participants for flumequine and oxolinic acid contents are satisfactory in terms of detection and identification.

Also the results of the quantitative evaluation have highlighted the accuracy of the quantitative results: only three unsatisfactory z-scores reported for all three materials. Regarding material 3 containing marbofloxacin, the worst z-score is 2.05; these results really underline the valid accuracy of the methods used for this compound. This proficiency test was the first one provided by the EU-RL Anses-Fougeres dedicated to the analysis of (fluoro)-quinolones in farmed trout. We thank the 40 laboratories, which a relatively large participation rate allowing to calculate low uncertainties of the assigned values for the three materials. Global satisfactory results were obtained for the qualitative step as well as for the quantitative evaluation of these quinolone residues in fish.

Acknowledgements

The EU-RL thanks the network of NRLs for participation in this PT.

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ANTIBIOTIC RESIDUES IN MILK DURING THE CHEESE MAKING PROCESS. PART I. EFFECT OF THE PASTEURIZATION STEP

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Abstract
Antibiotic residues in milk are not only a potential risk of toxicity to public health, but may also represent a serious problem for the dairy industry. When antibiotic residues are present at any concentration, technological problems may occur in cheese factory due to possible alteration of the fermentation process. Depending on the desired cheese, milk may be pasteurized to reduce the number of spoilage organisms and improve the environment for the starter cultures growth. The information available on the thermostability of antimicrobials for the heat treatments used in the dairy industry is very scarce. The aim of this first part of the study was to estimate the stability of compound belonging to five antibiotic families on fortified milk samples subjected to pasteurization (63°C for 30 min). Each class of compounds was determined by HPLC-DAD or LC-MS/MS methods before and after pasteurization and the results obtained were comparing to calculate the loss of concentration expressed as degradation percentage due to heating.

Introduction
Antibiotics are widely used in veterinary practice to treat bacterial infections including mastitis in lactating cows. In case of insufficient withdrawal periods or increased or incorrectly administered dosage, antibiotic residues will occur in milk. As well as public health concern, when present in raw cheese milk, antibiotic residues may represent a technological problem due their partial or total inhibition of the growth of starter cultures and acidic productions. As a consequence, cheese may have an uneven texture and pasty body with abnormal flavours giving rise to costly economic losses for the dairy industry. In order to protect public health, European Union has regulated the maximum residue limits (MRLs) allowed in milk and other foodstuffs by means of Council Regulation 37/2010/EC. Furthermore, regulatory authorities have established withdrawal periods that the producers have to be obey before they send the milk from medicated cows to the dairy market. Unfortunately, the withdrawal periods do not guarantee the absence of antibiotic residues in milk, but should guarantee that concentrations are below the respective MRLs upon delivery. To date, the information available on the presence of antibiotic residues in cheese is very scarce. In particular, there is a lack of knowledge concerning safety values of antibiotic residues in milk destined to the dairy industry below which no technical consequences occur for cheese making. Milk for human consumption or cheesemaking is thermally treated to ensure its quality and conservation, to reduce the number of spoilage organisms and to improve the environment for the starter cultures. Very few studies have investigated the effect of heat treatments on drug residues in milk. The aim of this research was to evaluate the persistence of veterinary drug residues in milk subjected to the pasteurization process. The selected compounds belong to five drug families that are widely used in veterinary practice: β-lactams, macrolides, quinolones, sulphonamides and tetracyclines.

Materials and methods

Standards
The standards were obtained from Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany), and TRC Inc. (Toronto, Canada). Individual stock standard solutions (100 – 1,000 µg mL^{-1}) were prepared in methanol for quinolones, macrolides, sulphonamides and tetracyclines, and in H_2O/ACN 75/25 (v/v) for β-lactams except ceftiofur (DMF).

Spiked Milk Samples and Heat Treatments
Milk samples from non-treated animals were used as blank samples and for the preparation of spiked samples. Each antibiotic family was separately added to milk subsamples at three concentration levels (0.5, 1 and 2 x MRL). Blanks and fortified subsamples were further divided into two aliquots: one aliquat was heated in a thermostatic bath at 63°C for 30 min prior to analysis, while the other aliquot was analysed without thermal treatment. The effect of pasteurization on drug residues in milk was carried out comparing heated with not heated samples for each fortification level.

a. β-lactams
Sample preparation. A portion of 10 g of milk was centrifuged at 4,000 rpm at 4°C for 10 min. The defatted sample was extracted with acetonitrile (20 mL). After centrifugation, an aliquot of the supernatant (6 mL) was evaporated to dryness under

a stream of nitrogen at 40°C. The residue was dissolved in 0.4 mL of water/acetonitrile (95:5 v/v) mixture, centrifuged at 14,000 rpm for 10 min and then analysed by LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using a TSQ Quantum triple quadrupole equipped with a Finnigan SurveyorTM HPLC system (Thermo Scientific, San Jose, CA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved using a Waters XSelect HSST3 column (150 mm x 3.0 mm, 5 µm). Gradient elution was performed using a binary mobile phase of (A) 0.05% formic acid in water and (B) acetonitrile, from 95% A up to 90% B. The flow rate was 0.2 mL min^{-1} and the injection volume 20 µL.

b. Macrolides

Sample preparation. A portion of 1 g milk was extracted with 2 mL acetonitrile and 15 mL citric acid-sodium phosphate solution at pH 3.5 containing 0.1 M EDTA. After shaking and centrifugation, a second extraction with 15 mL of solution was carried out. The total extract was loaded on Oasis HLB SPE cartridge. The analytes were eluted with methanol and the eluted was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 1 mL of methanol/ acetonitrile/ water (1:1:1 v/v/v) containing 0.2% formic acid and injected into the LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using a Quattro Premiere XE mass spectrometer equipped with a UPLC Acquity system (Waters, Milford, MA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Waters HSS T3 column (100 mm x 2.1 mm, 1.8 µm). Gradient elution was performed using a binary mobile phase of (A) water/acetonitrile (95:5 v/v) containing 0.2% of formic acid and (B) 0.2% formic acid in acetonitrile, from 90% A up to 50% B. The flow rate was 0.45 mL min^{-1} and the injection volume 10 µL.

c. Quinolones

Sample preparation. Two g of milk were extracted with 2 mL acetonitrile and 20 mL McIlvaine-EDTA buffer at pH 4. After centrifugation, the extract was purified with Oasis HLB SPE cartridge. The analytes were eluted with methanol containing 0.1% of formic acid, and the eluted was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 0.5 mL of methanol/water (50:50 v/v) and injected into the LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using an API 5500 QTrap tandem mass spectrometer detector (Applied Biosystem, Foster City, CA, USA) equipped with a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Poroshell 120 EC-C18 column (50 mm x 3.0 mm, 2.7 µm). Gradient elution was performed using a binary mobile phase of (A) 0.1% formic acid in water and (B) methanol, from 95% A up to 90% B. The flow rate was 0.4 mL min^{-1} and the injection volume 5 µL.

d. Sulphonamides

Sample preparation. Two g of milk were extracted with ethyl acetate. After centrifugation, the extract was evaporated to dryness under a stream of nitrogen at 45°C. The residue was dissolved in 1.0 mL of methanol/water (75:25 v/v) and defatted with 1 mL of *n*-hexane. The defatted extract was finally injected into the LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using a API 3000 triple quadrupole mass spectrometer (AB Sciex Instruments, Foster City, CA, USA) equipped with a Series 200 micro pump system (Perkin Elmer, Boston, MA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Waters C18 column (100 mm x 2.1 mm, 5.0 µm). Gradient elution was performed using a binary mobile phase of (A) 0.1% formic acid in water and (B) methanol, from 90% A up to 70% B. The flow rate was 0.2 mL min^{-1} and the injection volume 5 µL.

e. Tetracyclines

Sample preparation. Five g of milk were extracted with 20 mL of a succinic acid 0.1 M at pH 4 and 20 mL of methanol. After shaking and centrifugation, the extract was purified with the metal chelating affinity chromatography (MCAC). The eluted was then analysed by HPLC-DAD.

HPLC- DAD analysis. Analysis was performed using a HPLC 1100 system coupled to a 1260 Diode Array Detector (Agilent Technologies, Santa Clara, CA, USA). The adsorption wavelength used for the UV-vis spectra was 360 nm with a slit of 4 nm. Chromatographic separation was achieved on a Supelcosil LC-8 DB column (250 mm x 4.6 mm, 5.0 µm). Gradient elution was performed using a binary mobile phase of (A) aqueous oxalic acid 0.01 M/THF (99:1 v/v) and (B) acetonitrile/methanol (50:50 v/v), from 70% A up to 50% B. The flow rate was 1 mL min^{-1}min and the injection volume 100 µL.

Results and discussion

According to their use in lactating cows and upon the availability of MRLs in milk, we selected 35 compounds belonging to five families of antibiotics. We decided to study the heat effect on these compounds at three levels of concentration taking into account the MRL of each compound established in milk (0.5, 1 and 2 x MRL). The pasteurization process was carried out on blanks and fortified samples at low temperature and for long time (63°C for 30 min) in a thermostatic bath. This is a conventional combination of temperature and time, easily reproduced experimentally in a laboratory with a thermostatic bath. The dairy industry uses however now a higher temperature and shorter time conditions (72°C for 15 s). Furthermore, in order to check if a proper pasteurization occurred, the alkaline phosphatase activity was determined. The figures below show the degradation percentages at each level of concentration for each compounds that were calculated comparing data from heated and not heated samples. Figure 1 show the degradation percentages obtained for β-lactams antibiotics. In accordance with Roca *et al*. (2011), the heat treatment did not cause significant decreases in the levels of penicillins despite their labile β-lactam ring. On the other hand, cephalosporins presented a greater instability to heating, probably due to the nature of their substituents at C-3 and C-7 which play an important role in whether the β-lactam bond is hydrolysed or broken. The cephalosporins cephapirin and cephacetrile show a higher degree of degradation (40% and 30%, respectively), because their ester bonds are unstable.

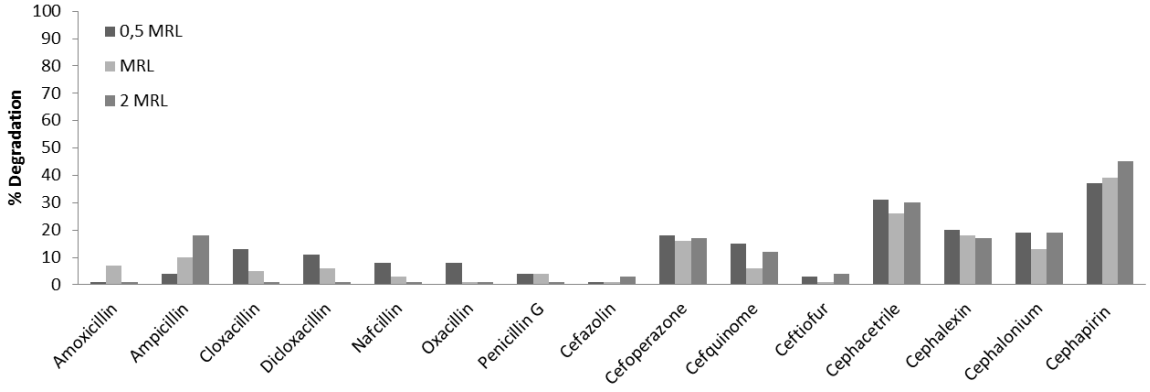

Figure 1. Degradation percentages of β-lactams in thermally treated milk samples at 0.5, 1 and 2 x MRL.

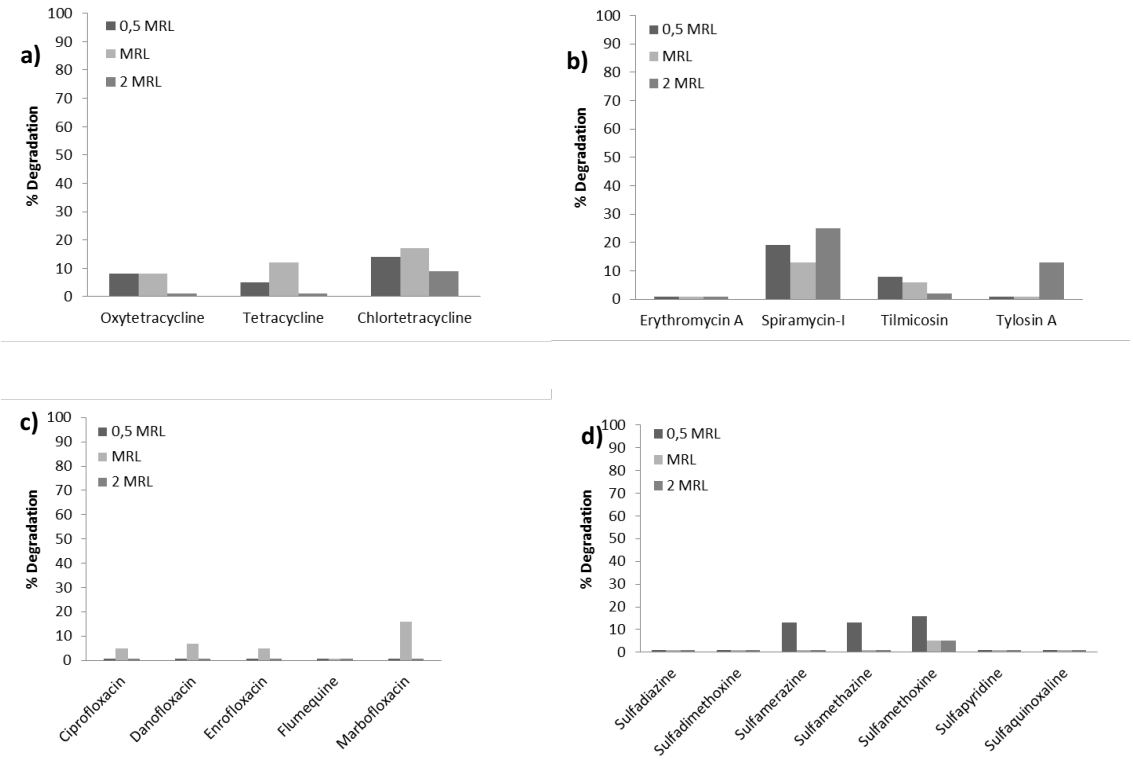

Figure 2. Degradation percentages of a) tetracyclines b) macrolides c) quinolones and d) Sulfonamides in thermally treated milk samples at 0.5, 1 and 2 x MRL.

In Figure 2 the results obtained for the other antibiotic families are reported. The degradation percentage for these compounds was always below 20% suggesting their high stability at the heat treatment. Zorraquino *et al*. (2011) calculated antimicrobial activity losses of macrolides in milk at different heat treatments using a bioassay. For pasteurization at 63°C for 30 min, they obtained a very low reduction of antimicrobial activity (21% erythromycin and 13% spiramycin). We did not find published studies reporting the thermostability of these compounds in milk using chromatographic techniques.

Conclusions

The pasteurization process carried out at 63°C for 30 min did not cause a significant loss of antibiotic residues in milk. In fact, the degradation percentages obtained were below 20% for the most of antibiotics selected in all the spiked samples tested. So, the heat treatment could not prevent these antimicrobial substances from reaching consumers and the dairy industry. It is necessary to implement control measures through the milk production chain and to evaluate if the fixed MRLs for antibiotics in milk guarantee a good cheese manufacturing.

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GAS CHROMATOGRAPHY-COMBUSTION-ISOTOPE RATIO MASS SPECTROMETRY (GC-C-IRMS) AS A TOOL TO DISTINGUISH BETWEEN ENDOGENOUS AND EXOGENOUS BOLDENONE AND NORTESTOSTERONE IN URINE

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Abstract
In the present study a gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) method was developed for the analyses of boldenone and nortestosterone in livestock urine with the aim of being able to differentiate between the endogenous or exogenous origin of these steroids. The developed method was based on an existing method for testosterone and estradiol, therefore allowing the simultaneous analyses of these four steroids. Urine sample clean up methodology consisted of C_{18} SPE clean up, hydrolysis, liquid-liquid extraction, a second C_{18} SPE clean up, followed by acetylation of the steroids. Final clean-up was performed using UPLC fractionation, collecting the α- and β-epimers of the four steroids and dehydroepiandrosterone (DHEA, as endogenous reference compound) in five separate fractions. Based on *bovine* urines samples spiked with internal standards for all four steroids, recoveries of the entire sample preparation method ranged between 28% and 50% (based on GC-MS analyses). At present time no urine samples with endogenous boldenone or nortestosterone were available in order to determine endogenous $\Delta\delta^{13}$C-values by GC-c-IRMS, however, analyses of urine samples from treated bovines with boldenone or nortestosterone esters showed $\Delta\delta^{13}$C-values >12‰ (relative to DHEA).

Introduction
The use of exogenous steroids as growth promoters for fattening purposes in livestock is prohibited. In case of administration of synthetic natural occurring steroids, it is difficult to determine whether the origin of these steroids is endogenous or exogenous. Gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) has been shown to be a useful analytical technique in order to distinguish between the endogenous and exogenous origin of steroids. Exogenous steroids are generally synthesized from plant material having different carbon isotope composition compared to endogenously produced steroids, resulting in $^{13}C/^{12}C$ isotope ratios for exogenous steroids generally lower than -30‰ while values for endogenous steroids are usually above -28‰ (van Renterghem *et al*., 2012). However, external factors, such as diet, can influence the isotope ratios of endogenous steroids among individuals, therefore isotope ratios of the target steroids are compared to a steroid precursor, also called endogenous reference compound (ERC). For certain steroids deviation of isotope ratio by more than 4‰ compared to the isotope ratio of the ERC is indicative of external administration of the steroid.

GC-c-IRMS methods have been developed for the differentiation between the endogenous or exogenous origin of steroids such as testosterone and estradiol in livestock urine (van Rossum, 2015; Janssens *et al*.,2013), whereby the main metabolites in *bovine* urine of the steroids (the α-epimers) are measured. These methods include several sample preparation and clean up steps, such as C_{18} clean up, hydrolysis, liquid-liquid extraction, acetylation and LC fractionation, prior to GC-c-IRMS analyses. Besides testosterone and estradiol, there are other steroids that can occur naturally in livestock (*porcine*, *bovine* and *equine* urine), *e.g*. boldenone and nortestosterone (Scarth *et al*., 2009). It is therefore of importance that new GC-c-IRMS based methods are developed in order to detect illegal administration of these two steroids. There are, however, species-specific differences in the pharmacokinetics of steroids, *e.g*. *bovines* metabolize β-epimers and excrete them as α–epimers, while *porcines* lack this enzyme and excrete the steroids as β-epimers. Therefore, the objective of this study was to develop a GC-c-IRMS method in order to differentiate between the endogenous or exogenous origin of α- and β-boldenone and α- and β-nortestosterone in livestock urine. The methodology is based on an existing validated GC-c-IRMS method for testosterone and estradiol (van Rossum, 2015, with DHEA as ERC) with the intention of extending this method with boldenone and nortestosterone.

Materials and Methods

Standards and chemicals
Standards of α-testosterone (α-T), α-estradiol (α-E2), α-nortestosterone (α-NT), β-nortestosterone (β-NT), β-boldenone (β-Bol) and dehydroepiandrosterone (DHEA) were obtained from Steraloids and β-testosterone (β-T) and β-estradiol (β-E2) from Sigma. The internal standards β-Bol-d3, β-NT-d3, β-T-d3, and β-E2-d3, and α-boldenone (α-Bol) were provided by EURL.

Sample preparation

Sample preparation and clean-up of livestock urine samples for boldenone and nortestosterone was based on the testosterone and estradiol methodology (van Rossum, 2015) (see Figure 1 for the workflow). Briefly, urine samples were spiked with internal standards followed by the addition of 2 M acetate buffer at pH 5.2. The samples were subsequently loaded on a C_{18} cartridge for a primary clean up. After washing steps with water and methanol/water (10/90 v/v), the target compounds were eluted with methanol. After methanolysis (addition of methanolic HCl, 3 M) for 2 h at 60°C and hydrolysis (addition of glucuronidase and buffer pH 7.4) overnight at 37°C, the samples were extracted twice with pentane. The concentrated pentane fraction was redissolved in methanol/water and subsequently loaded on a second C_{18} cartridge. After wash steps with water and methanol/water (40/60 v/v), the target compounds were eluted with methanol/water (80/20 v/v). The samples were evaporated and transferred to derivatisation vials with ethanol. For the acetylation of the steroids, the samples were evaporated to dryness, and pyridine and acetic anhydride were added and the samples were heated at 60°C for 1 h.

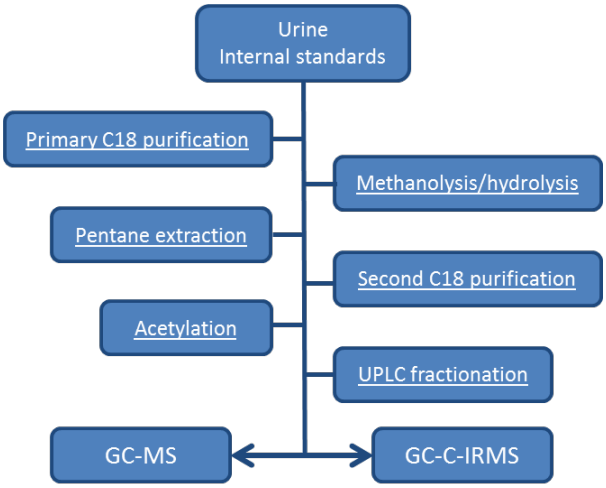

Figure 1. Workflow of the sample preparation of steroid analyses in urine by GC-C-IRMS.

The samples containing acetylated-steroids were fractionated using an Acquity UPLC i-class system (Waters) connected to a PDA detector and QDA MS detector. The collected fractions were evaporated and dissolved in isooctane, and prior to GC-C-IRMS (Thermo trace GC ultra connected to a Thermo MAT253 MS) analyses, steroids were quantified by GC-MS analyses (Varian GC CP-3800 connected to Varian MS 1200L). The GC-MS was equipped with a VF-17MS column (30 × 0.25 mm ID, 0.25 µm film thickness) and the following temperature ramp was used: 110°C for 1 min, 1°C min^{-1} to 285°C and hold for 0.5 min, 20°C min^{-1} to 340°C. The flow was set at 1.0 mL min^{-1}, and the injector, transfer liner, and ion volume temperatures were 250°C, 330°C, and 250°C, respectively. The following masses were monitored for steroid quantification: boldenone epimers *m/z* 147, nortestosterone epimers *m/z* 256, testosterone epimers *m/z* 228, estradiol epimers *m/z* 314, DHEA *m/z* 270. All labelled internal standards (d3) were monitored at mass 3 units higher compared to the unlabelled standards.

The GC-c-IRMS (Thermo trace GC ultra connected to a Thermo MAT253 MS) was equipped with a VF-1MS column (60 × 0.25 mm ID, 0.25 µm film thickness) and the following temperature ramp was used: 100°C for 2 min, 40°C min^{-1} to 235°C and hold for 2 min, 35°C min^{-1} to 330°C and hold for 4.77 min, 1°C min^{-1} to 331°C and hold for 0.28 min, 25°C min^{-1} to 348°C and hold for 2.32 min. The flow was set at 1.2 mL min^{-1} and the injector temperature was 250°C. The combustion furnace temperature was set at 950°C, and *m/z* 44, 45, and 46 were monitored.

Results and Discussion

Instrumental optimization

Under the above mentioned GC-MS conditions from the existing methodology, boldenone and nortestosterone epimers are separated from testosterone, estradiol and DHEA. The ions *m/z* 147 and 256 were added to the MS method for boldenone and nortestosterone quantification, respectively. Also, the GC conditions during the GC-C-IRMS analyses allowed for the separation of the target steroids and the ERC. As satisfactory separation of all the steroids was obtained, no further optimization of the GC parameters was performed.

GC-c-IRMS analyses cannot distinguish between co-eluting compounds, therefore removal of interfering matrix is a crucial step in ensuring high quality data. UPLC fractionation provides as a final clean up step separating the steroids from the matrix prior to GC-c-IRMS analyses. An UPLC-fractionation method was developed to separate not only α- and β-boldenone and α- and β-nortestoterone, but also α- and β-testosterone and α- and β-estradiol as well as DHEA from the sample matrix. An Acquity BEH C_{18} column (1.7um, 2.1 × 100 mm) and acetonitrile/water (10/90 v/v and 0.1% formic acid) and acetonitrile/water (90/10 v/v and 0.1% formic acid) were used as mobile phases A and B, respectively. Standard mixtures containing all the target steroids were injected in order to optimize the separation of all the steroids from the matrix. A mobile phase gradient, listed in Table 1, achieved this separation of the individual steroids from each other and from the matrix (Figure 2). Fractionation windows were set up based on the width of the peaks and was slightly enlarged to ensure that the entire peak was collection should a shift in retention time occur (Figure 2). A total of five fractions were collected: Fraction 1 contained α- and β-boldenone and α-nortestosterone; Fraction 2 contained β-nortestosterone and α-testosterone; Fraction 3 contained β-testosterone; Fraction 4 contained DHEA, and Fraction 5 contained α- and β-estradiol (Figure 2).

Table 1. Gradient program for the UPLC fractionation

Time (min)	Mobile phase A (%)[1]	Mobile phase B (%)[2]	Flow (mL min^{-1})
0	100	0	0.6
4	80	20	0.6
9	70	30	0.6
17	55	45	0.6
19	30	70	0.6
22	0	100	0.6
22.1	100	0	0.6

[1] *acetonitrile/water (10/90 v/v and 0.1% formic acid)*
[2] *acetonitrile/water (90/10 v/v and 0.1% formic acid)*

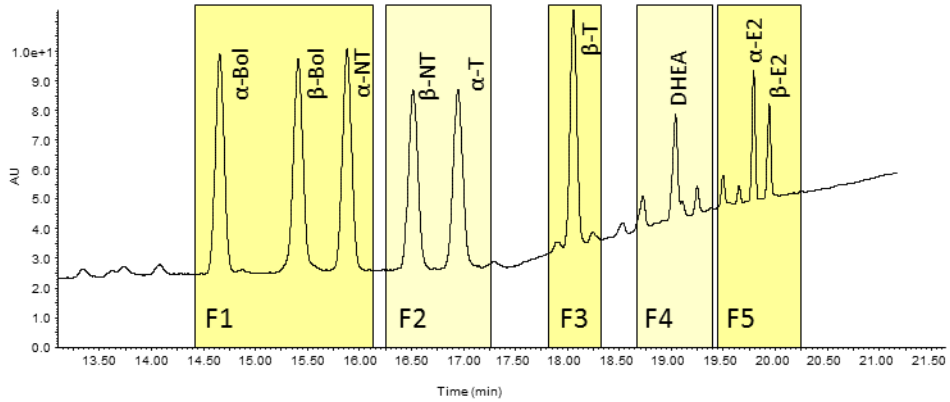

Figure 2. PDA chromatogram of the separation and fractionation of a standard mixture containing acetylated α- and β-Bol, α- and β-NT, α- and β-T, α- and β-E2, and DHEA. F1-5 refers to the collected fractions 1-5.

Sample preparation optimization

The existing methodology for the GC-c-IRMS method for testosterone and estradiol was tested for boldenone and nortestosterone (see Figure 1 for workflow). The sample preparation methodology, as described above, was performed on standard solutions of α- and β-boldenone and α- and β-nortestosterone. Recoveries of the boldenone and nortestosterone epimers were calculated after individual clean up steps, *i.e.* after the first C_{18} clean up, after the pentane extraction, and after the second C_{18} clean up. All the samples were derivatised prior to GC-MS analyses. After the first C_{18} clean up > 94% of the spiked amount was recovered for all steroids, after the pentane extraction >90% of the spiked amount was recovered, while after the second C_{18} clean up >72% of the spiked steroids was recovered. After both C_{18} clean ups a second additional eluting fraction was collected, however, no boldenone or nortestosterone were detected in these fractions indicating that all the boldenone and nortestosterone were eluted in the first eluting fraction from the C_{18} cartridges. All the sample preparation steps were also tested in the presence of urine matrix. On average 88% of the spiked boldenone and nortestosterone epimers to urine were recovered in samples. These results show that the existing extraction and clean up method for testosterone and estradiol can be used for the boldenone and nortestosterone epimers. Subsequently *bovine* urine samples (n=4) were spiked with the four internal standards and the samples were worked up as described above including UPLC fractionation and were analysed by GC-MS. The recoveries for the four internal standards ranged between 28% (β-E2-d3) and 50% (β-NT-d3).

Application of the GC-c-IRMS method

The screening of several *bovine* urine samples for boldenone and nortestosterone by GC-MS provided no positive results and therefore, at the present time, no reference $\Delta\delta^{13}$C-values for endogenous boldenone and nortestosterone could be determined by GC-c-IRMS. However, urine samples were available from treated *bovines* with boldenone undecylenate and nortestosterone fenylpropionate. α-Boldenone and α-nortestosterone, along with DHEA, were detected in urine samples from the treated bovines, and the δ^{13}C-values of α-boldenone, α-nortestosterone, and DHEA are listed in Table 2. The corresponding $\Delta\delta^{13}$C-values for α-boldenone and α-nortestosterone in the urine samples were 12.10‰ and 12.90‰, respectively. For other steroids (e.g. testosterone and estradiol) a threshold $\Delta\delta^{13}$C-value of 4‰ is generally used to differentiate between endogenous (<4‰) and exogenous (>4‰) origin of steroids (van Rossum, 2015; WADA Technical Report, 2014). Whether 4‰ is an appropriate threshold for boldenone and nortestosterone is unclear. However, when using a threshold of 4‰, the detected α-boldenone and α-nortestosterone in the urine samples from the treated animals ($\Delta\delta^{13}$C-value is >12) would be

considered as of exogenous origin. Regardless, data on endogenous $\Delta\delta^{13}$C-values is essential in determining the threshold differentiating the endogenous or exogenous origin of these steroids.

Table 2. δ^{13}C-values of α-boldenone, α-nortestosterone, and DHEA and $\Delta\delta^{13}$C-values for α-boldenone and α-nortestosterone in urine samples from treated bovines.

Treatment	δ^{13}C (‰)[1]			$\Delta\delta^{13}$C (‰)[2]	
	α-boldenone	α-nortestosterone	DHEA	α-boldenone	α-nortestosterone
boldenone undecylenate	-30.27		-18.17	12.10	
nortestosterone fenylpropionate		-31.04	-18.14		12.90

[1] $\delta^{13}C\ (‰) = [(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}]/(^{13}C/^{12}C)_{standard} \times 1000$
[2] $\Delta\delta^{13}C\ (‰) = \delta^{13}C_{ERC} - \delta^{13}C_{TC}$

Conclusions

In this study a GC-c-IRMS method was developed that could be used in order to differentiate between the endogenous or exogenous origin of steroids in livestock urine. The developed method allows for the simultaneous analyses of boldenone, nortestosterone, testosterone, and estradiol. Due to the separation of the α- and β-epimers of all target steroids, this method cannot only be applied to *bovine* and *equine* urine, but also to *porcine* urine samples. Although no threshold for $\Delta\delta^{13}$C-values currently exist for boldenone and nortestosterone distinguishing endogenous from exogenous steroids, need to be determined, analyses of urine samples from treated bovines showed a clear deviation of the δ^{13}C-values compared to the ERC, resulting in $\Delta\delta^{13}$C-values >12‰.

Acknowledgements

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ANTIBIOTIC RESIDUES IN MILK DURING THE CHEESE MAKING PROCESS PART II. EFFECT OF THE SKIMMING STEP

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Abstract
Antibiotic residues in milk are not only a potential risk of toxicity to public health, but may also represent a serious problem for the dairy industry. When antibiotic residues are present at any concentration, technological problems may occur in cheese factory due to possible alteration of the fermentation process. Unfortunately, the scientific literature concerning the evaluation of cheese-making processes with respect to the concentration of drug residues in milk is insufficient. Depending on the desired fat content in the final product, some cheeses may be obtained from semi-skimmed or skimmed milk. The aim of the second part of the study was to evaluate the distribution of antimicrobial residues between polar and non-polar constituents of milk obtained from the skimming process. First, the efficiency of skimming was evaluated on *bovine* whole milk in three different ways: at 4°C for 6 h, at 20°C for 6 h and with centrifugation at 3,000 rpm and 4°C for 10 min. The higher percentage of cream was obtained at 4°C for 6 h (15%); this combination of time and temperature was then selected and applied to fortified milk samples with drugs belonging to five families of antibiotics (β-lactams, macrolides, quinolones, sulphonamides and tetracyclines). In order to estimate the loss of concentration due to the skimming process, the analyses were performed on treated and not treated milk samples and each class of compounds was determined by HPLC-DAD or LC-MS/MS methods.

Introduction
Antibiotics are widely used in veterinary practice to treat bacterial infections including mastitis in lactating cows. In case of insufficient withdrawal periods or increased or incorrectly administered dosage, antimicrobial residues will occur in milk. When present in raw cheese milk, antibiotics residues may represent a public health concern, but also a technological problem due their partial or total inhibition of the growth of starter cultures and acidic productions. As consequence, cheese may have an uneven texture and pasty body with abnormal flavours giving rise to costly economic losses for the dairy industry. In order to protect public health, European Union has regulated the maximum residue limits (MRLs) allowed in milk and other foodstuffs by means of Council Regulation 37/2010/EC. Furthermore, regulatory authorities have established withdrawal periods that the producers have to be observed before to send the milk from cows medicated with antibiotics to the dairy market. Unfortunately, the periods do not guarantee the absence of antibiotic residues in milk, but only that their concentrations remain below the respective MRLs. There is a lack of knowledge concerning the safety values of antibiotic residues in milk below which no technical consequences occur during cheese making. Furthermore, the scientific literature is insufficient to assess the effect of the processes carried out in the dairy industry on the concentration of drug residues in milk. For this, it could be of interest to study the distribution of drug residues over the different components of milk obtained during the cheese-making process. Available literature suggests that the distribution of drugs between polar and non-polar constituents of milk appears to be a function of the concentration and route of the administered drug as well as its polar characteristics. It is clear that additional studies are needed in this area. In this preliminary work, we studied the loss of concentration of drug residues in spiked milk subjected to the skimming process in order to estimate drugs distribution between the aqueous layer and the cream layer. The selected compounds belong to five drug families that are widely used in veterinary practice: β-lactams, macrolides, quinolones, sulphonamides and tetracyclines.

Materials and methods

Standards
The standards were obtained from Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany), and TRC Inc. (Toronto, Canada). Individual stock standard solutions (100 - 1000 µg mL^{-1}) were prepared in methanol for quinolones, macrolides, sulphonamides and tetracyclines, and in H_2O/ACN 75/25 (v/v) for β-lactams except for ceftiofur (DMF).

Skimming and Spiked Milk Samples
Whole milk samples from non-treated animals were used to test the efficiency of different skimming conditions in terms of percentage of cream produced. The following conditions were tested: a) 20°C for 6 h in a thermostatic bath, b) 4°C for 6 h in a refrigerator room and c) centrifugation at 3,000 rpm and 4°C for 10 min. For the preparation of spiked samples, each antibiotic family was separately added to milk subsamples at 2 x MRL concentration. Subsamples were further divided into two

aliquots one of which was subjected to the selected skimming process (at 4°C for 6 h). All samples, skimmed and not skimmed, were then analysed as follow.

a. Beta lactams

Sample preparation. An amount of 10 g milk was extracted with acetonitrile (20 mL). After centrifugation, a 6-mL aliquot of the supernatant was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 0.4 mL of water/acetonitrile (95:5 v/v) mixture, centrifuged at 14,000 rpm for 10 min and then analysed by LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using a TSQ Quantum triple quadrupole equipped with a Finnigan SurveyorTM HPLC system (Thermo Scientific, San Jose, CA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved using a Waters XSelect HSST3 column (150 mm x 3.0 mm, 5 µm). Gradient elution was performed using a binary mobile phase of (A) 0.05% formic acid in water and (B) acetonitrile, from 95% A up to 90% B. The flow rate was 0.2 mL min^{-1} and the injection volume 20 µL.

b. Macrolides

Sample preparation. A portion of 1 g milk was extracted with 2 mL of acetonitrile and 15 mL of citric acid-sodium phosphate solution at pH 3.5 containing 0.1 M EDTA. After shaking and centrifugation, a second extraction with 15 mL of this solution was carried out. The total extract was loaded on Oasis HLB SPE cartridge. The analytes were eluted with methanol and the eluate was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 1 mL of methanol/ acetonitrile/ water (1:1:1 v/v/v) containing 0.2% formic acid and injected into the LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using a Quattro Premiere XE mass spectrometer equipped with a UPLC Acquity system (Waters, Milford, MA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Waters HSS T3 column (100 mm x 2.1 mm, 1.8 µm). Gradient elution was performed using a binary mobile phase of (A) water/acetonitrile (95:5 v/v) containing 0.2% of formic acid and (B) 0.2% formic acid in acetonitrile, from 90% A up to 50% B. The flow rate was 0.45 mL min^{-1} and the injection volume was 10 µL.

c. Quinolones

Sample preparation. A sample of 2 g milk was extracted with 2 mL of acetonitrile and 20 mL of McIlvaine-EDTA buffer at pH 4. After centrifugation, the extract was purified with Oasis HLB SPE cartridge. The analytes were eluted with methanol containing 0.1% of formic acid, and the eluate was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 0.5 mL of methanol/water (50:50 v/v) and injected into the LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using an API 5500 QTrap tandem mass spectrometer detector (Applied Biosystem, Foster City, CA, USA) equipped with a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Poroshell 120 EC-C18 column (50 mm x 3.0 mm, 2.7 µm). Gradient elution was performed using a binary mobile phase of (A) 0.1% formic acid in water and (B) methanol, from 95% A up to 90% B. The flow rate was 0.4 mL min^{-1} and the injection volume 5 µL.

d. Sulphonamides

Sample preparation. An amount of 2 g milk was extracted with ethyl acetate. After centrifugation, the extract was evaporated to dryness under a stream of nitrogen at 45°C. The residue was dissolved in 1.0 mL of methanol/water (75:25 v/v) and defatted with 1 mL of n-hexane. The defatted extract was finally injected into the LC-MS/MS.

LC-MS/MS analysis. Analysis was performed using an API 3000 triple quadrupole mass spectrometer (AB Sciex Instruments, Foster City, CA, USA) equipped with a Series 200 micro pump system (Perkin Elmer, Boston, MA, USA). Data were acquired in positive electrospray ionization mode using multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Waters C18 column (100 mm x 2.1 mm, 5.0 µm). Gradient elution was performed using a binary mobile phase of (A) 0.1% formic acid in water and (B) methanol, from 90% A up to 70% B. The flow rate was 0.2 mL min^{-1} and the injection volume 5 µL.

e. Tetracyclines

Sample preparation. A portion 5 g milk was extracted with 20 mL of a succinic acid 0.1 M at pH 4 and 20 mL methanol. After shaking and centrifugation, the extract was purified using metal chelating affinity chromatography (MCAC). The eluate was analysed using HPLC-DAD.

HPLC-DAD analysis. Analysis was performed using a HPLC 1100 system coupled to a 1260 Diode Array Detector (Agilent Technologies, Santa Clara, CA, USA). The adsorption wavelength used for the UV-vis spectra was 360 nm with a slit of 4 nm. Chromatographic separation was achieved on a Supelcosil LC-8 DB column (250 mm x 4.6 mm, 5.0 µm). Gradient elution was performed using a binary mobile phase of (A) aqueous oxalic acid 0.01 M/THF (99:1 v/v) and (B) acetonitrile/methanol (50:50 v/v), from 70% A up to 50% B. The flow rate was 1 mL min^{-1} and the injection volume 100 µL.

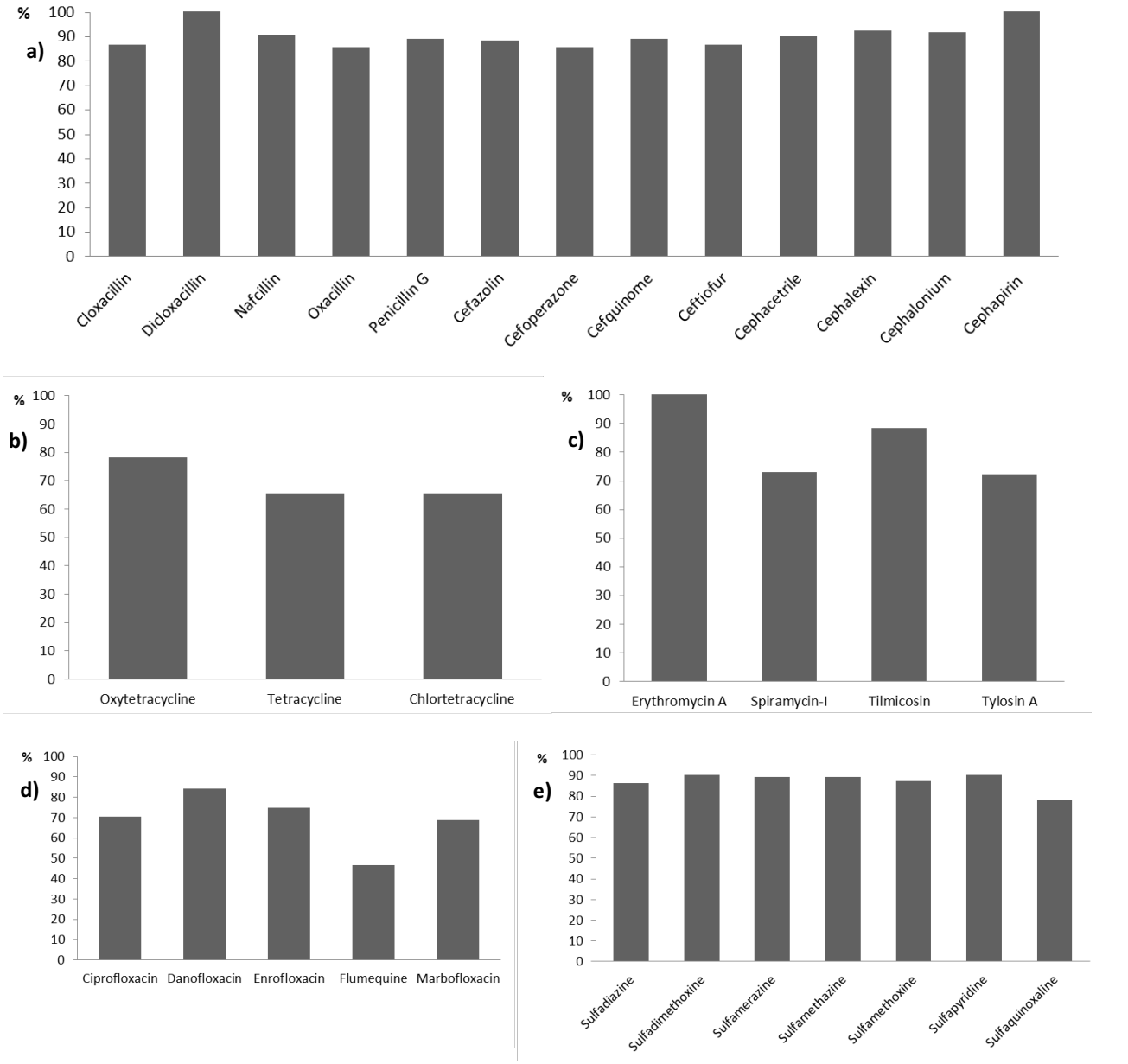

Figure 1. Recoveries obtained for a) β-lactams b) tetracyclines c) macrolides d) quinolones and e) sulfonamides in milk samples subjected to the skimming process carried out at 4°C for 6 h on milk samples spiked at 2 x MRL.

Results and discussion

In this study, we first estimated the efficiency of the separation between polar and non-polar constituents of milk applying three different skimming conditions: a) 20°C for 6 h, b) 4°C for 6 h and c) centrifugation at 3,000 rpm and 4°C for 10 min. The first mode was carried out in a thermostatic bath and was selected to simulate what is done routinely in dairies. The second skimming mode was tested in a refrigerator room to evaluate if low temperature improves the separation between the lipid layer and the aqueous layer. Finally, the third condition represent the defatting step performed in our analytical methods for the antibiotic residues determination in milk. The percentages of cream obtained from the tested skimming processes were the following: a) 3.5%, b) 15% and c) 7.2%. Considering the obtained results, we selected the second treatment as the most suitable to appreciate a difference on the concentration of antibiotics between skimmed and not skimmed milk. According to the use in lactating cows and to the presence of MRLs in milk, we selected about 30 compounds belonging to five antibiotics

families. Milk samples were fortified at a concentration equal to 2 x MRL and the analyses were performed before and after skimming. Figure 1 summarizes the data obtained for the selected antibiotic compounds.
The difference in concentrations between whole milk and skimmed milk, expressed as percentage of recovery, was below 15% (recoveries in excess of 85%) for most of the compounds. A different distribution between the cream layer and the aqueous layer was found for tetracyclines and quinolones because their higher loss of concentration in skimmed milk (recoveries less than 80%). In particular, flumequine showed a low recovery (50%) probably due to a high affinity for the non-polar component of milk.

Conclusions

In this work we examined the effect of a widely used process in the dairies, namely the skimming process, on the concentration of antibiotic residues in milk. In the skimming, the cream layer obtained is enriched with fat and is removed from the rest of milk. Skimmed milk are widely used in the diary market of Norther Italy, in particular it is involved in the manufacture of cheeses known for quality and economic importance as the Parmiggiano Reggiano and Grana Padano cheeses. In order to evaluate the distribution of antibiotics between the polar and non-polar components of milk, we subjected spiked milk samples to a skimming process carried out at 4°C for 6 h. The analyses were performed on skimmed and not skimmed milk in order to determine a difference on antibiotics concentration. The results indicated that antibiotics belonging to β-lactams and sulphonamides families were mainly distributed in the aqueous layer while quinolones and tetracyclines showed a higher affinity for the fat content of the cream layer. Unfortunately, scientific literature on the subject is not available for the comparison of the data. This is preliminary work, more studies on the distribution of antibiotics over milk components such as cream, curd and whey will be provided.

Acknowledgements

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VALIDATION OF A SCREENING AND CONFIRMATORY METHOD FOR TIAMULIN IN LIVER BY LC-MS/MS

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Abstract
The antibiotic tiamulin is a semisynthetic derivative of pleuromutilin. Pleuromutilins are mainly used in feed or water medication for gastrointestinal disease. In the residue control the marker residue for tiamulin in pig liver is "the sum of the metabolites that may be hydrolysed to 8-α-hydroxymutilin". A screening and confirmatory method validated according to Commission Decision 2002/657/EC for the analysis of the marker residue is presented. The focus points have been to reduce the amounts of organic solvents used in the extraction procedure and to substitute the solvents incriminating to the environment. The MRL in liver is 500 µg kg^{-1} and the validation resulted in a CCα of 588 µg kg^{-1}. The method is accredited and is applied to the Danish National Residue Control Plan.

Introduction
In 2015 the amount of pleuromutilins sold for pigs in Denmark was 7,880 kg active compound, and tiamulin accounts for nearly 100 %. These figures in Table 1 shows, that tiamulin is widely used in the Danish pig production, and is at the "top five" of the most popular antibiotics used in pigs.

Table 1. Use of pleuromutilins in Danish pig production (Vetstat)

Year	Kg active component	% of total use of antibacterials
2013	8,963	9.9
2014	8,121	9.4
2015	7,880	9.7

In 2014 the Danish pig producers committed themselves to reduce the consumption of tetracyclines by 50% by the end of 2015 (Danmap 2014, Landbrug og Fødevarer). To ensure animal welfare while reducing the amount of tetracyclines used, one of the strategies mentioned was to replace tetracyclines with tiamulin (Landbrug og Fødevarer). The figures for 2015 are not yet published, but so far a reduction of tetracycline use was observed and the consumption of tetracyclines was reduced by 11% from 2014 to 2015 (Vetstat).

Due to the widely use of tiamulin, a method to measure the marker residues of tiamulin was therefore of interest for the Danish monitoring program. Tiamulin cannot be included in a multimethod for antibiotics due to the need of hydrolysis to monitor the residues. A single method is therefore required. The former published AOAC-method for determination of tiamulin residues in swine liver (Markus *et al*., 1993) has a high consumption of unwanted solvents such as dichloromethane for extraction. Consequently, the object of the method development was to develop a LC-MS/MS method suitable for routine analysis of tiamulin in swine liver and with focus at the substitution of harmful organic solvent.

The AOAC-method includes a hydrolysis step which could not easily be validated since tiamulin is not hydrolysed directly to 8-α-hydroxymutilin (Tarbin *et al*.). Therefore, the hydrolysis step of the AOAC-method has not been changed. The clean-up procedure has been optimized to minimize solvent consumption and to avoid the solvents most incriminating for the environment.

Materials and Methods

Standards, solvents and reagents
8-α-hydroxymutilin was supplied by Novartis (now Elanco). Water, acetone, heptane, sodium hydroxide, hydrochloric acid, methanol, formic acid and acetonitrile. All used organic solvents were of analytical grade. OASIS HLB SPE columns from Waters. Extraction solution was prepared by mixing 1,800 mL acetone and 30 mL 0.5 M HCl.

Sample preparation
Excess of fat was removed from the liver sample. The liver was blended and an amount of 1 g was weighed into 50 mL plastic tube. Samples for matrix matched calibration curve were spiked. The samples were extracted with 10 mL extraction solution (Acetone:0.5M HCl). The samples were whirly mixed and then shaken for 10 min. After centrifugation the supernatant was

transferred to another plastic tube and the extraction is repeated using 5 mL extraction solution. The supernatants were combined and 2 mL 0.5 M HCL and 10 mL of water were added. The samples were evaporated to 10-15 mL at 45°C. After evaporation 1 mL of water and 1 mL 7 M NaOH were added. The samples were shaken, and then incubated in water bath at 45°C for 20 min. After cooling the samples in cold water, 5 mL of water and 1 mL concentrated HCl were added. The samples were shaken and 2 mL of Heptane was added. The samples were shaken for 10 min. After centrifugation, the heptane phase was removed and discarded. The OASIS HLB columns were conditioned successively with methanol and water. After sample loading, the columns were rinsed with 3 mL 15% methanol in water. Samples were eluted with 5 mL of methanol. The samples were evaporated to dryness. The dry residue was dissolved in 300 µL of methanol. After shaking the extract for 10 min, 700 µL of water was added and the samples were successively shaken and high-speed centrifuged before injection 10 µL into the LC-MS/MS-system.

Apparatus and data acquisition
The LC-MS/MS system consisted of a Thermo Fischer Accela LC equipped with a Luna PFP(2) column (50x200 mm, 3 µm) coupled to a TSQ Quantum Access Mass spectrometer with an ESI interface. The mobile phases were 0.1% formic acid in water and 0.1% formic acid in methanol. The LC-MS/MS analysis is done within 10 min.

Results and discussion
During method development different problems were observed. In the final step, problems with precipitation in the samples after dissolving the dry residue were seen. The problem was solved by including a heptane wash of the cooled samples before adding HCl to the extracts. Heptane was chosen over hexane due to less toxicity. As a part of the validation the absolute recovery of the method was calculated found to be only 31%. The heptane washing step partly accounts to the loss of analyte, but it was concluded that the low recovery was acceptable because the CV% was low (9%), indicating that the recovery was stable; similar the LOD at 50 µg kg^{-1} which is far below MRL, is a reason to accept a low absolute recovery. When the method is run routinely it is important to avoid fat precipitating in the samples resulting in blocked capillaries in the LC-MS/MS-system. Due to the low absolute recovery spiked standard curves are obligate.

Linearity
Linear regression analysis was performed by plotting peak area versus analyte concentration. The linearity was examined in the range from 0-1,500 µg kg^{-1}. Residues were calculated and all residues above LOQ were below 20%. It was found that the calibration curve was linear in the range from 0-1,500 µg kg^{-1}.

Specificity
The specificity of the method was examined and it was found specific according to retention time and ion ratio for all samples at levels at or above ½ MRL.

Matrix effects were studied in 20 blank samples distributed in three validation series. A part of the extract was spiked and compared to the response of a solvent standard at the same level. The results are shown in Table 2.

Table 2. Study of matrix effects. Area of spiked extracts and solvents standards at same level. Number of spiked extracts and number of injection of solvent standard are specified in brackets ().

Series	Area (mean) Spiked extract	CV% Area Spiked extract	Area (mean) Solvent standard	CV% Area Solvent standard	Ratio Area extract/area solvent standard
1	541380 (5)	4	652166 (5)	4	0.83
2	434545 (8)	6	481399 (6)	5	0.90
3	725071 (7)	6	841721 (5)	6	0.86

The mean area is in all three series lower in spiked extracts than in solvent standards at the same level, indicating that there is observed some ion suppression. The ratio of response between matrix and solvent standard was between 0.83 and 0.90 meaning there is some degree of ion suppression.

Two transitions are monitored routinely, namely *m/z* 337>139 and *m/z* 337>301. The signal to noise ratio is significantly better in one of the transitions compared to the other. The transition with less noise is used in screening series.

Precision and recovery
Precision of the method was determined as repeatability and within-laboratory reproducibility at 3 levels. The analyses are done as 6 double determinations in four days by two technicians. According to 2002/657/EC the coefficient of variation (CV) for the repeated analysis under reproducibility conditions, shall not exceed the level calculated by the Horwitz Equation. In

Table 3, the repeatability and within-laboratory reproducibility are shown. At all three levels examined, the CV% of the within-laboratory is below the reproducibility calculated with the Horwitz equation.

The relative recovery was determined and found to be acceptable.

Table 3. Overview of the results of the validation

Level	CV% repeatability	CV% within-laboratory reproducibility	Reproducibility CV (%) Horwitz equation	Relative recovery %
250 µg kg^{-1}	7.0	10.7	19.7	90
500 µg kg^{-1}	10.5	11.4	17.8	94
750 µg kg^{-1}	8.6	10.1	16.7	94

Detection limit and decision limit

The decision limit was calculated by the calibration curve procedure. The result is seen in Table 4 below.

Limit of detection and limit of quantification was calculated as well from 20 samples spiked to the concentration 150 µg kg^{-1} as 3 and 6 times the standard deviation. The detection capability has been set to the limit of detection. A cut-off value will be established when sufficient data material is available.

Table 4. Limit of detection (equal to detection capability), Limit of quantification and decision limit (CCα).

LOD (CCβ)	LOQ	CCα
50 µg kg^{-1}	100 µg kg^{-1}	588 µg kg^{-1}

Stability of samples

The samples were found to be stable in the autosampler at 5°C for at least 5 days.

Results from the Danish National Residue Control Plan

The method has been applied to the national control plan in 2014 and 2015. A total of 204 samples have been analyses. There were no detected residues above CCβ in the analysed samples.

Conclusions

A method meeting the requirements of Commission Decision 2002/657/EC has been developed. The decision limit (CCα) is 588 µg kg^{-1} and the limit of quantification is 100 µg kg^{-1} and is far below the MRL (500 µg kg^{-1} in liver). The method measures the marker residue of tiamulin, 8-α-hydroxymutilin and is both simple and robust, making the method suitable for routine analysis at a control laboratory. Furthermore, the method development has focused at, and solved the safety requirement according to the Danish Working Environment Act. The method has been applied to the Danish national plan in 2014 and 2015. And is an important supplement to the LC-MS/MS multi methods used in the control, since tiamulin accounts for one of the five most used antibiotics in Denmark. A total of 204 samples have been analysed. There were no detected residues above CCβ in the analysed samples.

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TOWARDS RESPONSIBLE USE OF ANTIMICROBIALS WITHOUT LOSS OF A GOOD UDDER HEALTH

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Abstract

On dairy farms, the majority of antimicrobials is used for the treatment of udder health problems. In a project in Flanders, Belgium, four dairy herds were assisted towards a more prudent use of antibiotics by advising on udder health. Evidence based and a well-founded advice on preventive measures and approach of individual problem cows was given. On average, the defined daily dose of antimicrobials per animal decreased with 20 % and that of critically important molecules decreased by half, while a good udder health was maintained. For selective dry-cow therapy, 3 protocols were tested. Cows that did not receive antibiotic treatment at dry off, did not have more clinical mastitis cases after calving nor had a higher somatic cell count than treated cows. We propose selective dry-cow therapy based on last three individual somatic cell counts with a cut-off at 100,000 and 150,000 cells mL^{-1} for cows and heifers, respectively, absence of clinical mastitis during the previous lactation and a milk production < 15 kg, besides the use of internal teat sealers and a good dry cow management. Counselling farmers and applying selective dry cow therapy can lead to decreased use of antimicrobials and in particular of critically important molecules without loss of a good udder health.

Introduction

On dairy farms, the majority of antimicrobials is applied as intramammary treatment as part of udder health management (Scherpenzeel *et al*., 2014). In fact, mastitis is the most important disease on dairy farms. The disease can reveal itself as a clinical infection with visible signs as abnormal milk, a swollen udder (quarter) and/or a sick cow with fever, loss of appetite, and in severe cases even death of the animal may follow. Besides the clinical form, subclinical mastitis is often present. In this case, no abnormalities are visible. The infection is detectable by *e.g*. an elevated somatic cell count (SCC) and a positive bacteriological culture result of a milk sample. A cow is considered infected when the SCC of a composite milk sample is above 250,000 cells mL^{-1}, a heifer when the SCC is above 150,000 cells mL^{-1}. On a regular basis, the bulk milk somatic cell count (BMSCC) is analysed to consider the udder health status of the herd, as is required by law.

The vast majority of clinical and subclinical mastitis cases is caused by intramammary infection with bacteria. The origin of these bacteria can be the cows environment like manure, bedding material, etc. ("environmental pathogens") or the micro-environment of the udder like skin of the teat and teat apex, streak canal, milk of chronically infected cows, etc. The latter are referred to as "contagious pathogens" as the main route of infection is through transmission from cow to cow. Within the mastitis pathogens, a distinction is made between major and minor pathogens. Major pathogens like *Escherichia coli*, *Streptococcus uberis* and *Staphylococcus aureus* are able to cause clinical mastitis and a considerable elevation of SCC, while minor pathogens like *Staphylococcus species* (non-aureus staphylococci) and *Corynebacterium bovis* cause only a moderate SCC increase and seldom clinical mastitis.

In '69, a five-point control program was published to deal with udder health problems on dairy farms by prevention of new intramammary infections and by effective cure of existing infections (Neave *et al*., 1969). In this respect, a clean environment and a good milking technique are essential to prevent new intramammary infections. To cure existing infections, intramammary treatment of all cows with long-acting dry cow antibiotics at the end of lactation was suggested at that time (dry cow treatment). The latter preventive measure has been adopted by many farmers in Belgium as well as in many other countries, to ensure a healthy udder in the subsequent lactation. However, treating all cows at dry off regardless of their infection status (blanket dry cow treatment), might result in unnecessary use. In fact, a considerable number of cows will have a healthy udder at dry off, so blanket dry cow therapy can (partially) be catalogued as metaphylactic or even preventive use of antimicrobials, which is to be discouraged.

Tackling udder health issues by increasing preventive measures and drying-off healthy cows without antibiotics might support responsible use of antimicrobials. In the presented study performed in Flanders, Belgium, four dairy herds were assisted towards a more prudent use of antibiotics by advising on udder health and by introducing selective dry cow therapy. The effect of selective dry cow treatment on the udder health in the next lactation was assessed, as well as the potential reduction in antimicrobial consumption induced by increased attention on preventive measures.

Materials and Methods

Selection of herds

Four dairy herds, geographically divided over Flanders, were included in this project. Three of them were research herds (combined with commercial activities), the fourth herd was a fully commercial dairy farm. The herds had on average 63 adult cows (range 51 – 80) and participated in the Dairy Herd Improvement (DHI) program. The 305-d milk yield ranged between 8,000 and 11,000 kg. Milking was done by an automated milking system (2 herds) or a conventional milking parlour (2 herds).

Milk sampling and laboratory analysis

Milk sampling was performed for all clinical mastitis cases, defined as any visual abnormality of the milk or the udder. Milk from some subclinical cases was sampled, with subclinical mastitis defined as SCC of a composite milk sample above 250,000 cells mL^{-1} for cows and above 150,000 cells mL^{-1} for heifers. Quarter level milk samples were taken of every cow not earlier than ten days before dry off and within four days after calving. Sampling was performed mostly by the farmers and sometimes the farm's veterinarian, after providing information on a correct sampling procedure.

All quarter milk samples (clinical and subclinical mastitis, before dry off and post-calving) were transported to the lab, where they were submitted to standard culturing followed by susceptibility testing of major pathogens, both performed as described earlier (Supré *et al*., 2014). Additionally, the quarter milk samples of each cow at dry off were pooled to a composite milk sample and examined with real-time PCR targeting mastitis pathogens (Pathoproof PCR, Thermofisher).

Udder health monitoring

Monitoring udder health was done by an initial herd check, in which the milking technique, the hygiene of lactating and dry cows and of young stock, and the general herd management was scrutinized. The initial herd check was followed by evaluation of DHI data on a monthly basis combined with regular visits during the 18-month study period (October 2013 – March 2015). On every occasion, the basic principles of good udder health management (as proposed in the five-point control program) were emphasized, including the recommendation to cull chronically infected cows (based on cow level SCC from DHI data). Based on the result of bacteriological culturing of the clinical and subclinical milk samples, an approach for each problem cow (treatment, culling or dry off) and/or adaptation of preventive measures on herd level was proposed to the farmer after consulting the farm's veterinarian. In subclinical mastitis cases during lactation, the cow level approach was based on the culture result in combination with cow factors. The approach could be "immediate treatment" (in lactation), "postponed treatment" (at dry off), "culling" (when prognosis was bad), or "wait-and-see" (with minor pathogen infections).

Farmers registered all clinical mastitis cases in their herd management software of choice.

The udder health during the study period, as assessed by the BMSCC and the number of clinical mastitis cases, was compared to an equal period of time in the past (October 2011 – March 2013). BMSCC was determined by flow cytometry (Fossomatic 5000, Foss Electric, Hillerød, Denmark). A clinical case that was recorded less than two weeks apart from a previous case in the same quarter was not considered a new case and therefore excluded from the analysis.

Antimicrobial usage

Treatment protocols. The choice of antimicrobials was based on the AMCRA guidelines for prudent use of antimicrobials. By AMCRA (the Belgian Centre of Expertise on Antimicrobial Consumption and Resistance in Animals), all antimicrobials are divided in 3 categories based on their relevance to human medicine and their ability to induce antimicrobial resistance, as derived from the OIE guidelines (www.AMCRA.be). The basic molecules are included in the "yellow" category (*e.g*. penicillins and $1^{st}/2^{nd}$ generation cephalosporins), the most critical molecules in the "red" category (fluoroquinolons and $3^{rd}/4^{th}$ generation cephalosporins for parenteral treatment), and in between an "orange" category exists (Table 1). Within the "orange" category, molecules are split up to "basis orange" and "critical orange", as in Belgium, $3^{rd}/4^{th}$ generation cephalosporins for local (intramammary) use are not classified as red but as orange (though critical).

Table 1. Classes of antimicrobials as defined by AMCRA (www.AMCRA.be)

Class	Examples
Yellow	Penicillins, 1^{st} and 2^{nd} generation cephalosporins
Orange	Aminopenicillin, lincosamids, macrolids, tetracyclins, rifaximin
- Of which critical orange	3^{rd} and 4^{th} generation cephalosporins for local treatment
Red	3^{rd} and 4^{th} generation cephalosporins for parenteral treatment, fluoroquinolons

In the event of a clinical mastitis case, farmers were advised to treat immediately (after sampling). The treatment protocol of choice was derived from (historical) culture data on the farm, and was proposed by the herd veterinarian. When treatment was applied for a subclinical case, the treatment protocol was based on culture results of that case.

In general, the attempt was to preferably use "yellow" class products for (sub)clinical mastitis cases, if the type of infection and the severity allowed it. Regular consultancies took place between each herd veterinarian and the project veterinarian, to discuss the treatment protocol on each herd based on the culture results, susceptibility testing for that farm and the AMCRA guidelines. The herd veterinarian took the final treatment decision.

Data collection and processing. Farmers registered all drug usage in their herd management software. The antimicrobial consumption was quantified as defined daily doses animal (DDDA) and expressed as the number of standardized treatment days a cow received per year (Stevens *et al*., 2016). Therefore, data from the management software of each herd were exported to excel files, to determine the total amount for each antimicrobial administered to adult dairy cows. This total usage over the 18-month study period was put in a benchmark system, which took the number of adult cows per herd, the on-label dosage and the approximate weight of an adult dairy cow (600 kg) into account to calculate the DDDA. One dry cow injector accounted for one day-under-treatment per injector as proposed by Scherpenzeel *et al*. (2014); as a result drying off one cow accounted for 4 treatment days.

The DDDA during the study period (October 2013 – March 2015) was compared to the DDDA in an equal period in the past (October 2011 – March 2013).

(Selective) dry cow treatment

Cows had to fulfil several requirements to be dried off without long-acting antimicrobials. When these were not met, long-acting antimicrobials were used intramammarily at dry off. The type of dry cow injector used was adapted to the culture results of the herd. *E.g*. when Gram-positive bacteria were the main cause of intramammary infections, an injector with a Gram-positive spectrum was preferred; when the herd had a history of Gram-negative infections in early lactation, a broader-spectrum dry cow injector was chosen.

Selective dry cow treatment was introduced in three consecutive steps, going from a very strict to a more moderate protocol (Table 2). The protocols were based on cow-level factors (absence of clinical mastitis in the previous lactation, milk yield at dry off, somatic cell count, real-time PCR on composite sample) whether or not supplemented with quarter-level factors (somatic cell count and bacteriological culture on quarter milk samples). The rationale of working in three consecutive protocols was to persuade farmers, as they were reluctant to dry off cows without treatment, at first. The next step was taken when the farmers were convinced of the acceptable results of the previous step. Cows with a history of clinical mastitis in that lactation and cows with a milk production of > 15 kg at the time of dry off were always treated at dry off, as a basic rule in all three protocols.

The decision to use dry cow therapy was made on cow level, so when applied, all four quarters of the cow received long-acting antibiotics. Each cow (irrespective of antimicrobial treatment at dry off) received internal teat sealants.

Table 2: Inclusion criteria to dry-off without long-acting intramammary antimicrobial treatment.

	Criteria	**Protocol_1** **Oct. '13 – Jan . '14**	**Protocol_2** **Feb. '14 – Sep. '14**	**Protocol_3** **Oct. '14 – Mar. '15**
Cow level	Clinical mastitis	Absent in preceding lactation	Absent in preceding lactation	Absent in preceding lactation
	Milk Yield at dry off	< 15 kg	< 15 kg	< 15 kg
	Somatic cell count	< 150,000 cells mL^{-1} on each DHI test day in preceding lactation	< 150,000 (heifer) or < 100,000 cells mL^{-1} (cow) on each of the last 3 DHI test days	< 150,000 (heifer) or < 100,000 cells mL^{-1} (cow) on each of the last 3 DHI test days
	Pathoproof PCR[a]	Not performed	Absence of major pathogens	- [b]
Quarter level	Somatic cell count	< 100,000 cells mL^{-1}	Acceptable	- [b]
	Culture	Negative	Negative	- [b]

[a] *Real-time PCR targeting mastitis pathogens;* [b] *Performed but no inclusion criterion.*

Results and discussion

Without any legal regulation on antimicrobial consumption, it remains tempting for dairy farmers to use antimicrobials as an assurance to keep udder health save. This project aimed for two goals in order to change that mind-set on four dairy farms in Flanders, that is (1) finding a practical (and secure) way to apply selective dry cow therapy, and (2) achieving an overall more prudent use of antimicrobials by strict consultancy and monitoring of udder health.

Finding a practical way to select cows to treat or not to treat at dry off is not easy. In a trend-setting study in the Netherlands, the protocol was based on a single SCC at the last DHI test day of 250,000 cells mL^{-1} for cows and of 150,000 cells mL^{-1} for heifers (Scherpenzeel *et al*., 2014). However, the incidence of clinical mastitis postpartum increased, resulting in stricter guidelines. In Belgium, no regulations are in place to prohibit (blanket) dry cow therapy, so it is hard to convince farmers to

dry off cows without treatment. Even for the farmers included in this project, the reluctance to dry off cows without long-acting antimicrobials was clear. Building confidence was important so we decided to work in 3 consecutive steps, going from more to less stringent. In protocol_1, the basic rule (excluding cows with a history of clinical mastitis in that lactation and cows with a milk production of > 15 kg at the time of dry off from the non-treated group) was combined with other (rigorous) cow and quarter level factors: SCC of < 150,000 cells mL^{-1} at every DHI test day of the lactation, and a quarter level SCC < 100,000 cells mL^{-1} and a negative bacteriological culture in every quarter before dry off (Table 2). As a result, only a minority of cows were dried off without antibiotics in the first time slot (10.2%). Going to protocol_2, SCC at the cow level was only considered at the last 3 test days before dry off (instead of at all test days) and the quarter level SCC and culture result were judged less strict. Real-time PCR was performed on a composite sample and should reveal the absence of major pathogens. These criteria still left only 10.0% non-treated cows. In the next step (protocol_3) quarter level SCC, quarter level culture result and cow level real-time PCR were not taken into account to select cows, leaving 31.8% non-treated cows. Over the three protocols, 235 animals were dried off of which 16.2% without antimicrobials.

Although bacteriological culture before dry off was not taking into account in all three protocols, it was performed for research purposes over the total study period. Quarters that were culture negative at dry off remained culture negative over the dry period in 87.5% of the quarters of treated cows versus 77.1% of non-treated cows. The higher percentage of culture positive quarters in non-treated cows was attributed to infections with minor pathogens (*Staphylococcus species* or *Corynebacterium bovis*) rather than those with major pathogens. In fact, 2.3 versus 1.2 % infections with a major pathogen occurred in quarters of cows that did resp. did not receive dry-cow injectors compared to 10.2 versus 21.7% infections with a minor pathogen.

The SCC of non-treated cows was somewhat higher in early lactation compared to the end of the previous lactation, but still lower than for cows that received dry cow injectors. In fact, the geometric mean SCC increased from 38,600 cells mL^{-1} (the last three DHI test days before dry off) to 56,300 cells mL^{-1} (first three DHI test days of the early lactation) in non-treated animals, while it decreased from 155,200 cells mL^{-1} to 88,500 cells mL^{-1} in cows that received long-acting antibiotics at dry off (Figure 1).

The finding of Scherpenzeel and colleagues (2014) of more clinical mastitis cases in non-treated cows, was not confirmed. Over the four herds, 14.2 % of the cows that received dry-cow injectors got at least one case of clinical mastitis in the first 100 days of lactation, versus 13.2 % of the non-treated cows. An effect of internal teat sealants, used in every quarter of the present study, may explain this difference.

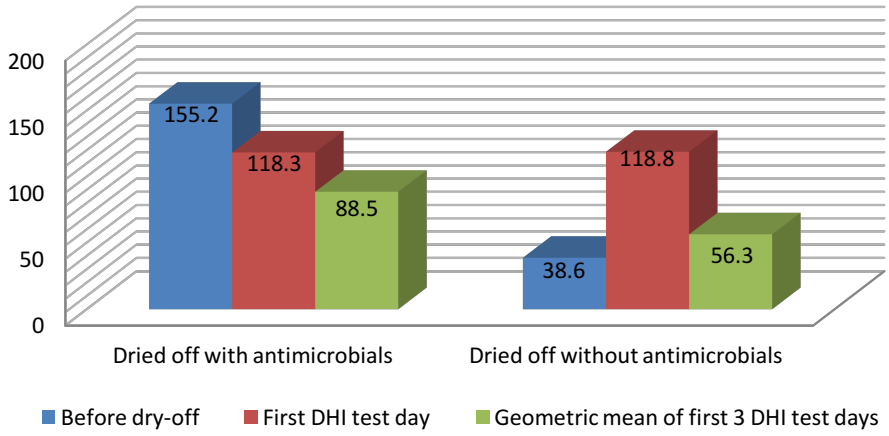

Figure 1: Individual somatic cell count (1,000 cells mL^{-1}) in early lactation of cows dried off with compared to without long-acting antimicrobials.*

During the study period, the BMSCC remained low or improved on each of the 4 herds with an average of 155,000 cells mL^{-1} (range 83 – 256,000 cells mL^{-1}).

Every animal received, on average, antimicrobial treatment during 6.4 days per year (range 5.1 – 7.7) compared to 8.0 days (range 5.8 – 9.9) in a comparable period before the study, resulting in a 19.7% overall reduction. The largest reduction of antimicrobial consumption on herd level was 41.1 % (herd A), one herd however had a slightly increased DDDA (+ 4.1 %, herd D) (Figure 2). The consumption of critically important molecules for systemic use ("red category") decreased by half (1.9 to 0.9 days). A shift from red and critical orange (overall 3.4 DDDA before to 1.3 DDDA during the study; - 62.2 %) to basic orange and yellow (overall 4.6 DDDA before to 5.2 DDDA during the study; + 11.3 %) was visible.

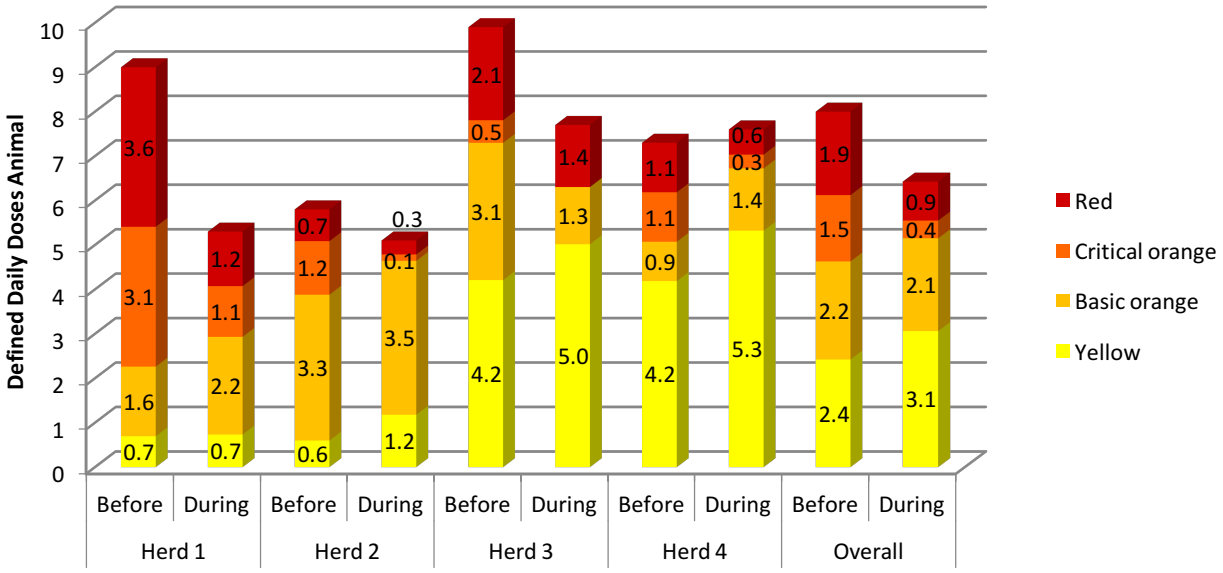

Figure 2: Antimicrobial usage on the four Flemish dairy farms expressed as defined daily doses animal, during (October 2013 – March 2015) versus before (October 2011 – March 2013) the study and categorized in red – orange (critical and basic) – yellow antimicrobial classes.

Conclusions

We propose selective dry cow therapy based on protocol_3 (Table 1), besides the use of internal teat sealers and a meticulous (dry cow) management. The protocol_3 seems to be (1) applicable in the field, (2) rendering an acceptable number of cows to be dried off without long-acting antimicrobials (i.e. one third), and (3) keeping udder health in the next lactation on a solid level. Extra tools, *e.g.* real-time PCR targeting mastitis pathogens on cow level or standard culture on quarter level, were not critical to obtain good results but might enhance trust and therefore, might help to extend the implementation of selective dry cow therapy. Selective dry cow treatment together with monitoring or improving udder health, can lead to a responsible and even decreased use of antimicrobials, especially of those critical molecules.

Acknowledgements

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IN VITRO GROWTH INHIBITION OF *BOVINE* INTRAMAMMARY STREPTOCOCCI AGAINST B-LACTAMS

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Abstract

Mastitis is the economically most important disease in dairy herds. Determining the causative agents of intramammary infections and testing its susceptibility to antimicrobials is a first step in justified treatment. However, the availability of veterinary clinical breakpoints for classifying pathogens as resistant or susceptible is a bottleneck. Also, little is known on epidemiological cut-off values. In Flanders, streptococci are identified as the most prevalent pathogens isolated from milk from intramammary infections. To provide an epidemiological insight in the growth inhibition of the *bovine* intramammary streptococcal population, disk diffusion results of β-lactams were compared. Inhibition zone diameters of 3,038 *Streptococcus uberis* and 1,299 *Streptococcus dysgalactiae* were examined through histograms. Comparison of the distribution of strains over the inhibition zone diameters for most β-lactams showed high similarity between the streptococci. For *Strep. dysgalactiae*, a Gaussian curve was also displayed for oxacillin, while *Strep. uberis* displayed a totally different pattern, a bimodal curve widely distributed with most strains located at the lowest diameters. The data point out that (1) streptococci show a species-specific epidemiology in growth inhibition against oxacillin, demanding species-specific epidemiological cut-off values, and (2) if oxacillin is used to predict cloxacillin susceptibility, clinical breakpoints might be necessary as well.

Introduction

Mastitis, in its clinical or subclinical form, remains the costliest disease on dairy farms worldwide. The majority of antimicrobials used on dairy farms is applied as intramammary treatment as part of udder health management (Scherpenzeel *et al*., 2014). The disease can reveal itself as a clinical infection with visible signs as abnormal milk, a swollen udder (quarter) and/or a sick cow with fever, loss of appetite, and in severe cases even death of the animal may follow. Besides the clinical form, subclinical mastitis is often present. In this case, no abnormalities are visible. The infection is detectable by *e.g*. an elevated somatic cell count and a positive bacteriological culture result of a milk sample. A survey on dairy herds in Flanders, the northern part of Belgium, revealed that 41 % of the cows had a subclinical intramammary infection (Piepers *et al*., 2007). For clinical mastitis on the other hand, the mean incidence rate in Flemish dairy herds was estimated at 7.4 quarter cases/10,000 cow-days at risk and showed a high between-herd variation (Verbeke *et al*., 2014). In The Netherlands, 26 cases per 100 cows per year were registered (Barkema *et al*., 1998). Along with the disease, farmers are confronted with production losses, animal discomfort, disturbance in the milking routine, and treatment costs.

In Flanders, the most frequently isolated major pathogens from subclinically infected cows are *Streptococcus uberis* and *Staphylococcus aureus* (MCC, 2015). *Strep. uberis*, *Escherichia coli*, *Staph. aureus* and *Strep. dysgalactiae* are isolated from clinical mastitis cases (MCC, 2015). Investigating the antimicrobial resistance pattern of a bacterium is frequently performed by means of the disk diffusion method in routine veterinary labs due to the practical and economic factors. To inform the farmer and/or veterinarian on the most appropriate choice of therapy, clinical breakpoints are required. There are often, however, unavailable for the particular combination of the pathogen/antimicrobial per host species. As an alternative for the division into susceptible, intermediate susceptible, or resistant, a bacterial population can be divided into "wild type" and "non-wild type" population based on the distribution of inhibition zone diameters.

In this paper, a description of the antimicrobial resistance profile of the most prevalent *Streptococcus* species in Flanders (*Strep. uberis* and *Strep. dysgalactiae*) is presented according to both clinical and epidemiological criteria.

Materials and Methods

Bacteriological culturing

Analyses were performed at the laboratory of the Milk Control Centre Flanders (MCC). This organization is authorized to perform the official analysis of bulk milk samples of the Flemish dairy herds, on milk quality and composition. Besides that, MCC runs a routine lab for bacteriological culturing of milk samples.

Quarter milk samples presented at the lab during a 32-month period (January 1st 2013 until August 31st 2015) were taken into account. The samples were send to the lab on voluntary basis by farmers or their veterinarian, and originated from (sub)clinical intramammary infections. If multiple samples from the same farm were submitted on the same day, this set of samples was referred to as one 'dossier'.

For standard culturing, the guidelines of the National Mastitis Council were followed (NMC, 1999). Briefly, a 0.01 mL loop of milk was spread on a quadrant of an aesculin blood agar plate (Oxoid, Basingstoke, UK) and incubated aerobically at 37°C for 36 ± 12 h. Milk samples showing abnormal milk were plated also onto a McConckey agar plate (Oxoid). A quarter was considered culture-positive when growth of one or more colonies was detected. Phenotypic features were examined after 24 and 48 h. Growth characteristics, Gram-staining and/or presence of growth on the McConckey plate were used to distinguish Gram-negative from Gram-positive bacteria. The catalase test was used to distinguish staphylococci (positive reaction) from the *Streptococcus-Enterococcus* group (negative reaction). Isolates from the *Streptococcus-Enterococcus* group were divided based on the aesculin reaction. Within the aesculin-negative cocci, the CAMP-test allowed to distinguish *Streptococcus dysgalactiae* from *Strep. agalactiae*. Within the aesculin-positive cocci, the growth characteristics (including colour), bile aesculin agar and NaCl 6.5% were used to differentiate *Strep. uberis* from other aesculin-positive cocci (enterococci, lactococci, and aerococci). If no conclusive identification could be made, the API® strep was carried out as prescribed by the manufacturer. A milk sample was defined as contaminated if > 2 different colony types were present.

Antimicrobial susceptibility testing

Not all strains of the species of interest (*Strep. uberis* and *Strep. dysgalactiae*) were subjected to susceptibility testing. Of each dossier (this is the set of milk samples from one farm arriving in the lab at the same day), one strain per species of interest was selected, namely the strain isolated in the quarter with the highest somatic cell count (data on somatic cell count not presented). This resulted in 3,038 *Strep. uberis* strains and 1,299 *Strep. dysgalactiae* strains.

Selected strains were subjected to the Kirby-Bauer disk diffusion method. In short, with the InoClic® system (i2a, Perols, France) used as described by the manufacturer, a clearly separate colony of the pathogen of interest was picked and suspended in 5 mL saline solution, resulting in 0.5 McFarland. The suspension was used for flooding the Mueller Hinton agar plates supplemented with 5% horse blood and 20 mg L^{-1} NAD (Biomerieux, France). Antibiotic impregnated paper disks were combined in a Gram-positive panel, and disks were applied with a dispenser. The antimicrobial agents were selected according to their occurrence in commercially available products for mastitis treatment and/or dry-cow therapy. In this study, the results for molecules from the β-lactam group were presented. These were ampicillin, amoxicillin/clavulanic acid, cephalonium, cefquinome, and oxacillin. Disks were purchased from i2a, except for cephalonium and cefquinome (Mast Group, Merseyside, UK). Disks contents were indicated in Table 1. After an overnight incubation at 35 ± 2°C, plates were read with the SIR scan Micro (i2a).

Evaluation of the inhibition zone diameters

The inhibition zone diameters were registered for each pathogen/antimicrobial combination. By using histograms, a graphical insight was given in the distribution of the tested strains over the inhibition zone diameters measured. When available, clinical breakpoints as provided by the Clinical and Laboratory Standards Institute (CLSI, 2008) or by the disk supplier were displayed in the histogram (see Table 1 and Figure 1).

Table 1. Overview of the characteristics of the antimicrobial impregnated disks.

Antimicrobial	Disk content	Clinical breakpoint (in mm)	Reference breakpoint
Ampicillin	10 µg	18	CLSI Veterinary
Amoxycillin + clavulanic acid	20/10 µg	13	CLSI Veterinary
Cephalonium	30 µg	13	Mast Diagnostics
Cefquinome	30 µg	18	Mast Diagnostics
Oxacillin	1 µg	-	-

Results and discussion

In this paper, antimicrobial susceptibility of the frequently isolated major pathogens *Streptococcus uberis* and *Strep. dysgalactiae* originating from milk samples of (sub)clinical mastitis in dairy herds in Flanders was presented. The samples were not originating from a random sample of herds and cows but were recovered during an approximate 2.5-year period in the routine lab. The followed protocol prescribed to select only one isolate of each pathogen of interest from each dossier for susceptibility testing, due to economic restrictions. This data set is thus a convenience sample. However, as a large number of strains is included from different herd types it will give an acceptable insight in the Flemish situation. Growth of a single colony from a 0.01-mL milk sample, on a single sampling occasion, was defined as a positive sample. This protocol provides high sensitivity but lower specificity compared to a proposed gold standard of triplicate milk samples (Dohoo *et al.*, 2011), which meets the objectives of most routine labs.

To examine the antimicrobial resistance of bacteria, a variety of methods is available. Routine labs strive for a quick test result and, more than in human medicine, veterinary labs are impeded by economic restrictions. Therefore, disk diffusion is most frequently used in these labs (CLSI, 2008), as is done in the lab described in this paper. For the disk diffusion method as

well as other tests, however, appropriate breakpoints are a prerequisite to differentiate susceptible from resistant bacteria and to guide a therapeutic approach (clinical breakpoints). Using the available veterinary clinical breakpoints for ampicillin, amoxicillin/clavulanic acid, cephalonium and cefquinome, we identified only a minority of resistant *Strep. uberis* and *Strep. dysgalactiae* strains. In fact, 99.8 – 100 % of the strains were susceptible to the tested β-lactams.

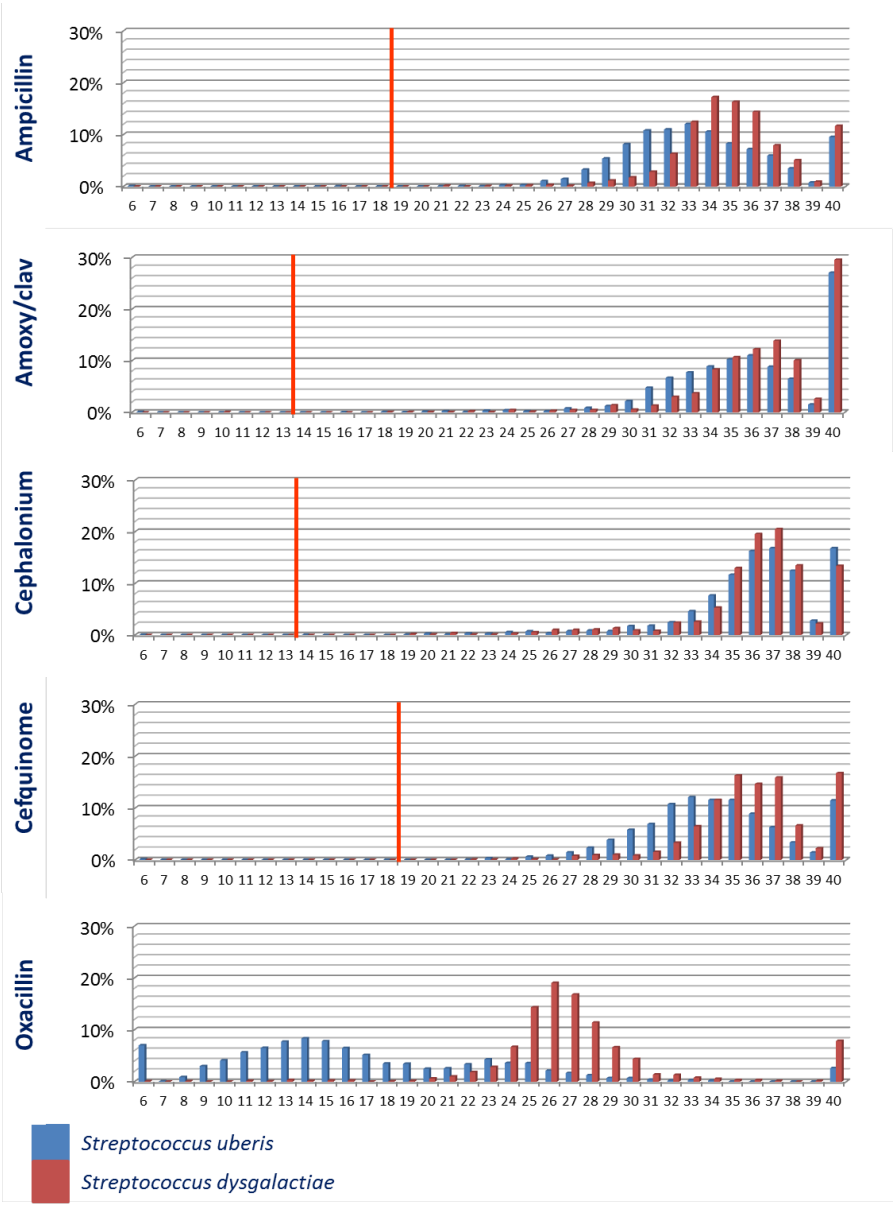

Figure 2. Distribution of strains of Streptococcus uberis *(n = 3,038) and* Strep. dysgalactiae *(n = 1,299) over the disk diffusion inhibition zone diameters. The red line indicates the veterinary clinical breakpoint.*

Because of the lack of a veterinary clinical breakpoints, no division in susceptible/resistant was made for the two streptococci against oxacillin. As an alternative, one could look at the epidemiological behaviour of the strains like the distribution of the tested strains over the inhibition zone diameters. Using this approach, the population can be divided into a “wild type” and a “non-wild type” population by epidemiological cut off values, where strains belonging to the non-wild type underwent an alteration leading to a potential diminished reaction to antimicrobials (EUCAST).

The epidemiological behaviour of this mastitis sub-population is shown in Figure 1. Histograms for all β-lactams, except for oxacillin, are very similar. For susceptibility of *Strep. dysgalactiae* to oxacillin, only a shift in mean inhibition zone diameter is visible; the majority of the strains still belonging to the putative wild type population. It is clear that for *Strep. uberis* against oxacillin, however, a bimodal curve was displayed with the majority of strains situated at smaller zone diameters (putative non-wild type). The pattern is clearly distinct from to the other antimicrobials tested, and the division of the population into

wild and non-wild type was ambiguous. In an earlier study in Germany using MIC tests, the distribution over the concentration seems also widespread over different MIC values measured (Germ-Vet, 2009).

In Belgium, cloxacillin is frequently used for (intramammary) mastitis treatment of lactating cows as well as for dry cow treatment, and its equivalent oxacillin is added to the antimicrobial panel at MCC upon request of the veterinarians. The use of oxacillin disks to examine streptococci could be discussed, as no clinical breakpoints nor epidemiological cut off values are available and, in addition, penicillin is the preferred molecule to test β-lactam susceptibility. It seemed however that when testing *Strep. dysgalactiae* against oxacillin, the strains followed a distribution comparable with the other β-lactams tested, indicating that using oxacillin disks might be possible for streptococci. *Strep. uberis* however displayed an exceptional curve. For other antimicrobials of the β-lactam group, this difference between the streptococcal species is not visible (Figure 1). Whether or not these interspecies incongruence is merely a laboratory observation or this has also an effect in the field, has to be determined. Obviously, veterinary cut off values (as well as clinical breakpoints) for mastitis pathogens should be defined with detail on species level, at least for *Streptococcus* species as it is a very heterogeneous group of organisms.

Conclusions

Clinical breakpoints as well as epidemiological cut off values are often unavailable for studying veterinary pathogens by disk diffusion or other methods, and in addition, epidemiological cut off values and clinical breakpoints are not (necessarily) linked (Schwarz *et al*., 2010). Analysing data based on epidemiological criteria is not the main objective of many routine labs. However, these routine labs often have access to a large amount of data which can be used to perform studies on specific populations. By using a high number of strains, a glance at the situation in the field of *bovine* mastitis in Flanders is provided in this study.

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SCREENING FOR VETERINARY DRUG RESIDUES AND STEROIDS IN MEAT USING HRMS AND A DATA-INDEPENDENT ACQUISITION MODE

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Abstract
A data-independent LC-HRMS^{E} screening approach for more than 250 compounds belonging to 28 different classes of veterinary drugs and steroids is presented. The sample preparation method is based on an LC-MS/MS screening used in routine. Compared to this LC-MS/MS method the LC-HRMS^{E} method allows screening and simultaneous confirmation of the identity of the detected compounds. Ten meat samples were spiked with a total of 50 veterinary drug residues or steroids at maximum residue level (MRL) or minimum required performance limit (MRPL). These samples were analysed in three different resolution settings both in positive and negative electrospray ionisation. Two of the three resolution settings were able to detect about 70% of the spiked compounds indicating that further optimisation of the method is necessary in order to have equivalent results as obtained with the LC-MS/MS screening. However, the LC-HRMS^{E} screening method has the added value that within the same analysis the identity of 50% of the detected compounds could be confirmed without reanalysing the samples.

Introduction
Where the use of (ultra) high performance liquid chromatography ((U)HPLC) coupled to low resolution (multistage) mass spectrometry (MS(/MS)) for (multi)residue analysis has become well established since the first articles on this topic were published in the nineties, the use of high resolution mass spectrometry (HRMS) in residue analysis is still very recent. Furthermore, when using LC-MS/MS, clear criteria for screening and identification are described by *e.g*. Commission Decision 2002/657/EC, but in the case of LC-HRMS identification criteria are still the subject of much debate.

High resolution mass spectrometry is gaining popularity and is most often used in profiling and/or "-omic" studies. Compared to low resolution mass spectrometry, HRMS has the advantage of measuring the accurate mass of all ionised compounds (compounds of interest and matrix compounds) and as such interfering compounds can be filtered out using mass clean-up techniques. Furthermore, by acquiring full scan data, retrospective analysis can be done on the obtained data. This last feature is used in the *in silico* analysis of prior analysed sample in order to investigate the presence of "newly discovered" compounds. Finally, with HRMS there is the possibility to work in the MS^{E} data-independent acquisition mode. In MS^{E} mode full scan accurate mass fragmentation spectra of all compounds present in a sample are recorded simultaneously with the accurate mass of the compound itself. Where this type of acquisition mode is typically used for structural elucidation of non-targeted analytes in different "-omic" approaches, this type of acquisition together with the other above described properties of HRMS offers an advantage for HRMS screening compared to LC-MS/MS screening as within one analysis the identity of the detected compounds may be confirmed.

Materials and Methods

Reagents and chemicals
Analytical reference standards of the >250 veterinary drugs and steroids belonging to 28 different classes were purchased at Sigma-Aldrich, Witega, J.H.Ritmeester, BOC Sciences, TRC, Dr. Ehrenstorfer, Tebu-Bio, WIV, Rikilt, Acros Chemicals or LGC Promochem. Acetonitrile and methanol were of LC-MS grade for the LC-MS/MS analysis and of UPLC grade for LC-HRMS^{E} analysis. Both grades were purchased at Biosolve (Valkenswaard, Nederland) as was the formic and acetic acid. Sodium sulphate was supplied by Merck (Darmstadt, Germany).

Sample preparation
Prior to sample preparation, meat samples were grinded and 5 g (±0.1 g) was weighed accurately into a polypropylene tube. Fifteen grams of sodium sulphate was added to dry the meat prior to extraction with two times 15 mL of acetonitrile/methanol (95/5, v/v). The supernatant was combined and 15 mL was evaporated to dryness at 40°C under nitrogen atmosphere. Finally, the dried residue was dissolved in 1 mL water/methanol/acetonitrile (50/25/25, v/v/v) and 0.05% acetic acid, filtrated through a 0.22 µm filter and transferred into an HPLC vial.

Sample preparation for screening experiment
In order to evaluate the screening approach using the data-independent acquisition mode of the high resolution mass spectrometer ten meat samples were each spiked with five veterinary drug residues or steroids at either MRL or MRPL, resulting in 50 compounds with concentrations between 0.3 and 200 µg kg^{-1}.

LC-MS/MS analysis
For LC-MS/MS analysis an Acquity UPLC© - Xevo TQ-S (Waters, Milford, MA, US) instrument was used. Of the final extract 5 µL was injected onto an Acquity UPLC© BEH C18 (150 x 2.1 mm, 1.7 µm) (Waters, Milford, MA, US) column. The flow rate was 0.4 mL min^{-1} and separation of the target analytes was achieved using the gradient elution program of Table 1. Compounds are ionised using electrospray positive or negative mode and for each compound 1 MRM transition was monitored during the screening analysis. Confirmation of the identity was obtained by re-injection of the sample, monitoring at least two MRM transitions and fulfilling the identification criteria mentioned in Commission Decision 2002/657/EC.

Table 1. Gradient elution program.

Time	A: H_2O and 0.05% acetic acid	B: Acetonitrile/methanol (50:50, v/v) and 0.05% acetic acid
0	100	0
2.45	100	0
14.45	5	95
18.95	0	100
23	100	0

LC-HRMS^E screening
High resolution screening was performed on an Acquity UPLC© - Synapt G2 S (Waters, Milford, MA, US) instrument. Screening capability of three different resolutions were compared: sensitivity mode (≈ 15,000 FWHM), resolution mode (≈ 20,000 FWHM) and high resolution mode (≈ 30,000 FWHM). For each resolution setting, samples were analysed in positive and negative electrospray and in each run samples were analysed using the data-independent MS^E mode where two simultaneous full scan acquisitions were recorded: one using no collision energy (low energy) and one using a collision energy ramp 8-40 eV (high energy). Chromatographic conditions were equal to the ones used during LC-MS/MS analysis (see above).

HRMS^E database construction
On an earlier occasion an LC-HRMS^E database containing information on more than 250 veterinary drugs and steroids belonging to 28 different classes was constructed. To construct this database, standards of each of the veterinary drugs and steroids were analysed in MS^E resolution mode both in positive and negative electrospray ionisation. For each compound the molecular formula, exact mass, ionisation mode, retention time and MS^E fragments were added to the database using Microsoft Excel®. This resulted in a database containing 341 entries.

Results and discussion
The obtained extracts were split into two vials and one aliquot was analysed with the LC-MS/MS screening method containing 261 MRM transitions divided into different time windows. The second aliquot was analysed with the LC-HRMS^E screening method where each aliquot was injected once in positive electrospray and once in negative electrospray mode and this for the three investigated resolution settings (≈ 15,000, ≈ 20,000, ≈ 30,000 FWHM).

For the samples analysed with the LC-MS/MS screening method 261 MRM transitions per sample had to be verified in order to identify the compounds spiked in ten meat samples. Doing so two compounds, medroxyprogesterone spiked at 1 µg kg^{-1} and 2-mercaptobenzimidazole spike at 2.5 µg kg^{-1}, out of 50 could not be detected. In one sample a peak was found at the same retention time as narasin, although this compound was not spiked.

The LC-HRMS^E screening / confirmation workflow starts with Chromalynx™ (Waters, Milford, US). This software package deconvolutes the HRMS^E data and compares the found masses to the exact mass of the compounds in the constructed database, taking into account the retention times as mentioned in the database. For screening purposes, a tolerance of 10 mDa was used on the accurate mass and 0.5 min on the retention times mentioned in the database. Once all samples were processed with Chromalynx™, the POSI±IVE™ software (Waters, Milford, US) selects only the compounds that were detected during the Chromalynx™ processing and automatically creates a Targetlynx™ (Waters, Milford, US) file. This file contains for all analysed samples the integrated chromatograms of the parent ion, recorded in the low energy trace, and the chromatograms of the MS^E fragments (as mentioned in the database), recorded in the high energy trace. Furthermore, Targetlynx™ compares the retention time and the ion ratios of the different fragments to these of the same compound as detected in a standard mixture analysed in the same sequence as the samples.

A compound was suspected to be present in the samples (screening) when a peak with the same accurate mass could be detected within a tolerance of 10 mDa and the retention time was equal (± 0.2 min) to the retention time of that compound in the standard mixture. Table 2 gives an overview of the obtained results for the screening in the different resolution settings.

Table 2. Results for screening and confirmation of the LC-HRMS^E data in the different resolution settings.

	Sensitivity mode	Resolution mode	High resolution mode
Screening			
Number of correctly found compounds during screening (%)	34 (68%)	35 (70%)	23 (46%)
Confirmation			
Percentage of positive screened compounds confirmed (%)	19 (56%)	19 (54%)	7 (30%)

Sensitivity mode (≈ 15,000 FWHM) and resolution mode (≈ 20,000 FWHM) gave comparable results and were able to detect about 70% of the spiked compounds. The presence of the compounds that were not detected with the screening workflow were controlled manually by extracting the accurate mass of the precursor ion within a window of 20 mDa. None of these compounds could be detected indicating that the fact that these compounds were not detected during the screening was not because of the software, nor because of the applied criteria (10 mDa, 0.5 RT) but because of sensitivity issues. Looking further into detail learned that the concentration of these compounds was between 0.3 and 50 µg kg^{-1} with the majority having a concentration ≥ 2.5 µg kg^{-1}. Knowing that the screening workflow was able to detect eleven compounds with concentrations ≤ 2.5 µg kg^{-1} it is clear that it is not the concentration of the compound that explains why certain compounds are not detected but rather the extraction or ionisation efficiency As seen during the LC-MS/MS screening, also the LC-HRMS^E screening, both in sensitivity as in resolution mode, gave one false positive result for narasin. In high resolution mode the time-of-flight tube of the Synapt G2 S is used in "W-mode", where in sensitivity and resolution mode it is used in "V-mode". By prolonging the flight path resolution is increased but sensitive decreases. This is also confirmed in the screening results as less compounds were detected in this mode.

The advantage of working with the data-independent acquisition mode is that simultaneous to the acquisition of the accurate mass of the compounds present in the sample, a high energy trace is recorded which contains fragmentation data of all ionised compounds. Using the above described POSI±VE screening workflow not only results in chromatograms with the accurate masses of the compounds found, but also returns chromatograms of the fragment ions present in the database and allows simultaneous confirmation of the detected compounds. To confirm the identity of the detected compounds following identification criteria for HRMS analysis were used: mass deviation ≤ ± 5ppm, RT ≤ 0.2 min, at least one fragment ion with ion ratio deviation within tolerance compared to analysed standard. As such at least 50% of the compounds detected during the screening in sensitivity or resolution mode could simultaneously be confirmed. For the other compounds either the detected mass deviation was ≥ 5ppm, no fragments were detected, ion ratios deviated more than 50% (mostly because obtained fragments had low abundance) or a combination of these. Consequently, a dedicated MRM-analysis needs to be done in order to unequivocally confirm the presence of these compounds.

Conclusions

A data-independent high resolution MS screening method and workflow was presented. This screening method and workflow allows the simultaneous screening and confirmation of veterinary drugs and steroids in meat samples. Best results were obtained in either sensitivity mode (≈ 15,000 FWHM) or resolution mode (≈ 20,000 FWHM) which allowed to detect 70% of the spiked compounds during screening. Increasing the resolution of the LC-HRMS to ± 30,000 FWHM drastically reduced the sensitivity which resulted in less than 50% of the spiked compounds being detected. A further in-depth look at the obtained screening results learned that the obtained false negative results were caused by sensitivity issues for certain compounds and further optimisation of extraction/clean-up or ionisation efficiency is needed.

The major advantage of the above described data-independent LC-HRMS^E approach is that it allows simultaneous confirmation of the identity of the compounds detected during the screening without reanalysis. This was demonstrated above where the identity of at least 50% of the compounds could be confirmed without reanalysing the samples, this in contrast to the routinely used screening method using LC-MS/MS where each suspected sample needs to be reanalysed using a dedicated MRM.

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DEVELOPMENT AND VALIDATION OF A MULTI-RESIDUES CONFIRMATORY METHOD FOR DETERMINATION OF MACROLIDES, LINCOSAMIDES AND PLEUROMUTILINES BY HPLC-MS/MS

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Abstract

A rapid and sensitive method was developed for the determination of multi-class antibiotics residues (eight macrolides, three lincosamides and two pleuromutilins) in meat by HPLC-MS/MS. Sample preparation included acetonitrile extraction, followed by SPE extraction with C18 cartridges. Methanol was used for elution with subsequent evaporation. The final extract was injected on reversed phase HPLC column with 20 min run duration. The mass spectrometer was operated in positive electrospray mode and multiple reactions monitoring mode. Isotopically-labelled standards were used for better quantification accuracy. At least two transitions were registered for each compound.

The method was validated according to national guide RMG 61. The whole validation study consisted of four individual experiments with two factors: operator and storage time before LC-MS/MS analysis. The expanded uncertainty for spiramycin was 32% in range of 2-160 µg kg^{-1} and 24% in range of 161-320 µg kg^{-1}; for erythromycin 34% in range of 10-40 µg kg^{-1} and 19% in range of 41-320 µg kg^{-1}; for tylosin 33% in range of 1-20 µg kg^{-1} and 18% in range of 21-160 µg kg^{-1}; for lincomycin 31% ranging from 1 to 160 µg kg^{-1}; for valnemulin 67% in range of 1-20 µg kg^{-1} and 22% in range of 21-160 µg kg^{-1}. The expanded uncertainty for tilmicosin, tylvalosin, tulathromycin, clarithromycin, clindamycin, pirlimycin and tiamulin was less than 43%.

Introduction

Macrolides' history of using dates back almost 50 years. It is one of the most interesting and promising classes of antibiotics. The basis of its chemical structure is macrocyclic lactone ring to which are attached one or more carbohydrate moieties as side chains. The antimicrobial activity of these compounds is due to a violation of protein synthesis at the stage of translation in cells of susceptible bacteria that leads its high effectivity against Gram-positive and Gram-negative cocci. Moreover, they are the most efficient medicine against diseases caused by Mycoplasma species. Macrolides are one of the safest classes of medical drugs.

Antibiotic residues may have direct toxic effects on consumers, *e.g*. allergic reactions in hypersensitive individuals, or may indirectly cause problems through the induction of resistant strains of bacteria.

Therefore, simple and reliable analytical methods are required to monitor these drug residues in the edible tissues of livestock animals. There is a number of analytical techniques such as ultraviolet (UV), high performance liquid chromatography (HPLC), capillary electrophoresis, various electrochemical detections, near infrared (NIR) and LC/MS used for determination of antibiotics with lactone ring, but they are usually restricted to the number of compounds which could be determined simultaneously.

LC-MS/MS was the technique of choice due to its high specificity and sensitivity. Monitoring of two product ions gives sufficient data to confirm the identity of a substance found in the complex matrices. Consequently, a confirmatory method using LC-MS/MS would be a supplement to existing screening methods. This study aimed to develop a multi-residue method for screening and confirming macrolides, lincosamides and pleuromutilins residues in meat by triple quadrupole mass spectrometry after a simple liquid-liquid extraction.

Methods

Standards

Spiramycin, erythromycin, tylosin, clarithromycin, lincomycin, clindamycin, valnemulin and tiamulin were purchased from Sigma (MO, USA). Tilmicosin, tulathromycin, pirlimycin, tylvalosin, roxitromycin-d7, azithromycin-d3, clindamycin-d3 and valnemuline-d6 were obtained from TRC (Canada). Troleandomycin was acquired from Santa Cruz (USA).

Instrumentation

The HPLC system was HP 1200 (Agilent Technologies, USA), consisted of a quaternary pump, a vacuum degasser, column compartment and an autosampler. The separation was achieved on a Pursuit C_{18} (5 µm, 150x2 mm) analytical column (Agilent Technologies). The triple quadrupole mass spectrometer 5500 QTRAP (Applied Biosystems/MDS Sciex, USA) was coupled to HPLC using an electrospray ionization interface in positive ionization mode (ESI+). Data acquisition was conducted

with help of Analyst 1.6.1 software (Sciex, Canada). Parameters for multiple reaction monitoring (MRM) transitions are presented in Table 1.

The injection volume was 20 µL and the analysis was carried out with gradient elution using solvent A (water containing 0.5% formic acid) and solvent B (acetonitrile) at a flow rate 0.2 mL min^{-1}. The gradient was 100 % A till 1 min, then went from 100% A to 5% A for 10 min, then changed from 5 % to 100 % for 0.1 min and then equilibrated for 9 min at 100% A. The ESI/MS/MS conditions were set as ionspray voltage (IS) 5,500 V and collision gas (CAD) 6 psi. The heater gas temperature (TEM) was set at 500°C. MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of triple-quadrupole mass spectrometer. MRM experiments were performed using a dwell time 50 ms and the collision energy, depending on the compound (shown in Table 1). Two transitions were used for the identification of each analyte but only one most intense was used for quantification.

Table 1. MS/MS for MRM transitions.

№	Analyte	Precursor ion (*m/z*)	Transition products (*m/z*)	Retention time (min).	Collision energy (V)/ Collision cell exit potential (V)
1	Spiramycin	422,2 (+)	100,9/174,1	7,04	22/16, 25/28
2	Erythromycin	734,5 (+)	158/576,3	8,09	35/21, 27/32
3	Clarithromycin	748,5 (+)	590,3/158	8,78	25/24, 33/24
4	Tulathromycin	807,0 (+)	577,3/158	6,42	33/27, 48/8
5	Tilmicosin	869,6 (+)	174,2/696,3	7,53	55/23, 53/35
6	Tylvalosin	1042,6 (+)	229/174	9,58	35/25, 33/28
7	Troleandomycin	814,5 (+)	200,2/628,1	9,35	33/17, 27/20
8	Tylosin	916,5 (+)	772,1/174	8,20	49/22, 39/36
9	Lincomycin	407,2 (+)	359,2/126	6,05	28/28, 35/14
10	Clindamycin	425,2 (+)	377,1/126	7,41	25/26, 35/12
11	Pirlimycin	411,2 (+)	112/363	7,21	31/18, 21/16
12	Valnemulin	565,4 (+)	263/147	8,96	21/30, 51/26
13	Tiamulin	494,3 (+)	192/118,9	8,71	33/54, 57/26
14	Roxitromycin-d7	844,7 (+)	686,4	8,79	31/30
15	Azithromycin-d3	752,7 (+)	594,5	7,09	39/27
16	Clindamycin-d3	428,4 (+)	380/129	7,40	26/26, 35/15
17	Valnemuline-d6	571,6 (+)	269,2/153,2	8,96	23/30, 51/26

Sample preparation

Samples were homogenized and 1 g of each sample was weighed into plastic tube. Samples were fortified by addition of 50 uL of Internal Standard Mixture (concentration 1 µg mL^{-1}). Tubes were vortexed during 1-2 min and left for 10 min in dark place at room temperature. 4 mL of ACN were added to each sample. Tubes were shaken for 15 min following centrifugation during 15 min at 4,000 rpm and 4° C. Liquid layer was transferred into clean plastic tube and evaporated to the volume of 200 uL. Obtained extract was reconstituted in 2 mL of water and carried on C18 cartridge Oasis HLB, previously conditioned with 2 mL of water and 2 mL of methanol. Then cartridge was washed with 2 mL of water and dried under vacuum for 10 min. Eluting was carried out with 3 mL of methanol into new plastic tube. Eluate was then evaporated to the volume of 200 uL in the stream of nitrogen at 40° C. Samples were reconstituted in Solvent A and centrifuged during 15 min at 1,500 rpm and 4°C. Upper layer was transferred into vial for LC-MS/MS analysis.

Validation

The method was validated for pork according to RMG 61-2010 “State system for ensuring the uniformity of measurements. Accuracy, trueness and precision measures of the procedures for quantitative chemical analysis. Methods of evaluation”. The whole validation study consisted of four individual experiments with two factors: operator and storage time before LC-MS/MS analysis. One pork muscle sample was used. The validation levels were 2, 10, 40, 160, 320 ng mL^{-1} for spiramycin, 10, 40, 160, 320 ng mL^{-1} for erythromycin and 1, 5, 20, 80, 160 ng mL^{-1} for other analytes. Quantification was performed using individual matrix calibration curve for each series with same concentration levels for each analyte. In order to prove the specificity and the lack of susceptibility to matrix interferences, several blank samples fortified with internal standard were additionally analysed in each series. The in-house validation study was performed within four weeks.

The specificity of the method was demonstrated as no interfering peaks were observed at the retention time of macrolides, lincosamides and pleuromutilins in a variety of blanks and by analysing structurally related compounds like the labelled internal standards which can be separated from the analytes. The recovery, corrected by matrix calibration and the use of internal standards, lies in the range of 45-74% for all analytes in the validated concentration range and are given in Table 2.

Results

MRLs used in the Russian Federation are presented in Table 2. Measurement range and expanded uncertainty are given in Table 3.

Table 2. MRL and recovery of macrolides, lincosamides and pleuromutilins.

Analyte	MRL (µg kg^{-1})	Recovery (%)
Spiramycin	250	55
Erythromycin	200	74
Clarithromycin	-	65
Tulathromycin	-	52
Tilmicosin	50	69
Tylvalosin	50	67
Troleandomycin	-	64
Tylosin	100	57
Lincomycin	100	61
Clindamycin	100	70
Pirlimycin	100	55
Valnemulin	50	45
Tiamulin	100	65

Table 3. Validation parameters.

Analyte	Measurement range (ng mL^{-1})	Expanded uncertainty (%)
Spiramycin	2-160	32
	160-320	24
Erythromycin	10-40	34
	40-320	19
Tilmicosin	1-100	30
	100-160	18
Tylosin	1-20	33
	20-160	18
Tylvalosin	5-80	32
	80-160	16
Tulathromycin	1-40	43
	40-160	22
Clarithromycin	1-80	37
	80-160	22
Lincomycin	1-160	31
Clindamycin	1-20	37
	20-160	14
Pirlimycin	1-80	26
	80-160	14
Valnemulin	1-20	67
	20-160	22
Tiamulin	1-160	28

Conclusion

Fast and reliable LC-ESI-MS/MS multi-residue method allowing simultaneous determination of eight macrolides, three lincosamides and two pleuromutilins in meat was developed and validated. The validation was carried out according to national guide RMG 61-2010. The methods have been successfully applied for determination of tylosin in honey (Progetto Trieste proficiency testing sample).

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TARGET AND POST-TARGET ANALYSIS OF ANTIBIOTICS IN ANIMAL TISSUE BY UHPLC-HRMS

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Abstract
A very rapid and simple method is proposed for the screening of antibiotics belonging to seven families in muscle and kidney from different animal species by UHPLC-HRMS (Exactive-Orbitrap™). A generic sample preparation using a solvent extraction combined with dispersive solid-phase extraction (DSPE) clean-up was used. Three different solvents were tested during the method development, and a comparative study with QuEChERS™ method was carried out. Ionization parameters were optimised using statistical design of experiments (DoE) to select settings that are suitable for all molecules.
The analytical method was validated according to Commission Decision 2002/657/EC and the guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines of the CRLs (20/1/2010). Specificity, CCβ, cut-off factor Fm and threshold value T were determined for both matrices. A database of about 90 authorized antibiotics was created to carry out target and post-target analysis. The method is being applied to samples from the regulatory control programme and it has just been included in the scope of accreditation of the Public Health Laboratory of Valencia (Spain). Tetracyclines, sulphonamides and quinolones are the most frequently found antibiotics above CCβ.

Introduction
A wide variety of antibiotics is available for therapeutic and prophylactic purposes for food-producing species. EU countries are required to test for the presence of antibiotics and other veterinary residues under their National Plans. The Regulation 37/2010/CE, includes about 90 antibacterial substances in Table 1 with the established MRLs for different animal products and species (Commission Regulation (EU) No 37/2010). Microbiological tests have provided an inexpensive first approach to sample testing that meet the demand of a high number of samples, but the lack of specificity and sensitivity has led to new approaches for the screening in the official control of these compounds.

Liquid-chromatography coupled to mass-spectrometry, in particular LC-MS/MS technology, has been used for both screening and confirmatory purposes (Gaugain-Juhel *et al*. 2009; Granelli *et al*. 2007). The development of new systems of liquid-chromatography coupled to high resolution mass spectrometers is a very challenging alternative that provides not only a target approach to control a very wide range of compounds, but also a post-target strategy to widen the scope of our methods when single stage full scan is applied (Kaufmann *et al*. 2013; Leon *et al*. 2012). This paper describes a very rapid and simple screening method to monitor 39 target antibiotics belonging to seven families by UHPLC-HRMS and the post-target approach to monitor all the compounds included in Table 1 of the Commission Regulation (EU) No 37/2010. The target antibiotics, their molecular formula, theoretical *m/z*, diagnostic ion and retention time are listed in Table 1.

Materials and Methods

Chemicals and reagents
Acetonitrile and methanol were of HPLC and LC-MS grade. Formic acid (purity 98-100%) and water were of hypergrade quality. Octadecyl-functionalized silica gel (C18) dispersant sorbent was obtained from Sigma-Aldrich (Barcelona, Spain). All commercial standards were of high purity and were obtained from Sigma-Aldrich (Barcelona, Spain), Witega (Berlin, Germany), Toronto Research Chemicals Inc. (Toronto, Canada), Acros from Thermo Fisher Scientific (Geel, Belgium).

Sample preparation
Extracts from kidney and muscle samples were obtained with a fast and simple procedure, using water and acetonitrile for the extraction followed by a dispersive solid phase extraction step with octadecyl-functionalized silica gel (C18) sorbent. Aliquots of 1.0 g were weighed in a 50-mL polypropylene tube after homogenization of the tissues. As appropriate, the analytes and internal standards (ISTDs) were added at this stage in order to prepare blank and CCβ quality control samples. A volume of 100 µL ISTD working solution, 2 mL of hypergrade water and 8 mL of acetonitrile were added to real samples and mixed for 10 min in a head-over-head mechanism. Quality control samples were processed together with the real samples. After that, the mixture was then centrifuged at 5000 rpm for 5 min at 15°C. Supernatant was then loaded into a 50 mL centrifuge tube containing the dispersive C18 sorbent followed by 5 min of mechanic shaking. Afterwards, the mixture was centrifuged

Table 1. High-resolution mass-spectrometry parameters and retention time.

Family	Antibiotic	Internal standard	Molecular formula	Theoretical *m/z*	Diagnostic ion		Retention time (min)
Macrolides	Spiramycin	Roxithromycin	$C_{43}H_{74}N_2O_{14}$	842.51455	$[M+2H]^{+2}$	422.26427	5.25
	Neo spiramycin		$C_{36}H_{62}N_2O_{11}$	698.43481	$[M+2H]^{+2}$	350.22496	4.94
	Tylosin		$C_{46}H_{77}NO_{17}$	915.51970	$[M+H]^+$	916.52643	7.07
	Erythromycin		$C_{37}H_{67}NO_{13}$	733.46124	$[M+H]^+$	734.46852	6.95
	Tilmicosin		$C_{46}H_{80}N_2O_{13}$	868.56658	$[M+H]^+$	869.57332	5.95
Lincosamides	Lincomycin	Roxithromycin	$C_{18}H_{34}N_2O_6S$	406.21376	$[M+H]^+$	407.22104	3.66
Sulfonamides	Sulfatiazol	Sulfadimetoxine-d6	$C_9H_9N_3O_2S_2$	255.01417	$[M+H]^+$	256.02089	3.50
	Sulfapyridine		$C_{11}H_{11}N_3O_2S$	249.05775	$[M+H]^+$	250.06447	3.59
	Sulfametoxipiridacine		$C_{12}H_{14}N_4O_4S$	310.07412	$[M+H]^+$	311.08085	4.42
	Sulfametazine		$C_{12}H_{14}N_4O_2S$	278.08429	$[M+H]^+$	279.09102	4.23
	Sulfaquinoxaline		$C_{14}H_{12}N_4O_2S$	300.06865	$[M+H]^+$	301.07537	5.85
	Sulfadimetoxine		$C_{12}H_{14}N_4O_4S$	310.07412	$[M+H]^+$	311.08085	5.68
	Sulfadiazine		$C_{10}H_{10}N_4O_2S$	250.05245	$[M+H]^+$	251.05972	3.22
	Sulfamerazine		$C_{11}H_{12}N_4O_2S$	264,06864	$[M+H]^+$	265.07537	3.75
	Sulfacetamide		$C_8H_{10}N_2O_3S$	214.04176	$[M+H]^+$	215.04849	2.71
Quinolones	Oxolinic acid	Norfloxacine-d5	$C_{13}H_{11}NO_5$	261.06427	$[M+H]^+$	262.07100	6.00
	Flumequine		$C_{14}H_{12}FNO_3$	261.08067	$[M+H]^+$	262.08740	6.92
	Danofloxacin		$C_{19}H_{20}FN_3O_3$	357.14942	$[M+H]^+$	358.15615	4.54
	Ciprofloxacin		$C_{17}H_{18}FN_3O_3$	331.13322	$[M+H]^+$	332.14050	4.42
	Difloxacin		$C_{21}H_{19}F_2N_3O_3$	399.14000	$[M+H]^+$	400.14672	4.70
	Enrofloxacin		$C_{19}H_{22}FN_3O_3$	359.16507	$[M+H]^+$	360.17180	4.49
Tetracyclines	Chlortetracycline	Demeclocycline	$C_{22}H_{23}ClN_2O_8$	478.11429	$[M+H]^+$	479.12157	5.13
	Doxycycline		$C_{22}H_{24}N_2O_8$	444.15381	$[M+H]^+$	445.16054	5.80
	Oxytetracycline		$C_{22}H_{24}N_2O_9$	460.14818	$[M+H]^+$	461.15546	4.01
	Tetracycline		$C_{22}H_{24}N_2O_8$	444.15327	$[M+H]^+$	445.16054	4.18
	Epi-tetracycline		$C_{22}H_{24}N_2O_8$	444.15327	$[M+H]^+$	445.16054	3.73
	Epi-oxytetracycline		$C_{22}H_{24}N_2O_9$	460.14818	$[M+H]^+$	461.15546	4.01
	Epi-chlortetracycline		$C_{22}H_{23}ClN_2O_8$	478.11429	$[M+H]^+$	479.12157	4.39
Beta-lactams	Amoxicillin	Amoxicillin-d4	$C_{16}H_{19}N_3O_5S$	365,10509	$[M+H]^+$	366,11182	2.87
	Ampicillin	Penicillin G-d7	$C_{16}H_{19}N_3O_4S$	349.11018	$[M+H]^+$	350.11690	4.49
	Penicillin G		$C_{16}H_{18}N_2O_4S$	334.09928	$[M-H]^-$	335.10600	7.06
	Penicillin V		$C_{16}H_{18}N_2O_5S$	350.09419	$[M-H]^-$	351.10092	7.60
	Oxacillin		$C_{19}H_{19}N_3O_5S$	401.10509	$[M-H]^-$	402.11182	7.62
	Cloxacillin		$C_{19}H_{18}ClN_3O_5S$	435.06612	$[M-H]^-$	436.07285	7.79
	Dicloxacillin		$C_{19}H_{17}Cl_2N_3O_5S$	469.02715	$[M-H]^-$	470,03387	8.11
	Cefalexin		$C_{16}H_{17}N_3O_4S$	347.09453	$[M+H]^+$	348.10125	4.27
	Nafcillin		$C_{21}H_{22}N_2O_5S$	414.12549	$[M-H]^-$	415.13222	8.16
	Desfuroylceftiofur		$C_{14}H_{15}N_5O_5S_3$	429.02408	$[M+H]^+$	430.03081	4.82
	Ceftiofur		$C_{19}H_{17}N_5O_7S_3$	523.02846	$[M+H]^+$	524.03629	6.17
Internal standards	Roxithromycin		$C_{41}H_{76}N_2O_{15}$	836.52457	$[M+H]^+$	837.53185	7.68
	Sulfadimetoxine-d6		$C_{12}H_8D_6N_4O_4S$	316.11124	$[M+H]^+$	317.11851	5.64
	Norfloxacine-d5		$C_{16}H_{13}D_5FN_3O_3$	324.16460	$[M+H]^+$	325.17188	4.30
	Demeclocycline		$C_{21}H_{21}ClN_2O_8$	464.09864	$[M+H]^+$	465.10592	4.63
	Amoxicillin-d4		$C_{16}H_{15}D_4N_3O_5S$	369,12965	$[M+H]^+$	370,13692	2.85
	Penicillin G-d7		$C_{16}H_{11}D_7N_2O_4S$	341.14266	[M-H]-	342.14994	7.03

again for 5 min at 5000 rpm and 15°C. The whole liquid phase was transferred to a 15 mL polypropylene tube and was evaporated to dryness under nitrogen stream at 45°C. The dry residues were dissolved in 1,000 µL of a mixture water/methanol (90/10, v/v) 0.1% formic acid and 10 µL of the final extracts were then injected in the UHPLC-HRMS system.

UHPLC-HRMS analysis

The ultra-high performance liquid chromatography (UHPLC) system was an Accela™ Thermo Fisher Scientific (Bremen, Germany) equipped with a Kinetex C_{18} XB column (50 x 3.0 mm, 2.6 µm) from Phenomenex®. The flow rate was set at 400 µL min^{-1}. The mobile phase components were solvent A (0.1% aqueous formic acid) and solvent B (methanol containing 0.1% formic acid). The initial mobile phase proportion of 98% (A) was maintained for 0.30 min. After that, solvent A decreased linearly to 20% until 8 min and then decreased linearly to 1% in 0.10 min and was maintained for 0.90 min. Finally, solvent A changed to the initial percentage (98%) in 0.50 min and was kept at 98% for 5.50 min to equilibrate the column before the next injection. The total run time was 15 min. Acquisition and data processing were carried out with ThermoScientific TraceFinder™ 3.1 software.

Mass analysis was performed on the Orbitrap mass spectrometer Exactive™ analyser (Thermofisher Scientific, Bremen, Germany). The system was equipped with a heated electrospray ionisation probe (H-ESI II). Improvements in analyte response were achieved using a statistical design of experiments (DoE). DoE was previously applied to other methods in our laboratory (Roca *et al*. 2014, Coscollà *et al*. 2014).

The system operated at a resolving power of 50,000 FWHM and a maximum inject time of 500 ms. The detection was carried out with the following optimized operational parameters: spray voltage, 2.5 kV for negative mode and 3.0 for positive mode; sheath gas (N2, >95%), 40 arbitrary units (au); auxiliary gas, 10 au; skimmer voltage, 30V; capillary voltage, 50V; heater temperature, 300°C; and capillary temperature, 260°C. The value of mass tolerance was set at 5 ppm and no specific lock mass was used for internal mass axis correction (external mass calibration). For the automatic gain control (AGC) the "Balanced" setting was selected. The scan range acquisition was set from 80 to 1200 *m/z* and the injection was conducted in one run with switching polarity mode (ESI^+ /ESI^-).

Analyte detection in target analysis was based on the presence of the exact mass (± 5 ppm) within a time window of ± 30 s. To evaluate the results, relative response and relative retention time were taken into account, as different internal standards were used for each compound family.

Method validation

The validation of the method was performed according to Commission Decision 2002/657/EC and to the Guidelines for the validation of screening methods for residues of veterinary medicines (Anon., 2010). Detection capability CCβ was assessed and threshold value (T) and cut-off factor (Fm) were determined in muscle and kidney of several animal species. Specificity/selectivity was also studied by evaluating the analytical response for the blank samples.

To determine T, 20 blanks of muscle and 20 blanks of kidney from several species (muscle: *bovine*, *ovine*, *porcine*, *equine*, poultry and rabbit; kidney: *bovine*, *ovine*, *porcine* and *equine*) were tested with the described method. The mean of the relative responses B and the corresponding standard deviation were calculated. The threshold value T was obtained by applying the formula: $T = B + 1.64\ SD_b$. Then, the same blank samples were spiked at the screening target concentration (¼ MRL) for each matrix. The mean relative response M and the corresponding standard deviation were calculated. The cut-off factor Fm was obtained by applying the formula: $Fm = M - 1.64\ SD.$

Antibiotics Database

A screening database of about 90 allowed antibiotics (Table 1 in Regulation 37/2010/CE) was created using TraceFinder 3.1 software. The molecular formula, the theoretical accurate mass of the monitored ions, and the retention time (this only for target compounds) were included in this database. Identification and confirmation settings such as mass tolerance of 5 ppm, a threshold area of 5,000, a S/N ratio threshold of 5.0, RT window 30 s for the molecular ion and, a fit threshold of 90 %, a mass deviation of 5 ppm and a deviation of the intensity of 10 % for the isotopic pattern, were selected.

Post-target analysis

After target analysis, retrospective analysis of samples was carried out. The acquired raw data files were reprocessed to be compared with the database that included target and post-target compounds. Results were evaluated by applying the screening method in the software. Green flags for *m/z* value and isotopic pattern showed the presence of suspicious compounds or tentatively identified compounds.

Results and Discussion

Optimization of ion source parameters

The main factors affecting the ESI ion source efficiency are sheath gas pressure (SGP), auxiliary gas (AG), spray voltage (SV), capillary temperature (CT), capillary voltage (CV), skimmer voltage (SKV) and heater temperature (HT). These seven parameters were firstly investigated by a screening design (Plackett-Burman, PB) in search of the most influencing factors, which were subsequently optimised by a response surface methodology (Central Composite Design, CCD).

Plackett-Burman screening Design (PB). The parameters and ranges investigated in this screening were sheath gas pressure (20-60 au), auxiliary gas (10-20 au), spray voltage (2-4, KV), capillary temperature (100-450°C), skimmer voltage (10-50V), heater temperature (100-500°C) and capillary voltage (20-70 V). In this study, only CT presented a significant effect on the response of a few target compounds. The remaining six factors HT, SGP, SKV, AG, CV and SV had no significant effect. Besides, HT and SGP were also selected for the CCD experiment in order to obtain an accurate value of them.

Central Composite Design (CCD). To run the CCD experiments, the spray voltage was fixed at the standard values provided by the manufacturer for both, positive (3 KV) and negative (2.5 KV) modes; the auxiliary gas and skimmer voltage were fixed at an intermediate value of 10 au and 30 V, respectively, because they had both positive and negative effects. Capillary voltage was set at 50 V because only a positive effect was reported for this parameter. After the CCD experiment, the optimised factor settings obtained were: sheath gas pressure 40 au, capillary temperature 260°C and heater temperature 300°C.

Optimization of extraction solvents

Different extraction solvents were studied to find the composition necessary for a generic extraction method. Water/acetonitrile 20:80 (solvent 1), acetate buffer at pH 4.6/acetonitrile 20:80 (solvent 2) and Mcvaillne-EDTA Na_2 buffer/acetonitrile 20:80 (solvent 3) were tested solvents. The behaviour of the solvents was very similar for kidney and muscle. The number of compounds that improved their response and the number of extracted compounds were the main criteria for the selection of the solvent. There was hardly any difference between solvents 1 and 2, therefore, the selection criterion was the simplicity. Solvent 3 only improved the response for tetracyclines, due to the presence of EDTA Na_2 that chelates the calcium in the medium, but not all the compounds were detected.

Evaluation of the EN QuEChERS method

The EN QueChERS method was also tested and evaluated. It consists of acetonitrile as extraction solvent, $MgSO_4$, NaCl and buffering citrate salts to induce partitioning. For cleanup, a dispersive solid phase extraction combining a primary secondary amine (PSA), anhydrous $MgSO_4$ and C_{18} was used. The same criteria above mentioned were applied in this case. Only sulphonamides improved their response and not all the compounds were detected. The lack of water in the solvent improved the response for the less polar compounds.

Validation

Specificity/selectivity were clearly shown as very low responses were obtained in blank samples and in only some of the compounds. As Fm>B was obtained for all the analytes in both matrices, the number of false negative rate was below 5%. And besides, Fm>T, which means that the rate of false positive was also below 5%. Table 2 shows the CCβ, mean of relative response B, threshold value T and cut-off factor (Fm) for each compound in muscle and kidney.

Applicability

The method was applied to more than 100 field samples from the regulatory control programme and the “screen positive” results were confirmed and quantified by LC-MS/MS. Lincomycin, sulfathiazole, sulfadiazine, enrofloxacin, ciprofloxacin, 4-epi-chlortetracycline, chlortetracycline, oxytetracycline, tetracycline, doxycycline, amoxicillin and penicillin G were “screen positive” target analytes that were confirmed and quantified by LC-MS/MS. However, in the case of sulfacetamide and penicillin G, two “screen positive” results in *ovine* muscle and one “screen positive” result in *bovine* kidney, respectively, were not confirmed. On the other hand, about 50 “screen negative” samples were also analysed by LC-MS/MS and no false negative results were obtained. The screening target concentration was set at the quantification limit of the confirmatory method for practical reasons. Regarding the post-target or retrospective analysis, 50 samples were processed to test suspicious compounds. Trimethoprim in *ovine* muscle, dihydrostreptomycin in *ovine* kidney and spectinomycin in *ovine* and *bovine* kidney were tentatively identified.

Conclusions

A generic screening method for the simultaneous detection of several classes of antibiotics in muscle and kidney from different animal species was set up to be used in official control. The proposed method has just been included in the scope of accreditation of the Public Health Laboratory of Valencia (Spain). After “screen positive” results, confirmatory methods (LC-MS/MS) are being applied. Target and post-target strategies can be carried out and a very wide range of molecules can be determined potentially according to the number of compounds included in the database. The post-target approach makes easier to widen the scope of the method and can be applied in flexible scope schemes for the accreditation according to regulation ISO 17025.

Table 2. Screening validation parameters for animal muscle and kidney.

Antibiotic	Muscle				Kidney			
	CCβ (µg kg^{-1})	B	T	Fm	CCβ (µg kg^{-1})	B	T	Fm
Spiramycin	50	0.000	0.000	0.141	150	0.000	0.000	0.248
Neo spiramycin	50	0.000	0.000	0.153	150	0.000	0.000	0.237
Tylosin	25	0.000	0.000	0.012	25	0.000	0.000	0.074
Erythromycin	50	0.000	0.000	0.150	50	0.000	0.000	0.206
Tilmicosin	13	0.000	0.000	0.035	250	0.000	0.000	1.033
Lincomycin	25	0.000	0.000	0.630	375	0.035	0.262	22.062
Sulfatiazol	25	0.000	0.000	0.061	25	0.000	0.000	0.049
Sulfapyridine	25	0.000	0.000	0.312	25	0.000	0.000	0.227
Sulfametoxipiridacine	25	0.000	0.000	0.283	25	0.000	0.000	0.262
Sulfametazine	25	0.000	0.000	0.421	25	0.000	0.000	0.445
Sulfaquinoxaline	25	0.000	0.000	0.060	25	0.000	0.000	0.076
Sulfadimetoxine	25	0.000	0.000	0.594	25	0.000	0.000	0.578
Sulfadiazine	25	0.000	0.001	0.046	25	0.002	0.013	0.087
Sulfamerazine	25	0.000	0.000	0.238	25	0.000	0.000	0.144
Sulfacetamide	25	0.000	0.002	0.013	25	0.000	0.000	0.028
Oxolinic acid	25	0.011	0.041	1.488	38	0.002	0.012	1.268
Flumequine	50	0.000	0.000	1.631	250	0.001	0.008	4.914
Danofloxacin	25	0.000	0.000	0.638	50	0.000	0.000	1.262
Ciprofloxacin	25	0.000	0.000	0.553	50	0.009	0.085	1.100
Difloxacin	75	0.002	0.013	1.598	150	0.000	0.000	3.901
Enrofloxacin	25	0.002	0.020	0.518	50	0.000	0.000	1.435
Chlortetracycline	25	0,000	0.000	0.718	100	0.012	0.111	3.600
Doxycycline	25	0,011	0.097	1.366	100	0.021	0.202	6.065
Oxytetracycline	25	0,006	0.049	1.211	100	0.103	0.698	5.865
Tetracycline	25	0,011	0.126	1.753	100	0.000	0.000	9.604
Epi-tetracycline	25	0,006	0.052	0.970	100	0.004	0.035	4.559
Epi-oxytetracycline	25	0,000	0.000	0.521	100	0.065	0.314	3.033
Epi-chlortetracycline	25	0,000	0.000	0.332	100	0.000	0.000	1.365
Amoxicillin	12.5	0.000	0.000	0.161	12.5	0.000	0.000	0.107
Ampicillin	12.5	0.000	0.000	0.524	12.5	0.000	0.000	0.329
Penicillin G	12.5	0.000	0.000	0.110	6.25	0.002	0.015	0.167
Penicillin V	6.25	0.000	0.000	0.077	75	0.000	0.000	0.078
Oxacillin	75	0.000	0.000	0.594	75	0.000	0.000	0.707
Cloxacillin	75	0.000	0.000	0.341	75	0.000	0.000	0.480
Dicloxacillin	75	0.000	0.000	0.120	50	0.000	0.000	0.068
Cefalexin	50	0.000	0.000	0.859	75	0.003	0.032	0.551
Nafcillin	75	0.000	0.000	0.667	250	0.000	0.000	0.643
Desfuroylceftiofur	250	0.000	0.000	0.315	250	0.000	0.000	0.079
Ceftiofur	250	0.000	0.000	2.257	12,5	0.000	0.000	0.388

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EXCRETION PROFILE OF 17B,19-NORTESTOSTERONE AND ITS MAIN METABOLITE IN *BOVINE* URINE AFTER INTRAMUSCULAR ADMINISTRATION

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Abstract
The administration of the androgenic anabolic steroid 17β,19-nortestosterone (17β-NT), or its esters, to food-producing animals is prohibited within the European Union. Despite this ban, the 17β-NT is one of the most recurrent forbidden anabolic steroid used in meat producing animals. In order to protect the consumer's health from the exposure to the harmful residues of this compound, it is of extremely importance to improve the effectiveness of the control by increasing the knowledge about the urinary excretion profile of the administered steroids and of their metabolites and of endogenous steroids affected by the exogenous administration. For this purpose, an experimental study was designed to assess the levels of synthetic and endogenous androgen steroids in *bovine* urines following four intramuscular administrations of 17β-NT and the excretion profile of 17β-NT and its main metabolite, 17α,19-nortestosterone (17α-NT). Urine samples collected from *bovines* treated with a 17β-NT and non-treated *bovines* bred under strictly controlled conditions, were investigated for the presence of endogenous and synthetic androgens. For this purpose, an LC-MS/MS method was developed and validated in accordance with Commission Decision 2002/657/EC. The 17α-NT derivative was found in almost all urine samples collected during treatment (concentration interval 0.5 – 25.7 ng mL^{-1}). On the other hand, 17β-NT was never detected.

Introduction
The use of anabolic compounds is prohibited in food producing animals within the European Union (Council Directive 96/22/EC). Despite the ban, 17β,19-nortestosterone (17β-NT) is one of the most frequently misused anabolic steroids in meat producing animals. Detection of its illegal use is often a very difficult issue since the presence of endogenous 17β-NT or some of its metabolites in different species has been demonstrated over the past few years. Therefore, it is extremely important to improve the effectiveness of the Official Controls by increasing the knowledge about the urinary excretion profile of the administered steroids and of their metabolites and the urinary elimination of endogenous steroids affected by an illegal administration. Since few data are available on the excretion profile of 17β-NT in calves, an experimental study was set up to compare the excretion profile of 17β-NT and its main metabolite 17α,19-nortestosterone (17α-NT) in *bovines* after four intramuscular administrations of 17β-NT and in *bovines* bred under strictly controlled conditions. The aim of the experimental plan was to study the elimination patterns of the administered compounds and its main metabolite and to examine the effect of an exogenous administration on the endogenous steroids levels.

Materials and methods

Animals and experimental protocol
Ten Friesian male veal calves were farmed for 6 months under controlled experimental conditions and were fed a liquid milk replacer diet available on the market, usually employed in zootechnical practice conditions with *ad libitum* access to water. During the sixth months of breeding, ten animals received four intramuscular doses of 50 mg of 17β,19-nortestosterone laureate at day 1, day 8, day 15 and day 22 (Decadurabolin® 50 mg mL^{-1}, Intervet, Boxmeer, The Netherlands). Four animals were used as controls. Appropriate measures were taken to avoid any kind of cross contamination between the different animals.

Samples Collection
Urine samples of treated bovines were collected before each administration. After the first and fourth administration, urine samples were collected at 6 h, 12 h (only after the fourth dose), 24 h, 48 h and 72 h. After the second and third administration, urine samples were collected at 6 h. Urine samples were also collected at the 8^{th}, 14^{th} and 16^{th} day after the last dose. Urine samples of control bovines were collected in parallel. Urine samples were collected (taking care to avoid faecal contamination) waiting for spontaneous micturition and were immediately stored in the dark at -20°C until analysis.

Chemicals and Reagents
All solvents were HPLC or analytical grade and purchased from Riedel-de Haën (Seelze, Germany). Water was purified by Synergy UV System (Millipore, Bedford, MA, USA). Sodium acetate anhydrous and β-glucuronidase-arylsulphatase (*Helix*

pomatia) were obtained from Merck (Darmstadt, Germany), this latter was used as supplied. Acetic acid was obtained from Sigma-Aldrich (St. Louis, MO). Bakerbond C18 (500 mg, 3 mL) Solid Phase Extraction (SPE) were supplied by J.T. Baker (PA, USA). 17β-testosterone (17β-T), 17α-testosterone (17α-T), 17β,19-nortestosterone (17β-NT), 17α, 19-nortestosterone (17α-NT) and 17β-testosterone-d2 (17β-T-d_2) were provided by Rikilt, The European Union Reference Laboratory in Wageningen, The Netherlands.

Sample extraction
Samples of *bovine* urine (2 mL) were fortified with 6 mL of acetate buffer solution (0.15 M, pH 4.8). The pH of each sample was adjusted to pH 5.0 with 2 M acetic acid. Urine samples were then spiked with the internal standard 17β-T-d_2, and 50 µL β-glucuronidase-arylsulphatase enzyme solution (*Helix pomatia*) was added and incubated for 12 h at 37°C. The mixture was centrifuged and purified by solid phase extraction (SPE) using a C18 cartridge. After elution with methanol, the solvent was removed under nitrogen stream. The residue was dissolved in 100 µL methanol and injected into the LC-MS/MS system.

LC-MS/MS analysis
Analyses were carried out by using an LC system existing of a Perkin Elmer Series 200 Micro Pump (Perkin Elmer, USA) with a PE Series 200 autosampler. The chromatographic separations were obtained at room temperature using a reversed phase HPLC column Gemini C18 (150 x 2.00 mm I.D., 5 µm) and gradient conditions. The mobile phase was composed of 1 % acetic acid solution and acetonitrile and the flow rate was 0.250 mL min^{-1}. The API 3000 triple quadrupole mass spectrometer was set in positive electrospray ionization (ESI) mode with a source temperature of 450°C; ultra pure nitrogen was used as a curtain and collision gas, and ultra pure air was used as nebulizer and auxiliary gas. The collision energy (CE) and the declustering potential (DP) were adjusted in MRM mode (Multiple Reaction Monitoring) for each transition monitored in order to reach the highest sensitivity for all the analytes. The optimized parameters are reported in Table 1. The full identification of the analytes and the validation of the method was achieved according to the criteria of the Commission Decision 2002/657/EC (Commission Decision 2002/657/EC).

Calibration and quantitation
Urine samples, previously tested and shown to contain no residues of the compounds of interest, were spiked daily with 5.0 ng mL^{-1} internal standard 17β-T-d_2, followed by mixtures of androgens to obtain concentration intervals of 0.5-10.0 ng mL^{-1} for 17β-NT, 17α-NT and 0.5-100 ng mL^{-1} for 17β-T and 17α-T. A good linearity of calibration curves was confirmed for all the analytes of interest at all the concentrations checked, as proved by correlation coefficients, all in excess of 0.9980.

Method Validation
Method validation was performed according to the requirements of the European Decision 2002/657/EC (Commission Decision 2002/657/EC) concerning the performance of analytical methods and the interpretation of results. Parameters taken into account were: specificity/selectivity, linearity, precision, trueness, decision limit (CCα) and detection capability (CCβ). The selectivity/specificity of the present method was assessed directly onto the chromatograms obtained from the blank and fortified urine samples. The occurrence of possible extra-peaks was tested by monitoring the two MRM transitions characteristic for each investigated compound onto the blank matrix chromatograms, in the retention time window expected for the analyte elution. The criteria for molecular identification are those of liquid chromatography coupled to tandem mass spectrometry. This was achieved by comparison the relative retention times of the analyte peaks in samples with those of the standard analytes in methanol. All deviations were within a ± 2.5% tolerance. Moreover, two transitions of the molecular peak of the analyte with a signal-to-noise ratio greater than 3 were monitored. All ion ratios were within the recommended tolerances as required by Commission Decision 2002/657/EC (Figure 1). Matrix calibration curves were obtained by spiking blank urine samples with the four steroids at five concentration levels. Three sets of replicates on three different days for a total of five concentration points for each curve was used to assess linearity. Method recovery and precision were evaluated using the results of the matrix calibration curves: recovery was expressed as a percentage of measured to spiked concentration, and precision as relative standard deviation. The CCα was calculated as three times the signal-to-noise ratio for 20 representative blank *bovine* urine samples. The CCβ was calculated analysing 20 blank materials fortified with the analytes at the CCα. The CCα and CCβ values were 0.4 ng mL^{-1} and 0.5 ng mL^{-1} for all the compounds.

Table 1. Precursor and most abundant product ions and their optimized parameters

Analyte	Transition	Collision Energy (eV)	Declustering Potential (V)
17β,19-nortestosterone	275 > 109[a]	35	50
	275 > 145	30	50
17α,19-nortestosterone	275 > 109[a]	35	50
	275 > 145	30	50
17β-testosterone	289 > 109[a]	40	35
	289 > 97	30	35
17α-testosterone	289 > 109[a]	40	35
	289 > 97	30	35
17β-testosterone-d_2	291 > 99[a]	35	50

[a]*Most abundand product ion*

Table 2. 17β-NT, 17α-NT, 17β-T and 17α-T residues in treated bovine urine samples.

Treated bovines																
	1st		(2nd)	(3rd)	(4th)	8th	15th		22nd	(23rd)		(24th)	(25th)	29th	35th	37th
Sampling	T_0	T_6	T_{24}	T_{48}	T_{72}	T_0	T_0	T_6	T_0	T_6	T_{24}	T_{48}	T_{72}			
17β-NT concentration in urine sample (ng mL^{-1})																
Average	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C.I.*	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
>CCα	0/10	0/10	0/10	0/9	0/10	0/9	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/9	0/8
17α-NT concentration in urine sample (ng mL^{-1})																
Average	n.d.	5.2	7.8	7.2	7.3	3.7	5.0	2.4	6.3	4.6	7.9	6.3	4.0	5.1	3.6	3.2
C.I.*	--	0.5-13.8	1.0-25.7	1.1-19.3	0.9-17.4	0.8-7.8	0.8-16.7	0.7-7.4	2.4-23.2	0.7-14.2	1.6-18.4	0.4-18.3	0.6-13.6	1.7-16.3	0.6-11.1	1.1-6.6
>CCα	0/10	8/10	9/10	8/9	10/10	6/9	10/10	8/10	5/10	9/10	10/10	9/10	10/10	7/10	6/9	4/8
17β-T concentration in urine sample (ng mL^{-1})																
Average	2.2	4.3	1.2	1.2	1.5	2.3	3.7	1.5	3.4	2.1	n.a.	0.6	n.a.	1.9	1.0	1.4
C.I.*	0.5-6.9	0.7-9.4	0.6-1.5	0.5-2.8	0.5-5.5	0.5-6.0	0.7-12.0	0.8-2.4	1.3-6.6	1.8-2.2	n.a.-0.5†	0.6-0.7	n.a.-0.6†	1.0-3.3	0.5-0.7	0.9-2.2
>CCα	10/10	9/10	5/10	8/9	8/10	5/9	7/10	3/10	3/10	3/10	1/10	2/10	1/10	3/10	6/9	5/8
17α-T concentration in urine sample (ng mL^{-1})																
Average	27.7	40.5	18.8	21.1	19.1	17.6	37.1	9.4	18.1	9.6	11.2	9.6	6.6	14.0	8.1	9.0
C.I.*	9.2-55.5	10.0-87.7	8.0-41.0	3.4-56.2	3.5-36.2	2.5-40.8	11.0-56.6	1.9-31.8	1.2-55.4	0.9-33.3	2.1-21.3	2.6-28.6	0.9-14.0	0.9-46.7	2.7-15.0	1.5-23.9
>CCα	10/10	10/10	10/10	9/9	10/10	9/9	10/10	10/10	9/10	10/10	10/10	10/10	10/10	10/10	9/9	8/8

**C.I., Concentration Interval; n.a., not applicable; n.d., not detected; †, only one positive sample*

Results and discussion

Residues of 17β-NT were never detected (< CCα) in treated nor in control *bovine* urine samples. The main metabolite of 17β-NT, 17α-NT, was never detected in treated *bovine* urine samples collected before the first administration and in control *bovine* urine samples. In contrast, 17α-NT was detected in almost all urine samples collected during the treatment period. Its elimination rate increased after each injection reaching the highest value about 24-48 hour after each administration (Figures 2 and 3). Residues of 17α-NT were still found in 70 %, 66 % and 50 % of *bovine* urine samples collected eight, fourteen and sixteen days after the last dosing, respectively. The data reported in Table 2 were determined by averaging the urine concentration values of each hormone (17β-NT, 17α-NT, 17β-T and 17α-T) obtained from treated *bovines* at each interval time. In urine samples collected from two control bovines, the average concentration of 17β-T and 17α-T was 5.3 ng mL^{-1} and 31.8 ng mL^{-1}, respectively. The concentrations ranged from 0.5 ng mL^{-1} to 24.2 ng mL^{-1} for 17β-T and from 3.6 ng mL^{-1} to 90.7 ng mL^{-1} for 17α-T. These preliminary results showed higher levels of 17β-T and 17α-T in control *bovine* urine samples with respect to those found in treated *bovine* urine samples (the average concentration for 17β-T was 1.2 ng mL^{-1} an the concentration ranged from 0.5 ng mL^{-1} to 3.9 ng mL^{-1}; the average concentration for 17α-T was 17.1 ng mL^{-1} and its concentration ranged from 6.6 ng mL^{-1} to 40.5 ng mL^{-1}), suggesting a possible suppression phenomenon of endogenous testosterone pro-

duction due to the exogenous androgens administration. Other authors (Scippo *et al*. 1993) also reported low levels of urinary endogenous testosterone in male *bovines* administered with natural hormones. These authors proposed a threshold level of urinary testosterone under which animals have to be considered potentially treated with natural hormones.

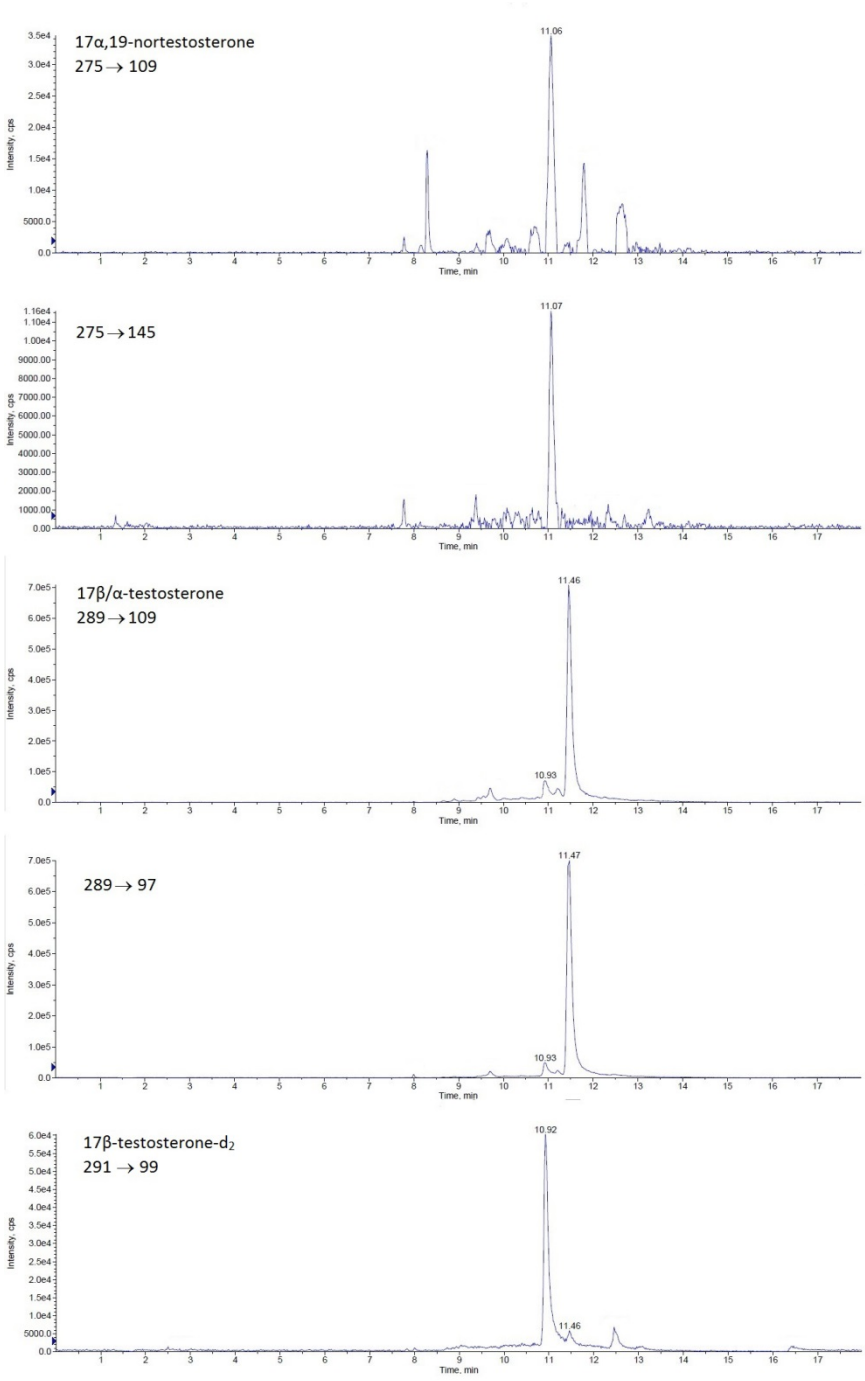

Figure 1. Extract ion chromatograms (XIC) of a urine sample from a treated bovine *found positive for 17α-NT.*

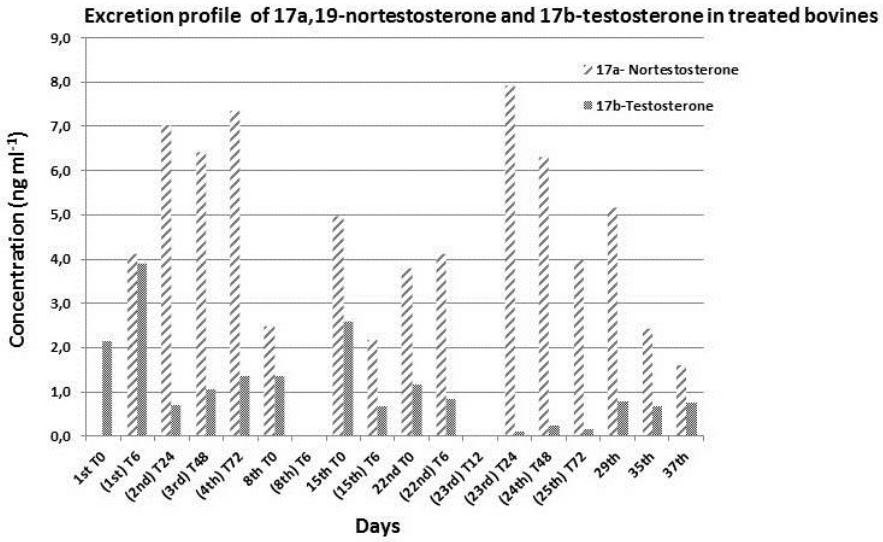

Figure 2. Excretion profile of 17α,19-nortestosterone (17α-NT) and 17β-testosterone (17β-T) in bovines *administered with four doses of 17β,19-nortestosterone.*

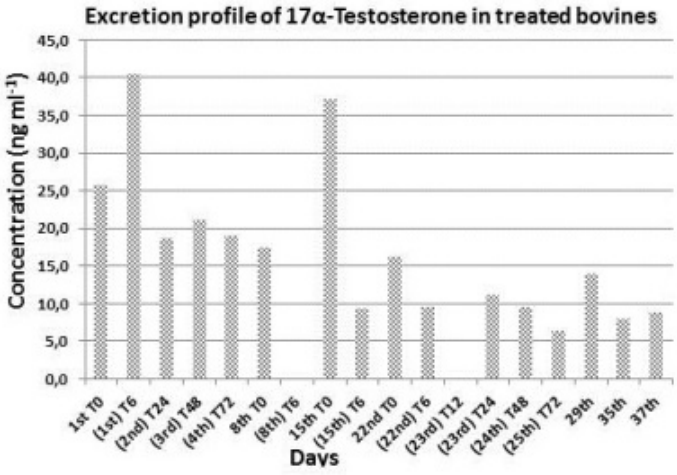

Figure 3. Excretion profile of 17α-testosterone (17α-T) in bovines *administered with four doses of 17β,19-nortestosterone.*

Conclusion

As reported by some authors, residues of 17α-NT can occur naturally in the urine of pregnant cows (Meyer *et al*., 1992), and both 17α-NT and 17β-NT can be detected in injured animals (Kennedy *et al*., 2009), as a consequence the detection of the illegal use of 17β-NT has become a difficult issue over the recent years. The aim of this work is to increase the knowledge about the excretion profile of 17β-NT and its main metabolite (17α-NT). Here, *bovines* were treated with four intramuscular administrations of 17β-NT laureate and levels of endogenous and exogenous androgens were compared in order to provide reliable data to discriminate treated from untreated *bovines*. Preliminary results showed the absence of 17β-NT residues in both treated and control *bovines*. In contrast, 17α-NT was present in almost all treated *bovines* in the treatment period. However, in some cases the absence of 17α-NT or its presence at very low level can make it very difficult to identify an illegal administration of the 17β-NT. Furthermore, preliminary results showed that the levels of the endogenous androgens, 17β-T and 17α-T, in control *bovines* were higher compared to those found in treated *bovines*. A larger number of control *bovines* have to be analysed to investigate whether the differences in endogenous androgens urinary levels found in treated and control *bovines* were due to physiological inter-individual variability or were related to exogenous hormone administration.

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NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ANALYSIS IN MILK BY QUECHERS AND LC-MS: LOW AND HIGH RESOLUTION DETECTION AND CONFIRMATION APPROACHES

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Abstract
We developed a simple method based on the QuEChERS approach for the determination of 10 non-steroidal anti-inflammatory drugs (NSAIDs) in milk samples using liquid chromatography and tandem mass spectrometry (LC-MS/MS) with an electrospray ionization source. The absolute recoveries for the extraction and clean-up steps are in the range 78-97%. The chromatographic separation is performed with a biphenyl column and mobile phases consisting of water and acetonitrile containing formic acid. MS/MS detection is performed with a triple quadrupole (QqQ) mass spectrometer. Furthermore, we explored the use of a hybrid high resolution mass spectrometer, combining a quadrupole and an Orbitrap mass analyzer, for detection of NSAIDs. Lower quantification limits for NSAIDs are achieved with the Q-Orbitrap detection, which is especially relevant for diclofenac, which has a maximum residue limit in milk of 0.1 µg kg^{-1}. Moreover, Q-Orbitrap provides very high selectivity, and thus it is particularly suitable for confirmatory analysis.

Introduction
Non-steroidal anti-inflammatory drugs have anti-inflammatory, analgesic and antipyretic properties. Gastric and intestinal disturbances are common side effects of NSAIDs, but allergies as well as other effects involving the hepatic, renal, hematopoietic, or central nervous systems may also occur.

In the European Union (EU), some NSAIDs are authorized for administration to food-producing animals, and to protect consumers, the European Commission (EC) has established maximum residue limits (MRLs) for several NSAIDs in food products of animal origin (EC, 2009). Furthermore, the EU Community Reference Laboratories have proposed recommended concentrations (RCs) for NSAIDs without MRL (CRL, 2007). These RCs are guidance values that laboratories need to achieve in order to ensure effective control. Table 1 shows MRL and RC values for NSAIDS in milk.

Table 1. Maximum residue limits (MRLs) and recommended concentrations (RCs) for NSAIDs.

Compound	Acronym	MRL in milk (µg·kg^{-1})		RC in milk (µg·kg^{-1})
		Bovine	*Caprine*	
5-Hidroxyflunixin	5-FLU	40		
Diclofenac	DCF	0.1		5
Meloxicam	MLX	15	15	
Phenylbutazone	PBZ			5
Oxyphenbutazone	OPB			5

Control laboratories require reliable and high throughput analytical methods. NSAIDs are a heterogeneous group of drugs with diverse chemical structures. This makes the development of NSAID multi-residue methods a challenging task, especially in terms of sample treatment.

Methods for analysing NSAID in milk samples are normally based on liquid chromatography (LC) coupled to mass spectrometry (MS), mostly using triple quadrupole (QqQ) instruments, although the use of gas chromatography coupled to MS has also been described (Gentile, 2007).

Few solvents have been proposed for NSAIDs extraction. Acetonitrile, whose effect on protein precipitation facilitates extraction, is widely used. However, methanol, as well as acetonitrile/methanol or acetonitrile/ethyl acetate mixtures are also employed. Since LC-MS is susceptible to matrix effects, and milk is quite a complex matrix, most methods include some clean-up of the extracts. Several strategies have been described, such as a liquid-liquid extraction with hexane, but most of the methods apply solid phase extraction (SPE) with different sorbents. There are also methods that do not include clean-up after the extraction step (Dubreil-Chéneau *et al*., *2011*; Van Pamel *et al*., 2015).

LC separation is usually performed with octadecyl columns and mobile phases are mostly based on acetonitrile/water mixtures at acidic pH. Regarding MS detection, most of the methods use a QqQ instrument with an ESI source, in positive or negative mode, depending on the compound, but also on the instrument.

The overall aim of this study was to develop a reliable and straightforward methodology for the analysis of NSAID residues in milk. Here, we present a new method based on the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) strategy (Anastassiades *et al*., 2003) and LC-QqQ-MS/MS to analyse 10 NSAIDs in milk. We also evaluated detection by HRMS/MS with a Q-Orbitrap instrument.

Materials and Methods

Reagents

Standards of ketoprofen (KTP), niflumic acid (NFL), flufenamic acid (FLF), meclofenamic acid (MEC), flunixin (FLU), 5-hydroxy-flunixin (5-FLU), oxyphenbutazone (OPB), phenylbutazone (PBZ), diclofenac (DCF) and meloxicam (MLX) were obtained from Sigma-Aldrich (Seelze, Germany). Meloxicam-D_3 (MLX-D_3), niflumic acid-$^{13}C_6$ (NFL-$^{13}C_6$), flufenamic acid-$^{13}C_6$ (FLF-$^{13}C_6$), phenyl-buta-zone-$^{13}C_{12}$ (PBZ-$^{13}C_{12}$), also from Sigma-Aldrich, were used as internal standards (IS).

Acetonitrile and methanol (HPLC quality) were obtained from Panreac (Barcelona, Spain). Acetonitrile of hypergrade quality was obtained from Merck Millipore (Darmstadt, Germany). Double deionized water of 18.2 MΩ·cm^{-1} was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Other chemicals were of analytical reagent grade.

Lichroprep RP-18 (25-40 µm) was obtained from Merck Millipore and Bondesil primary-secondary amines (PSA) 40µm-100µm were obtained from Agilent Technologies, (New Castle, USA). 0,22µm nylon membrane filters were obtained from Merck Millipore.

Instruments

LC-QqQ-MS/MS. The LC system consisted of an Agilent Technologies 1290 coupled to an Agilent QqQ 6460 mass spectrometer with electrospray ionization (ESI), used in both positive and negative modes. The ESI source was operated under the following conditions: capillary voltage: 3.0 kV (ESI-) and 3.5 kV (ESI+); sheath gas temperature: 375°C; gas temperature: 180°C; gas flow (N_2): 5 L·min^{-1}, sheath gas flow (N_2): 11 L·min^{-1}; nebulizer gas (N_2) pressure: 45 psi. Nitrogen was obtained from a Peek nitrogen generator (Air liquid, Paris, France). Instrument control and data processing were carried out using MassHunter B.07.00 software.

A gradient using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at a flow of 0.4 mL min^{-1} was used to separate the NSAIDs on a Phenomenex Kinetex Biphenyl column (2.6 µm, 100 x 2.1 mm); the column temperature was kept at 40°C and the injection volume was 20 µL. The gradient program was the following (time, %A): (0, 90), (10, 45), (10.1, 90), (14, 90). The total runtime was 14 min.

We monitored two transitions per compound and one transition for the internal standards.

LC-Q-Orbitrap-HRMS/MS. The HRMS instrument was a hybrid Q-Exactive from Thermo Scientific (Bremen, Germany). A Thermo Accela UHPLC system coupled to a Maylab Switch column manager and to the Q Exactive mass spectrometer was used. The chromatographic system was coupled to the MS with a Heated Electrospray Ionization Source II (HESI II). HESI II conditions were: spray voltage 3.5 kV (positive ionization) or 3 kV (negative ionization); sheath gas flow rate (N_2), 35 (arbitrary units); capillary temperature, 300°C; S-lens RF level, 50; heater temperature, 350°C. N_2 obtained from a nitrogen generator Zefiro (Clantecnologica, Seville, Spain) was employed as both the collision and damping gas.

Mass calibration for Orbitrap was performed daily to ensure a working mass accuracy lower than or equal to 5 ppm. Pierce LTQ Velos ESI Positive ion and Pierce LTQ Velos ESI Negative ion calibration solutions (Thermo Fisher Scientific, Rockford, IL, USA) were used to calibrate the mass spectrometer. The resolution was set at 70,000 (*m/z* 200, FWHM) at a scan rate of 2 Hz, and the automatic gain control (AGC) was set at 2e5 with a maximum injection time set at 100 ms.

XCalibur 2.2 and Trace Finder 3.2 (Thermo Fisher Scientific, San Jose, CA, USA) were used for UHPLC control and data processing, respectively.

A gradient using aqueous 0.1% formic acid (mobile phase A) and acetonitrile hypergrade with 0.1% formic acid (mobile phase B) at a flow of 0.3 mL min^{-1} was used to separate the NSAIDs on a Phenomenex Kinetex XB C18 column (1.7 µm, 100 x 2.1 mm); the column temperature was kept at 40ºC and the injection volume was 10 µL. The gradient program was the following (time, %A): (0, 70), (10, 45), (10.1, 70), (12, 70). Total runtime was 12 min.

Results and discussion

LC-QqQ-MS/MS

To achieve maximum sensitivity, we used the multiple reaction monitoring (MRM) mode for quantification and confirmation. The final selection of ESI polarity and MRM transitions for each NSAID was based on both sensitivity and selectivity criteria. The mass spectrometer was quick enough to switch polarity without compromising sensitivity. Two transitions of the precursor ion were selected to achieve enough identification points to confirm the identity of the analytes.

Although MS detection is a selective technique, good chromatographic separation is advisable. The chromatographic separation of NSAIDs is usually performed in reverse mode on C8 or C18 columns. In this study we assayed two chromatographic columns (Kinetex XB C18, 1.7 µm and Kinetex biphenyl, 2.1 µm, both solid core type). We used mobile phases based on water-acetonitrile containing formic acid. The biphenyl stationery phase has an enhanced aromatic selectivity compared to C18, and deeper interaction with the aromatic rings of the analytes is achieved. Some differences in the elution order of NSAIDs were observed between the columns. Since the NSAIDs peaks obtained with the biphenyl column showed a better symmetry than those obtained with the C18 column, the biphenyl column was selected for further assays. After adjusting gradient conditions for the biphenyl phase, the chromatographic separation takes a total run time of 14 min, including equilibration time.

Extraction

We carried out preliminary studies using the QuEChERS approach. The extraction step was based on acetonitrile containing 5% acetic acid to ensure protonation of carboxylic acids. We also added sodium chloride and magnesium sulphate. After shaking and centrifugation, the organic layer underwent a clean-up step by dispersive solid phase extraction (d-SPE). We compared two distinct dispersive media for the d-SPE step, C18 and PSA, and found better recoveries for the majority of analytes when using C18. When we tried to omit the clean-up step we obtained unsatisfactory results, with low signals for almost all analytes; thus, we conclude that the clean-up step should not be omitted.

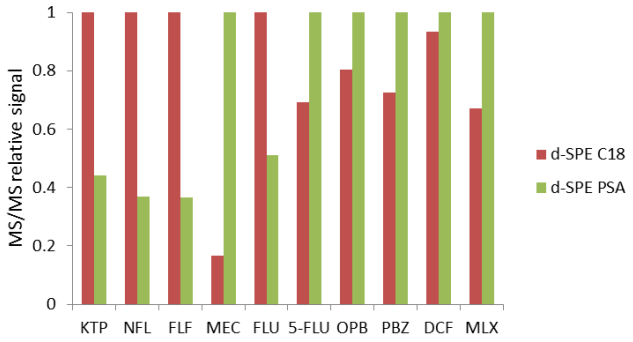

Figure 1. Extraction of NSAIDs from milk samples using QuEChERS. Clean-up by d-SPE: C18 vs PSA.

While the results achieved using the QuEChERS approach were satisfactory overall, MS signals for PBZ and OPB were low and poorly reproducible. Adding 0.006 M ascorbic acid to the acetonitrile extraction solution markedly improved the results of PBA and OPB, and with no relevant adverse effects on the signals of other NSAIDs.

Finally, we substituted sodium chloride with ammonium acetate in the extraction solution, and obtained lower baseline noises, thus allowing lower detection limits. The absolute recoveries for the QuEChERS method obtained at 2.5 µg·kg^{-1} are in the 78-96% range.

QuEChERS LC-QqQ-MS/MS method validation

We validated the QuEChERS LC-QqQ-MS/MS method in milk according to the European Commission Decision 657/2002/EC guidelines (EC, 2002). Calibration curves were obtained for each compound in the 2.5-25 µg·kg^{-1} range, except for 5-FLU (2.5-60 µg·kg^{-1}), using surrogate matrix matched standards (SMMS) and good linearity was achieved for all analytes.

Absolute recoveries for the extraction and clean-up steps were higher than 78%. Intermediate precision ranged from 1.7% to 16.9%. Trueness, determined by the spiked samples approach, was between 84-109%.

Selectivity was evaluated by analysing different blank samples, and no interferences at the retention time of the analytes were observed.

CCα and CCβ for MLX and FLU (expressed as 5-FLU) were determined around their MRL. For MLX CCα and CCβ were 15.8 µg·kg^{-1} and 16.5 µg·kg^{-1}, respectively, whereas for 5-FLU were 41.2 µg·kg^{-1} and 42.3 µg·kg^{-1}. However, for DCF, the method was not suitable to achieve its MRL (0.1 µg·kg^{-1}). We proceeded with DCF as for NSAIDs with no published MRL, which CCα were established at the lowest possible concentration level, by extrapolating the calibration curve. Results for CCα ranged from 0.4 to 1.5 µg·kg^{-1} for CCα and from 0.8 to 1.9 µg·kg^{-1} for CCβ.

LC-Q-Orbitrap- HRMS/MS

We explored the use of HRMS for analysing NSAIDs in milk samples. We used a hybrid Q-Orbitrap instrument (Q-Exactive) coupled to an UHPLC system. The chromatographic separation of NSAIDs was performed in the gradient elution mode on a Kinetex XB C18 column (100 x 2.1 mm, 1.7 µm), with water and acetonitrile-based mobile phases, both containing 0.1% formic acid, at 0.3 mL min^{-1}. Total run time was 15 min and retention times for NSAIDs ranged between 5.6 and 11 min.

For HRMS detection the working mode was product reaction monitoring (PRM) with a mass width for the precursor ion selection set at 1 Da and resolution set at 70,000 (*m/z* 200, FWHM). We selected mild conditions for collision energy, allowing both precursor and product ion monitoring.

SMMSs were injected in the LC-Q-Orbitrap system to test the performance of the method. Good linearity was obtained in the assayed concentration range (2.5-40 µg·kg^{-1}). Finally, we tested the performance of the UHPLC-MS/HRMS system at sub µg·kg^{-1} level. Figure 2 shows the chromatograms of a DCF standard in solvent at MRL level (0.1 µg·kg^{-1}) and of an extract of milk containing DCF at 0.1 µg kg^{-1}. The corresponding high resolution mass spectra of the product ions are also shown.

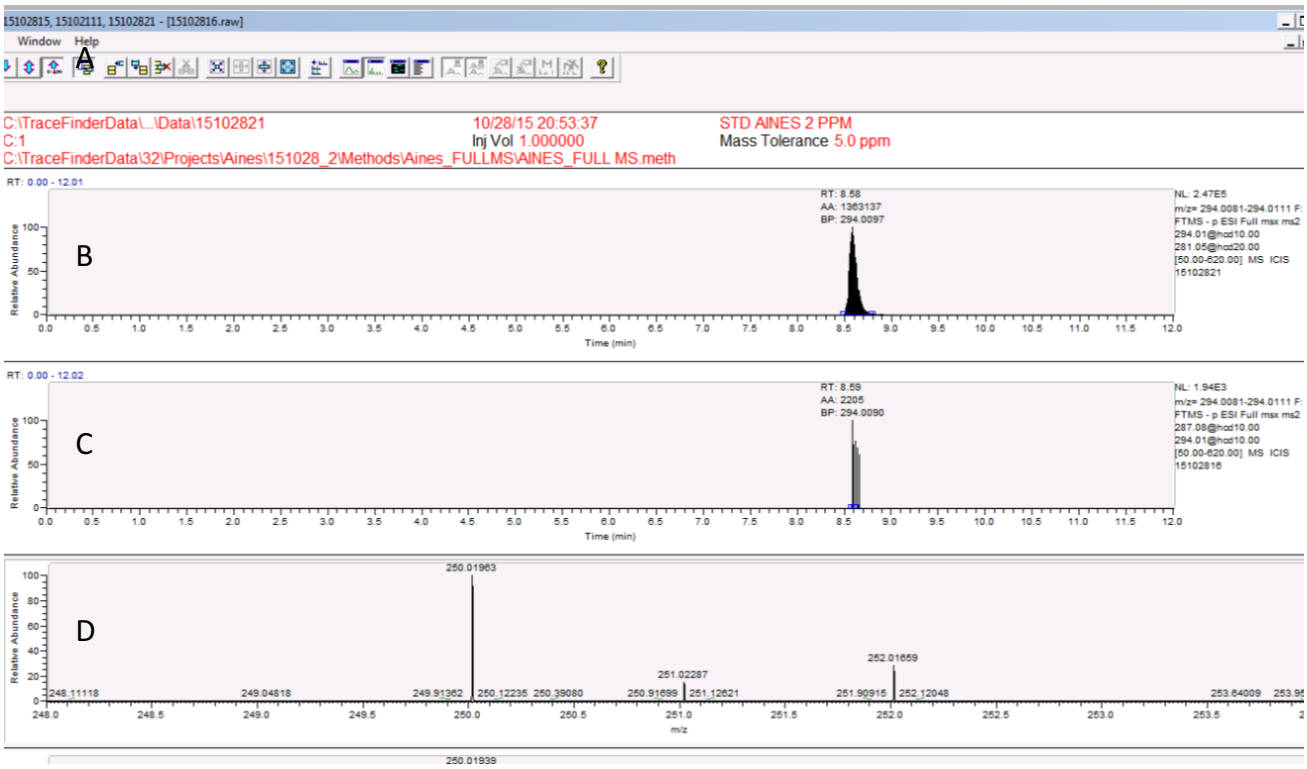

Figure 2. PRM HRMS chromatograms of a DCF standard in solvent at 0.4 µg·L^{-1} (A) and of an extract of blank milk spiked with DCF at 0.1 µg·kg^{-1} (B). C and D are the product ions HR mass spectra at the maximum of the peaks in chromatograms A and B, respectively.

Conclusions

The QuEChERS methodology has proved to be a reliable strategy for analysing NSAIDs in milk samples using LC-MS/MS, very useful for control laboratories with high workloads. On the other hand, the accuracy, resolution, and sensitivity provided by the Q-Orbitrap instrument are especially suitable for NSAIDs analysis at very low concentrations, as required for diclofenac.

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P35
(061)

PHARMACOKINETICS OF ABAMECTIN IN COMBINATION WITH MONEPANTEL IS NOT IMPACTED BY CYTOCHROME P450 INDUCTION

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Abstract
A recent report indicates that monepantel (marketed as Zolvix®) induces the activity of sheep cytochrome P450 enzymes, and in particular that of CYP3A, and consequently it is speculated that the co-administration of other anthelmintics such as ivermectin, with monepantel, will result in faster deactivation of the combination partner. Combination anthelmintics are desired in order to delay the onset of resistance.
We have conducted a pharmacokinetic study with monepantel, monepantel plus abamectin and abamectin alone in the Zolvix placebo formulation, using a 3-way crossover design, in sheep. Blood levels of monepantel, monepantel sulfone (the major metabolite of monepantel) and abamectin were determined using validated methods. Blood levels data were statistically evaluated for all analytes.
Our data clearly demonstrated the absence of drug-drug interactions, and additionally demonstrated bioequivalence for all analytes. Therefore, if induction of cytochrome P450 enzymes did occur, it was not at all clinically relevant, as there was no observable effect on the pharmacokinetic behaviour of abamectin and hence on the safety and efficacy of such a combination product.

Introduction
Monepantel is a new anthelmintic drug, marketed as Zolvix®, intended for the treatment of gastrointestinal roundworm infestations in sheep. Monepantel is rapidly transformed, by oxidation, via the sulfoxide, to the active metabolite monepantel sulfone, which is eliminated rather slowly through the faeces (Karadzovska *et al*., 2009). Metabolism *in vitro* and *in vivo* was studied with mass spectrometric methods (Stuchlikova *et al.,* 2013 and Stuchlikova *et al*., 2014). These researchers continued with investigations of monepantel effects on the cytochrome P450 induction, as these are the most relevant enzymes for Phase I metabolism (Stuchlikova *et al.,* 2015). They found that monepantel significantly increased all cytochrome P450-related activities, and increased expression of CYP3A24 mRNA in sheep, and proposed that that co-administration of other anthelmintics with monepantel may have serious pharmacological and/or toxicological consequences.

Co-administration of two different anthelmintics is an option to delay the development of resistance in nematodes, and indeed we have combined monepantel with abamectin, registered as Zolvix Plus for sheep. This product contains 25 mg mL^{-1} monepantel and 2 mg mL^{-1} abamectin in solution, formulated identically as Zolvix. This combination product has an extended spectrum of efficacy against gastrointestinal worms in sheep.

We have studied the pharmacokinetic profile of this combination product in sheep with direct comparison to the mono-products, each containing the single active drug in identical formulations.

Materials and Methods

Chemicals and Reagents
Monepantel (25 mg mL^{-1}) as Zolvix product was sourced commercially. Monepantel + abamectin (25 mg mL^{-1}+ 2 mg mL^{-1}) was prepared on a commercial scale by Vericore Ltd (now part of Elanco) and abamectin (2 mg mL^{-1}) was prepared on a laboratory scale by Novartis Animal Health Basel, Switzerland (now part of Elanco). All three formulations were clear solutions and contained the same excipients in the same proportions. The ratio of abamectin B1a/B1a was about 97/3 in all abamectin formulations. Analytical references standards of monepantel, monepantel sulfone and abamectin were sourced from Carbogen, Switzerland, and doramectin, which was used as an internal standard for abamectin analysis, was sourced from Sigma-Aldrich. All other laboratory chemicals and reagents were sourced commercially and were of analytical grade or better.

Animals and study design
The study was conducted as a 3-way cross-over design, with 18 Merino sheep, about 11 months old and 33-46 kg at the start, and equal numbers of each sex. Sheep were maintained in pens with roughage mix and pellets and ad lib access to water. The study was approved by the local animal ethics committee and conducted in compliance with GLP. The cross-over design is shown in Table 1 and there was eight-weeks interval between treatments.

Table 1. Study design

Group	Number	Phase 1	Phase 2	Phase 3
A	6	Monepantel	Monepantel + abamectin	Abamectin
B	6	Monepantel +abamectin	Abamectin	Monepantel
C	6	Abamectin	Monepantel	Monepantel + abamectin

Treatment
Each animal was weighed one day before each treatment and this bodyweight was used to calculate the volume of formulation (0.15 mL kg^{-1} BW, equivalent to 3.75 mg kg^{-1} monepantel and/or 0.3 mg kg^{-1} abamectin). The required volume was administered via disposable syringe slowly into the mouth of the animal.

Blood collection
Whole blood (about 5 mL) was collected from the jugular vein, into Vacuette tubes containing EDTA anticoagulant. The collection points were one day before treatment and then after treatment at 2, 4, 8, 12, 18, 24, 30, 36 hours and 2, 3, 4, 7, 10, 14, 21, 28 days. Tubes were stored frozen at about -20°C until analysis.

Analysis and analytical methods
Blood samples were analysed for monepantel + monepantel sulfone and/or abamectin B1a. Control and fortified blood samples (QC) were included in every analytical batch to assess method performance. Analytical methods were validated prior to use and a brief description is given in Table 2.

Table 2. Analytical methods.

Method	Monepantel	Monepantel sulfone	Abamectin B1a
Sample preparation	Protein precipitation followed by SPE		Addition of Internal standard, protein precipitation and SPE
LC	Achiral Waters Atlantis® T3 3 µm 2.1X 50 mm with Methanol/acetonitrile/water with ammonium bicarbonate		Phenomenex gemini 3 µm C18 100 x 4.6 mm. Methanol/water with ammonium formate
Detector	Sciex 4000 Q trap MS/MS with negative electrospray		Sciex 5500 Q trap with positive electrospray
MRM	472>186	504>186	890>305 916>331 (internal standard)
Calibration	Standards in pure solvent		Processed matrix matched standards with internal standard
LOQ (ng mL^{-1})	3 (validated)	3 (validated)	0.25 (validated)
Freezer Stability	12 months	12 months	7 months
Range (ng mL^{-1})	2-200	2-200	0.15 - 100

Statistical methods
Analytical data were subjected to the following transformation: values below LOQ were replaced by missing values.

For monepantel, monepantel sulfone, the sum (monepantel + monepantel sulfone) and abamectin B1a, the following PK parameters were calculated using non-compartmental analysis implemented on validated SAS macros:

Cmax, Tmax, AUC (0-t) where t is the last time point with levels $\geq$LOQ.

Additionally, 90% confidence intervals were calculated on log scale. Treatment group comparisons for Cmax and AUC(0-t) (relative bio-availability ratios) were calculated with corresponding 90% confidence intervals and tested for significance at 0.05. Bioequivalence was assessed, and two products were considered bioequivalent if the 90% confidence interval for the corresponding bio-availability ratio is entirely enclosed by the standard bioequivalence range of [0.80-1.25].

Results

In-life part
All animals completed the study in good health and the average weight gain was about 33% over the entire study period. Apart from one instance of self-resolving conjunctivitis (which is unrelated to any tested product) in one sheep, no adverse reactions were reported.

Analytical part

Blood samples were analysed for abamectin B1a within 2 months of collection and the mean accuracy and CV of QC samples in the range 0.25 to 100 ng mL^{-1} was 96-100% and 5.9-9.2% respectively indicating good performance.

Blood samples were analysed for monepantel and monepantel sulfone within 4 months of collection and the mean accuracy of QC samples in the range 3 to 200 ng mL^{-1} was 89-97% and 90-96% respectively. CVs were <9.4%. The method performed well for both analytes.

The eight-week wash-out period was acceptable as pre-treatment blood levels were <LOQ for all analytes and all treatment phases.

Monepantel blood levels, peaking at about 8 hours, depleted quickly, reaching LOQ by about 7 days, while for monepantel sulfone and abamectin, these blood levels fell to about LOQ by 28 days, after peaking at about 24 hours.

Pharmacokinetic (PK) analysis

The mean calculated PK parameters for each analyte per formulation are shown in Table 3. Graphical displays (on semi-log scales) of blood levels for each analyte are shown in Figures 1-3. The 90% confidence intervals for the relative bioavailability ratios for all analytes were enclosed by the range [0.8 – 1.25] with significances >0.05, indicating bioequivalence. The sum (monepantel + monepantel sulfone) was also assessed and yielded very similar results to monepantel sulfone alone (not displayed here).

Table 3. Geometric mean PK parameters.

Parameter	Formulation	Monepantel	Monepantel sulfone	Abamectin B1a
Cmax	Monepantel+abamectin	31.5	113.9	17.8
(ng mL^{-1})	Monepantel	30.9	103.8	-
	Abamectin	-	-	18.5
Tmax	Monepantel+abamectin	0.3	0.7	0.8
(days)	Monepantel	0.3	0.9	-
	Abamectin	-	-	0.8
AUC(0-t)	Monepantel+abamectin	35.8	500	62.4
(days.ng mL^{-1})	Monepantel	36.5	505	-
	Abamectin	-	-	66.3

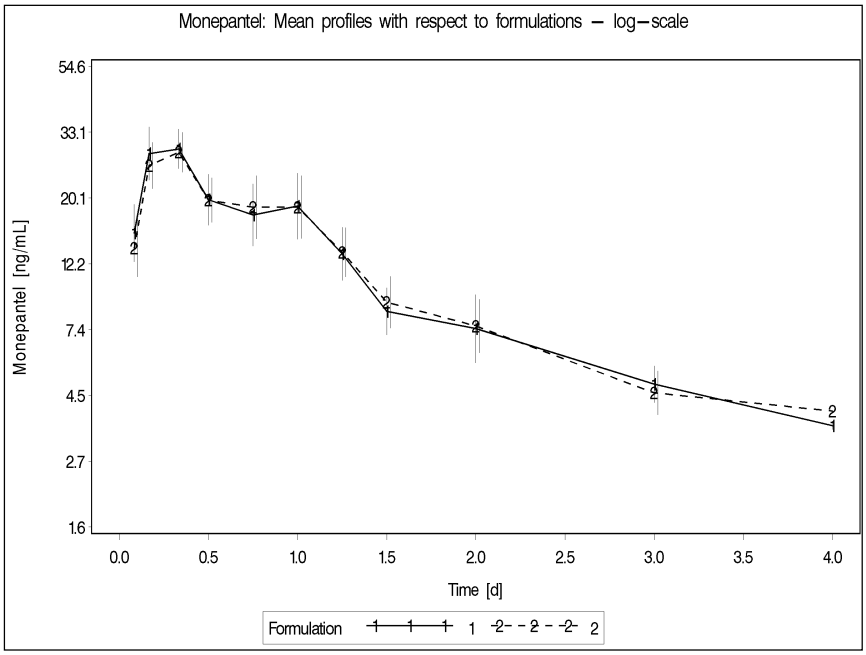

Figure 1. Mean blood profile of monepantel after administration of monepantel +abamectin (formulation1) and monepantel (formulation 2) Vertical bars indicate +/- two standard errors for the mean.

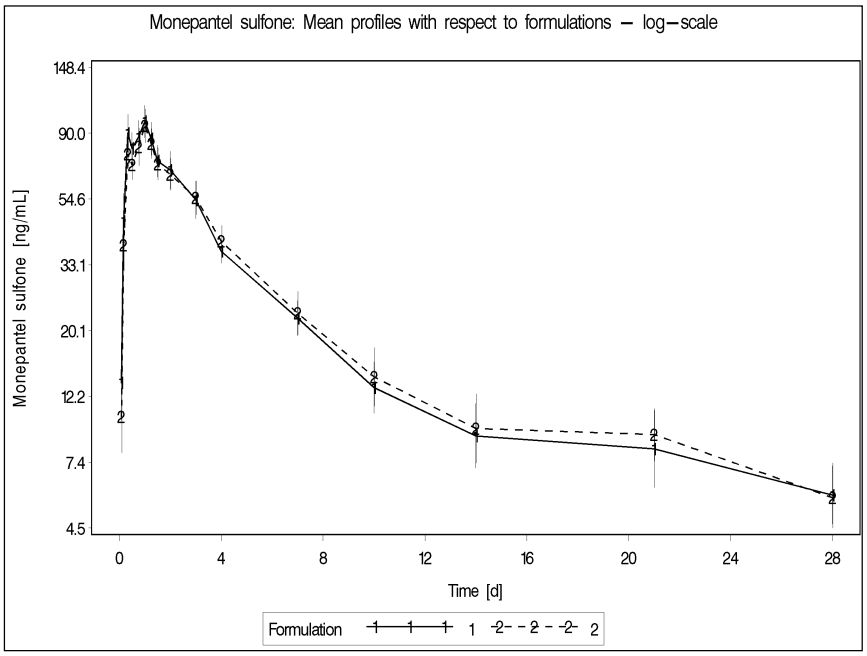

Figure 2. Mean blood profile of monepantel sulfone after administration of monepantel + abamectin (formulation1) and monepantel (formulation 2) Vertical bars indicate +/- two standard errors for the mean.

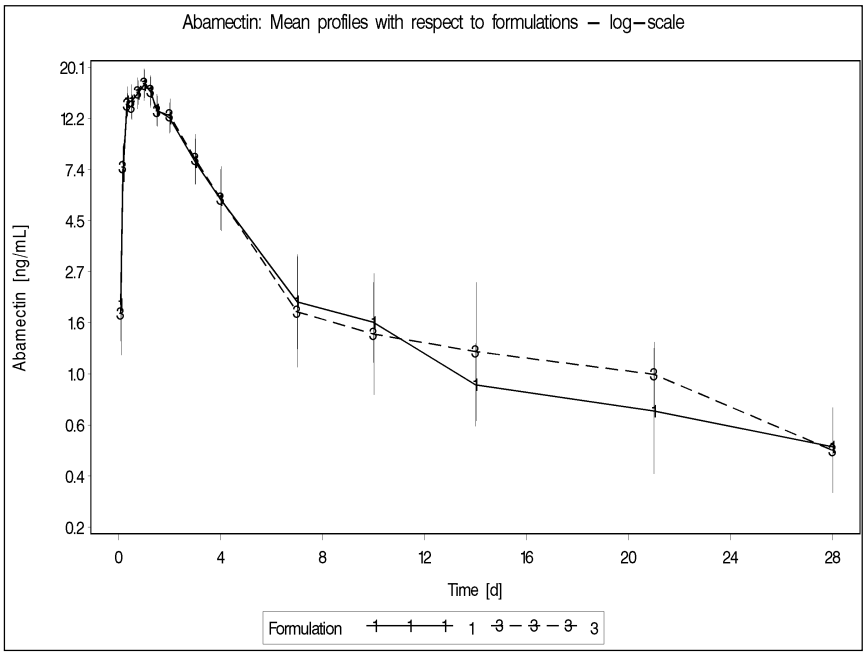

Figure 3. Mean blood profile of abamectin B1a after administration of monepantel + abamectin (formulation1) and abamectin (formulation 3) Vertical bars indicate +/- two standard errors for the mean.

Discussion

This study clearly demonstrates the absence of drug-drug interactions between monepantel and abamectin in sheep. It further demonstrates that monepantel and abamectin as a combination in a Zolvix-type formulation are bioequivalent to each of the drugs in the corresponding mono-formulation. Bioequivalence was demonstrated with respect to both AUC (0-t) and Cmax for monepantel, for monepantel sulfone, for the sum (monepantel + monepantel sulfone) as well as for abamectin. Moreover, there were no adverse or toxic effects on the target animals in this study, nor have any such effects been observed in efficacy studies (Rolfe *et al.,* 2013), which also demonstrated high efficacy against major gastrointestinal parasites present in sheep, including macrocyclic-lactone-resistant strains. Indeed, this combination is now a registered oral product for sheep in Australia and New Zealand

Conclusion

The presence of monepantel or monepantel sulfone does not affect the levels of abamectin found in the blood (no enhancement or depression) suggesting that any potential effect of monepantel on the cytochrome P450 liver enzymes that metabolise abamectin, is either non-existent or not perceptible and not at all clinically relevant.

Acknowledgements

The authors would like to thank Ms Anja Browning and Ms Yogini Patel for the analysis of blood samples and the validations of the analytical methods. Additionally, the authors are grateful to the staff of the Yarrandoo R&D Center (Elanco Australasia Pty Ltd) for the conduct of the animal part of the study.

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THE DETECTION OF TYLOSIN METABOLITES IN MANURE TO DETECT TYLOSIN TREATMENT AND TO DETERMINE ENVIRONMENTAL EXPOSURE

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Abstract

The monitoring of antibiotics mainly focuses on MRL enforcement in food products of animal origin. In order to extend the monitoring to enforce antibiotic use in general and to determine the exposure of antibiotics in the environment, research was done on manure. In this study, we studied the fate of some of the most frequently detected antibiotics in manure. High resolution mass spectrometry (Q-Orbitrap) was used to examine the presence of tylosin analogues in incurred manure samples. In addition to tylosin A and tylosin B (desmycosin), two hydroxy metabolites were detected at levels higher than tylosin A, the marker metabolite in tissues. This research demonstrated that it is importance to include metabolites and degradation products of antibiotics in the analytical methods for enforcement of antibiotic use and when studying the environmental exposure.

Introduction

The use of antibiotics in Dutch livestock in 2014 was 207 thousand kilograms of which 27 thousand kilograms macrolides/lincosamides (Authority Veterinary Drugs 2015). A commonly used macrolide is tylosin. It was found that pigs treated with radioactive labelled tylosin, excreted more than 90% of the activity through faeces. The majority of the excreted residues were tylosin D, tylosin A and dihydrodesmycosin (EMA 2007). Previously the use of manure as a tool for monitoring antibiotic use was described (Berendsen *et al*. 2015). In that study some interesting observations for tylosin were done (*e.g*. deviating ion ratios). An additional study revealed relatively fast degradation of tylosin in manure. The current study focused on the detection of tylosin metabolites and degradation products in manure. We searched for marker compounds to detect tylosin use, even after tylosin A was degraded, so to extend the detection window for tylosin treatment. Furthermore, we studied the tylosin metabolites and degradation products to determine their relevance in the environmental exposure of tylosin.

Materials and Methods

Chemicals and reagents

All reagents and solvents were of analytical or HPLC grade quality. Milli-Q water was prepared by using a Milli-Q system (Merck-Millipore) at a conductivity of at least 18.2 MΩ cm^{-1}. McIlvain-ethylenediaminetetraaceticacid (EDTA) buffer of 0.1 M was prepared by dissolving 74.4 g disodium-EDTA (Merck) in a mixture of 500 mL 0.1 M citric acid (Merck) and 280 mL 0.2 M phosphate buffer (Merck). The pH was adjusted at 4.0 and the volume was made up to 2 L. Lead acetate solution was prepared by dissolving 200 g lead(II)acetate (Sigma-Aldrich) in 1 L water. Tylosin A was purchased from Sigma-Aldrich (St. Louis, MO, USA), desmycosin was purchased by Toronto Research Chemicals (Toronto, Canada) and tylosin D by EDQM (European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France). The internal standard, azithromycin d_4 was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

Collection of faeces

In a previous study, during slaughter, faeces samples were taken directly from the intestinal tract from animals of randomly selected swine farms (Berendsen *et al*. 2015) These samples were immediately transported to the laboratory and stored at < -18°C. After analysis, negative and positive tylosin samples were selected for the current research.

Sample preparation

Incurred swine faeces samples were homogenized and aliquots of 2 g were weighed into 50 mL tubes. After adding the internal standard gamitromycin d_4, the samples were allowed to stand for 20 min. Then, 4 mL McIllvain-EDTA buffer was added and thoroughly mixed using vortex. Next, 1 mL acetonitrile was added and extracted for 15 min using a rotary tumbler. After that, 2 mL of a lead acetate solution was added and again thoroughly mixed using vortex and subsequently the sample was centrifuged for 10 min at 3,500 g. The supernatant was transferred to another tube and 13 mL 0.2M EDTA was added. The whole extract was loaded on a conditioned SPE column of 200 mg/6mL Strata-X RP (Phenomenex). After washing the column with 5 mL water and drying under vacuum, the compounds of interest were eluted with 5 mL methanol. The eluate was evaporated under a gently stream of nitrogen at 40°C. The residue was re-dissolved in 100 µL methanol and then diluted with 400 µL water. At last, the sample was filtered using a Whatman 0.45 µm filter and transferred to an LC-MS vial.

Quality control samples consisted of matrix matched standards of tylosin A, desmycosin and tylosin D in pig faeces in a range of 0 to 200 µg kg^{-1}.

Instrumentation

As LC system, a Thermo Scientific Dionex Ultimate 3000 Series was used. The chromatographic separation was performed on an Acquity U-HPLC BEH C18 column (100*2.1 mm; 1.7 µm). The column temperature was set at 40°C and the flow rate 0.3 mL min^{-1}. The mobile phase consisted of water (A) and methanol (B), both containing 2 mM ammonium formate and 0.16 mL L^{-1} formic acid. The linear gradient was: 0-1 min 0% B; 1-6 min increase to 20% B; 6-12 min increase to 100% B; 12-18 min hold at 100% B. Then decreasing to 100% A in 0.2 min and hold for 2.8 min. The total run time was 21 min and the injection volume 5 µL.

The LC system was connected to a Q-Exactive mass spectrometer (Thermo Scientific) equipped with a heated electrospray ionisation probe (HESI). The HESI parameters were: electrospray voltage 3.5 kV; capillary temperature 250°C; gas heater temperature 400°C; sheath gas flow rate 48 units; auxiliary gas flow rate 11 units; S lens RF level 50 units. The components were alternately detected in full scan mode and data dependent MS^2 mode (dd-MS^2) and in a second injection in the FS mode alternated with the all ion fragmentation mode (AIF). In the full scan mode the resolution was 70,000 (FWHM at *m/z* 200); the scan range was from *m/z* 80 to 1,200; the automatic gain control (AGC) was $3e^6$ and the maximum injection time (IT) was 100 ms. Fragmentation mass spectra of these precursors were recorded in the data dependent mode (dd-MS^2). For this mode, the resolution was set at 70,000 (FWHM at *m/z* 200); the AGC $1e^5$ and the IT 50 ms. The isolation window of the precursor in the quadrupole was 2 Da. The normalized collision energies (NCE) were set at 20, 50 and 80. Potential candidates of tylosin metabolites were included in an inclusion list. The settings for AIF were the same as for FS, but with addition of fragmentation using NCE of 20, 50 and 80.

Results

The reference standard tylosin A was measured in the full scan mode and dd-MS^2. The chromatogram is shown in Figure 1A. The retention time of tylosin A in full scan mode is 12.51 min and corresponded with the peak in dd-MS^2 and the exact mass of the compound observed was 916.5249, which is a 1.6 ppm difference from the theoretical mass of tylosin A. The product ion spectrum of tylosin A is presented in Figure 1C, showing product ions at *m/z* 916.5247, 772.4462 and 174.1121.

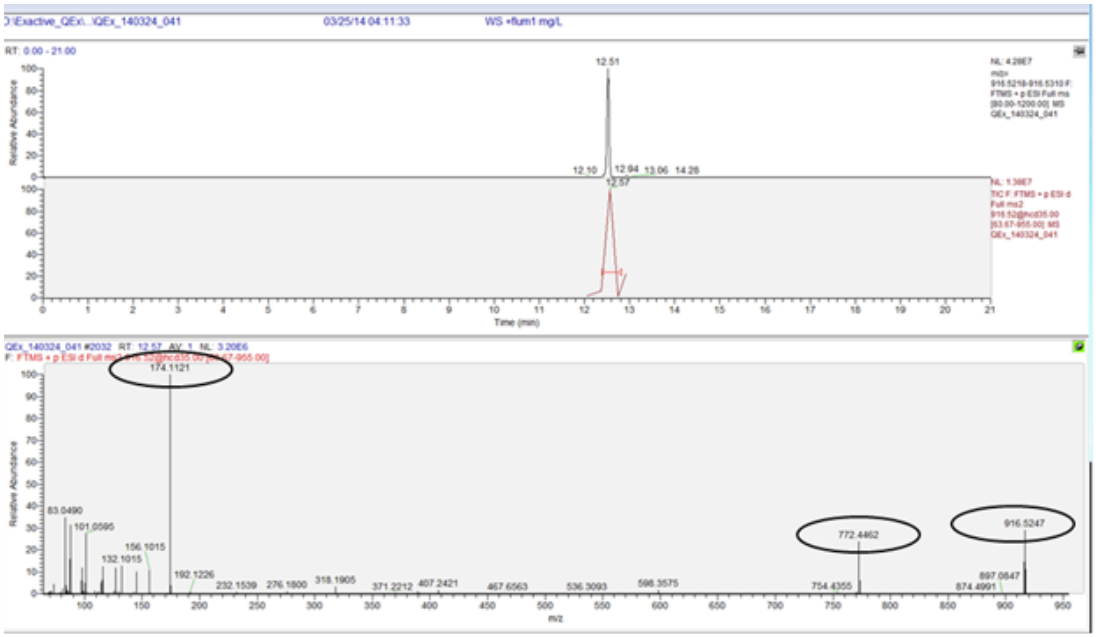

Figure 1. Chromatogram and mass fragmentation spectrum of standard tylosin A. Figure A is full scan chromatogram of exact mass of tylosin A; figure B is data dependent MS2 TIC chromatogram; figure C is mass fragmentation spectrum.

The proposed fragmentation scheme of tylosin A is given with the theoretical masses in Figure 2. The product ion at *m/z* 174.1121, corresponding with a molecular formula of C8H16NO3+ was the most intense product ion in the product ion spectrum. As this product ion was also visible for reference standards of tylosin B and tylosin D, it was hypnotized that this small product ion could be a marker for unknown tylosin metabolites or degradation products.

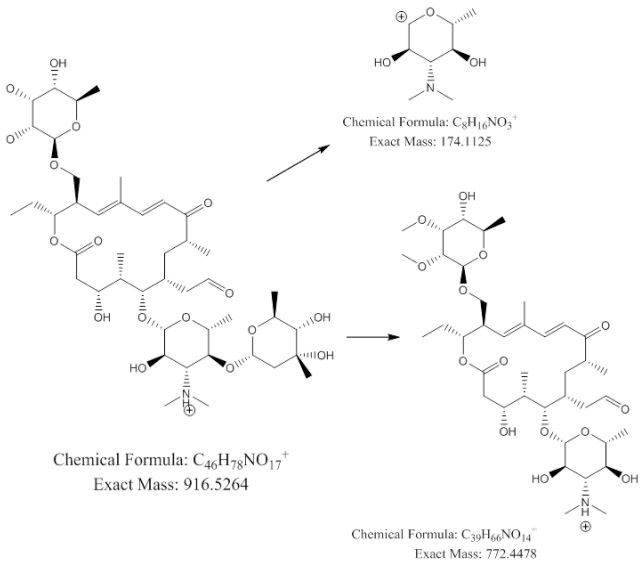

Figure 2 Mass fragmentation spectrum of tylosin A

A tylosin A incurred manure sample was analysed by LC-HRMS operated in full scan (FS) mode alternated with all ion fragmentation (AIF). The extracted ion chromatogram of the AIF mode for *m/z* 174.1125 is presented in Figure 3. Peaks were only detected in the range of 11 to 13 min, just around the actual RT of tylosin A. This indicates that *m/z* 174.1125 is a rather selective product ion and that the peak observed are likely from compounds that are related to tylosin A. From these peaks, the corresponding FS chromatograms and spectra were investigated to determine the exact mass of the relevant compounds. Also dd-MS^2 spectra were recorded to determine the product ions of the individual compounds. In this way, beside the parent compounds tylosin A and B, the hydroxy metabolites of these compounds were tentatively identified. For some small peaks, no indication was found.

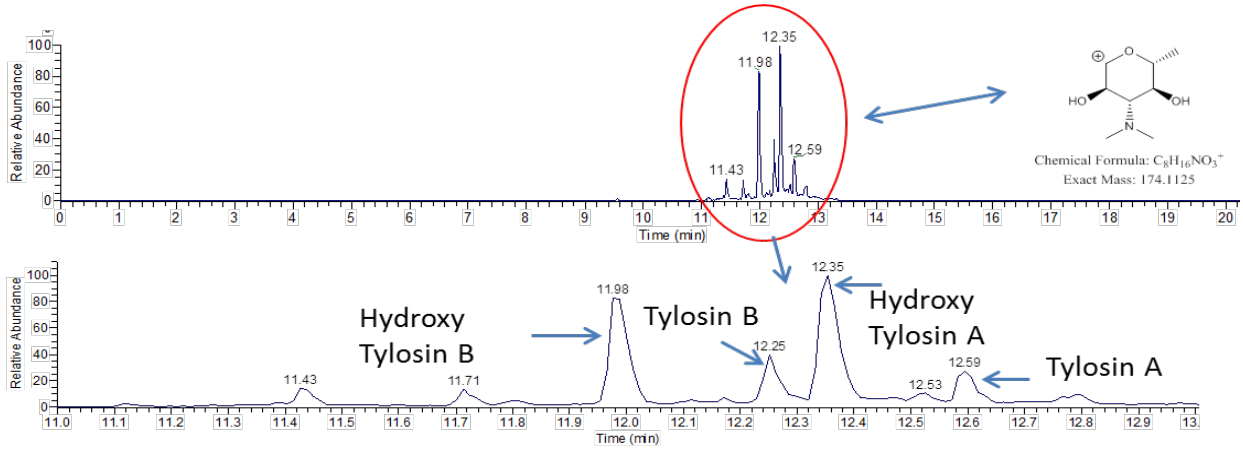

Figure 3 Extracted chromatogram in AIF mode of product ion m/z 174.1125 in an incurred manure sample.

Six manure samples were screened for tylosin and its proposed metabolites, including metabolites that were suggested in literature, being tylosin C, tylosin D, lactenocin, demycinosyltylosin (DMT), dihydrodesmycosin and 5-O-mycaminosyltylonolide (OMT). None of these metabolites were detected, with the exception of tylosin D with a concentration of 20 µg kg^{-1}. In all the samples, the concentration of tylosin A, the marker compound for tylosin in tissue is low; at maximum 26 µg kg^{-1}. The confirmation was based on the fragments in the MS^2 scan.

Hydroxy tylosin A and hydroxy tylosin B were quantified based on the response of the tylosin A respectively tylosin B in the matrix matched standard in the full scan mode. The quantitative results are presented in Table 1. Clearly, the hydroxy analogues were present at much higher levels compared to the native compounds. The confirmation of the hydroxy metabolites is considered tentative as no reference standard were commercially available.

Furthermore, a tylosin A isomer was observed by a change of the retention time of +0.1 min; the exact mass and the product ions were identical to tylosin A, but the relative abundances differed, which explains the differences in the ion ratio for tylosin as previously observed in manure samples. Paessens described the formation of iso-tylosin A as the result of an isomerization of conjugated double bonds under influence of light (Paessen *et al*. 1995).

Table 1. concentration of tylosin and metabolites in manure samples

Component	Concentration in manure (µg kg^{-1})					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Tylosin A	ND	ND	ND	ND	26	24
Tylosin A isomer	28	21	41	ND	460	500
Hydroxy tylosin A	430	390	90	ND	1700	1100
Tylosin B	ND	ND	24	ND	150	300
Hydroxy tylosin B	35	130	46	ND	420	570

ND = not detected

Clearly tylosin A is a minor contribution to the total tylosin related compounds in manure. Therefore, analysis of the hydroxy metabolites could be a strong tool in detecting tylosin treatments. Furthermore, these compounds should be included in the analysis when studying the exposure of tylosin to the environment.

Conclusions/discussion

The contribution of tylosin A in manure samples is relative small compared to its metabolites. Much higher are the concentrations of the hydroxy tylosin A and hydroxy tylosin B. An isomer of tylosin A was also observed which almost co-elutes with tylosin A itself. This explains small differences in the retention time and also accounts for deviating ion ratios observed. To monitor illegal and off-label use of tylosin, it is advisable to include these metabolites in the method as to obtain a longer detection window. Also, for research related to bacterial resistance and exposure to the environment, the contribution of the metabolites must be taken into account. In further studies, the microbiological activity of the hydroxy metabolites have to be researched.

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A COMPREHENSIVE METHOD FOR THE DETERMINATION OF 20 COCCIDIOSTATS RESIDUES IN VARIOUS FOOD MATRICES

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Abstract

Developing reliable analytical method with broad range of molecules and matrices is a challenge. We here present a versatile, LC-MS/MS based method for the determination of coccidiostat residues in food matrices. The procedure enables the determination of 20 coccidiostats in a wide range of matrices: muscle, liver, milk and eggs. Both ionophoric and chemical coccidiostats are in the scope of the method; this includes challenging analytes like the low retention of amprolium and the poor fragmentation of toltrazuril. Coccidiostats were extracted with aqueous acetonitrile solution, and purified on silica phase cartridges. The most demanding matrices liver and muscle required additional cleaning step with polymeric reversed-phase cartridges. Instrumental analysis was performed using an 8 min gradient with mobile phases consisting of ammonium formate and acetonitrile. Analytes were detected in selected reaction monitoring mode with both positive and negative electrospray ionisations. The validation, performed according to Commission Decision 2002/657/EC, proved that the method was fit for confirmatory analysis.

Introduction

Coccidiosis is a very contagious disease caused by single-cell parasites belonging to the genus *Eimeria*, infecting the gastrointestinal tract. This disease is a major problem which affects intensively farmed species such as poultry, pigs, sheep and rabbits. The application of anticoccidial agents is considered the method of choice to maintain animal health and welfare. According to Regulation 1831/2003/EC, eleven coccidiostats are authorized as feed additives, namely: decoquinate (DEQ), diclazuril (DIC), halofuginone (HAL), lasalocid (LAS), maduramicin (MAD), monensin (MON), narasin (NAR), nicarbazin (NIC), robenidine (ROB), salinomycin (SAL) and semduramicin (SEM). Coccidiostats such as amprolium (AMP) and toltrazuril (TOL) are licensed as veterinary drugs (Commission Regulation (EC) No 470/2009). Other coccidiostats: ethopabate (ETO), arprinocid (ARP), nequinate (NEQ), clopidol (CLO) are not to be used as feed additives or veterinary drugs.

The risk of occurrence of residues of coccidiostats in food of animal origin are related to unavoidable carry-over of coccidiostats from target to non-target feeds during industrial feed production and illegal usage of non-authorized coccidiostats or non compliance with withdrawal periods for authorized coccidiostats. To minimize risk of occurrence of residues of coccidiostats due to carry-over phenomena during feed production, EU has established maximum levels (ML) of authorized coccidiostats in non-target feed (Commission Regulation (EU) No 574/2011). The reason for introducing MLs was a conclusion that a certain degree of coccidiostats' contamination of feed is impossible to avoid. For the purposes of ensuring food safety, the maximum concentration of residues, which are permitted in food of animal origin, the so-called maximum residue limits (MRL) are established (Commission Regulation (EC) No 124/2009).

As a result of the above regulations, there is a need for reliable analytical methods for the determination of all regulated coccidiostats in wide range of matrices. Residues of coccidiostats are determined mainly using LC-MS/MS techniques. Multi-residue methods for the determination of coccidiostats in eggs (Olejnik *et al.* 2010; Galarini *et al.* 2011), muscle (Dubois *et al.* 2004; Moloney *et al.* 2012; Clarke *et al.* 2013) and milk (Nász *et al.* 2012) have been developed and published. Because of the complexity of liver, only a few methods for the determination of residues of coccidiostats in this analytical matrix were published (Rosén 2001; Olejnik *et al.* 2009; Ha *et al.* 2016). Some of the published methods was able to determine coccidiostats in more than one matrix, but none was able to determine residues of coccidiostats in liver and in other matrices. Our goal was therefore to develop and validate a reliable method capable to determine residues of 20 coccidiostats in a wide range of food products, such as milk, eggs, muscle and liver.

Materials and Methods

Chemical and materials

Amprolium, aprinocid, decoquinate, decoquinate-d5, diclazuril, ethopabate, ethopabate-d5, halofuginone hydrobromide, maduramicin ammonium, monensin sodium, narasin, dinitrocarbanilide, nigericin, robenidine hydrochloride, robenidine hydrochloride-d8, salinomycin sodium were purchased from Sigma-Aldrich (Germany). Dinitrocarbanilide-d8, toltrazuril, toltrazuril sulfone, toltrazuril sulfoxide were obtained from Witega (Germany), lasalocid sodium was purchased from Dr. Ehrenstorfer Labratories (Germany). Clopidol, clazuril, halofuginone hydrobromide 13C6, semduramicin sodium and methyldiclazuril were kindly donated by European Union Reference Laboratory in Berlin.

Acetonitrile, dimethylsulfoxide (DMSO), LC-MS grade were purchased from JT Baker (Germany), ammonium formate was obtained from Sigma-Aldrich (Germany), SiOH and Strata-X cartridges were obtained from Phenomenex (USA), acetone and ethyl acetate were bought from POCh (Poland), centrifugal filters 0.2 µm, Nanosep MF from Pall (USA). Ultra-pure water (resistance > 18mΩ) was generated by Mili-Q system (Milipore, France).

LC-MS/MS conditions

The LC-MS/MS system consisted of Shimadzu Nexera X2 UHPLC coupled with Shimadzu LC-MS 8050 mass spectrometer. The instrument was controlled by LabSolution SP2 software. Separation was performed using Agilent Zorbax Eclipse Plus C18 RRHD 1.8 µm, 2.1 x 50 mm chromatographic column in gradient mode with 0.01 M ammonium formate pH 4.0 (mobile phase A) and acetonitrile (mobile phase B) at the flow rate of 0.6 mL min^{-1}. Oven temperature was set to 40°C, injection volume was 5 µL, total analytical run was set to 8 min.

The analysis was performed using positive and negative heated electrospray ionization mode (HESI). The parameters of mass spectrometer were as follows: nebulizing gas was set at 3 L min^{-1}, heating gas and drying gas flowed both at 10 L min^{-1}, interface temperature was -300°C and heat block temperature -400°C. The capillary voltage was −3 kV and 4 kV for the negative and positive ionization mode, respectively. Selected reaction monitoring (SRM) mode was applied and each coccidiostat was analysed using two transitions, while for the internal standard one transition was monitored. Mass spectrometry conditions are summarised in Table 1.

Table 1. Mass spectrometry parameters

Analyte	Internal standard	Precursor ion	Product ions	Q1 Pre Bias (V)	Q3 Pre Bias (V)	Collision energy (eV)
	ESI (+) mode					
Amprolium	Halofuginone ^{13}C6	243.2	150.2/122.2	26	16/24	12/24
Arprinocid	Halofuginone ^{13}C6	277.9	143.0/108.0	29	29/22	26/51
Clopidol	Halofuginone ^{13}C6	192.0	101.2/87.1	10	20/18	28/30
Ethopabate	Halofuginone ^{13}C6	238.0	206.2/136.2	26	16/24	15/24
Halofuginone	Halofuginone ^{13}C6	416.1	138.1/100.1	21	25/20	20/23
Halofuginone ^{13}C6	-	422.0	138.0	21	20	23
Robenidine	Robenidine-d8	334.1	155.2/138.1	17	30/27	21/25
Robenidine-d8		342.0	159.2	30	17	21
Decoquinate	Decoquinate-d5	418.2	372.2/204.1	20	27/23	26/42
Decoquinate-d5	-	423.3	337.2	21	28	25
Lasalocid	Nigericin	613.3	577.3/377.2	30	30/28	34/37
				30		
Semduramicin	Nigericin	895.4	851.5/833.7	26	34/32	36/32
Monensin	Nigericin	693.3	675.4/479.3	34	26/24	40/53
Salinomycin	Nigericin	773.4	531.3/431.1	38	40/22	44/52
Maduramicin	Nigericin	934.5	647.5/629.4	28	34/32	22/27
Narasin	Nigericin	787.4	531.3/431.3	40	40/30	48/53
Nequinate	Decoquinate-d5	366.6	243.3/201.3			
Nigericin	-	747.5	703.5	36	38	54
	ESI (-) mode					
Toltrazuril	Dinitrocarbanilide-d8	424.4	424.4/42.1	-16	-29/-14	-5/-22
Toltrazuril sulfone	Dinitrocarbanilide-d8	439.4	371.0/42.1	-23	-24/13	-17/-22
Toltrazuril sulfoxide	Dinitrocarbanilide-d8	455.9	398.9/42.1	-16	-13/-17	-25/-13
Dinitrocarbanilide (Nicarbazin detection)	Dinitrocarbanilide-d8	301.9	138.2/108.2	-15	-22/-17	-15/-11
Dinitrocarbanilide-d8	-	309.1	141.2	-11	-23	-15
Diclazuril	Methyldiclazuril	406.9	336.0	-15	-21	-19
		404.8	334.2	-15	-21	-20
Clazuril	Methyldiclazuril	371.0	301.0	-11	-29	-17
		373.0	303.0	-11	-25	-27
Methyldiclazuril	-	418.8	321.1	-12	-30	-29

Sample preparation

A sample of 2.00 ± 0.01 g of milk, egg, liver or muscle was weighed into the polypropylene centrifuge tube and 20 µL IS solution was added. The sample was vortex-mixed and allowed to rest for 10 min. Then, 5 mL of 90% acetonitrile (80% acetonitrile in case of liver and muscle) was added and sample was vigorously mixed and centrifuged (3,500 rpm, 15 min). The supernatant was filtered using SiOH cartridge rinsed with 2 mL acetonitrile and washed with 1 mL acetonitrile prior to use. The extract was collected in a glass tube and allowed to stand in a freezer (-26°C) for at least 1 h. After freezing, the top layer of the extract was collected and evaporated to dryness (N_2, 45°C).

In case of liver and muscle, the dry residue was dissolved in 0.25 mL DMSO, transferred to clean tube with 2 portions of water (5 mL) and applied on Strata-X column conditioned with 2 mL methanol and 2 mL water. The cartridge was washed with 1 mL water and vacuum dried for 5 min. The analytes were eluted with 4 mL acetone: ethyl acetate (2:1, v:v) solution and evaporated to dryness (N_2, 45°C).

Finally, dry residue was reconstituted in 0.25 mL of acetonitrile: water (50:50, v:v), filtered through centrifugal filters (4,500 rpm, 5 min) and transferred to a vial.

Method validation

Validation was performed according to the criteria laid down by Commission Decision 2002/657/EC (Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC). Linearity calculated by preparing three series of matrix-matched calibration curves on five concentration levels and plotting ratios (analyte to analyte internal standard) versus concentration. For repeatability and reproducibility studies, three series (0.5, 1.0, 1.5 MRL/ML) were analysed (six samples for spiking level) and standard deviation and coefficient of variation was calculated for each level. Recovery of the method was assessed through the addition of known amount of coccidiostats to blank matrix. CCα was calculated as the concentration of 1.0 MRL/ML plus 1.64 times the SD, while CCβ calculated as CCα plus 1.64 times the standard deviation.

Results and discussion

LC-MS/MS conditions

Optimisation of chromatographic and mass spectrometry conditions resulted in sensitive and time-effective method. Usage of gradient program with ammonium formate and acetonitrile combined with C18 column was sufficient to overcome difficulties with low retention time of amprolium. Developed MS/MS conditions enabled to achieve sufficient sensitivity of all analytes. In case of toltrazuril, toltrazuril sulfone and toltrazuril sulfoxide, only one product ion was obtained.

Difficulties with toltrazuril fragmentation are well recognized. Similar problems are reported by other authors, they were also able to obtain only one precursor ion and one product ion (Ai *et al.* 2011). This problem has been fully resolved through application of atmospheric pressure chemical ionisation (APCI) technique; two product ions for toltrazuril were obtained (Martinez-Villalba *et al.* 2010). Unfortunately, application of APCI technique is challenging in case of other coccidiostats.

Sample preparation

Developing effective sample preparation is the most crucial step in method design. Appropriate sample preparation protocol should be easy and time-effective, but should also provide a recovery and precision as high as possible. Maintaining equilibrium between those factors is especially important in case of multi matrix methods, where different matrices could require a different approach.

Number of methods regarding determination coccidiostats in milk and eggs utilizes simple and quick sample preparation protocol. Usage of QuEChERS type protocol in case of milk and muscle samples were reported (Clarke *et al.* 2013). A method for egg samples without any sample purification, only extraction with MeOH, was developed (Shao *et al.* 2009).

We were able to develop an easy and time-effective sample protocol in case of milk and eggs, in which case the cleaning step consisted of SiOH cartridges followed by freezing. Modifying this protocol for muscle or liver samples was unsuccessful. Recovery and sensitivity were unsatisfactory for many analytes. Therefore, a more rigorous sample preparation was required. Recently, the application of ENVI-Carb SPE cartridges for determination polyether coccidiostats in liver was reported (Ha *et al.* 2016). Our experiments with ENVI-Carb material was not successful for liver samples either. Finally, we were able to achieve satisfactory results for liver samples when an additional cleaning step with Strata-X SPE cartridges was included (Figure 1).

Method validation

The developed method was validated for egg, liver and muscle and milk samples. Results of the validation experiment for egg and milk samples are shown in Table 3. Linearity and working range for all analytes for all matrices was acceptable as the values of R^2 were in excess of 0.98. The recoveries for the analytes were in the range of 90-120%. The coefficient of variance for repeatability was below 20%, in case of reproducibility value of CV was below 25% for all analytes in scope of the method. The decision limit and the detection capability values were satisfactory in all cases.

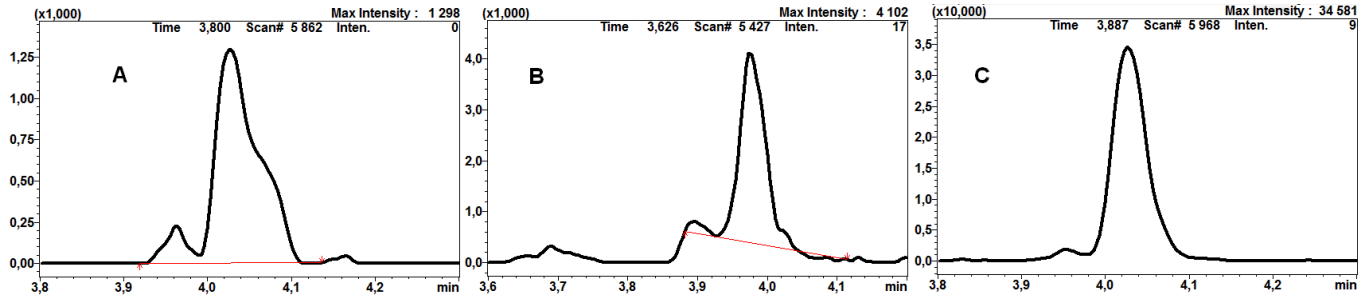

Figure 1. Chromatograms of salinomycin in spiked liver sample obtained using sample protocol for milk and eggs - cleaning step on SiOH cartridges followed by freezing (A), cleaning step on SiOH cartridges followed by freezing and d-SPE - 250 mg of ENVI-Carb (B), sample protocol optimized for liver and muscle - SiOH cartridges and freezing followed by Strata-X cartridges (C).

Table 3. Validation results of the developed method for the determination of coccidiostats in eggs and milk.

Analyte	Eggs					Milk				
	MRL/ML (µg kg^{-1})	Reproducibility CV(%)	Recovery (%)	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	MRL/ML (µg kg^{-1})	Reproducibility CV (%)	Recovery %	CCα (µg kg^{-1})	CCβ (µg kg^{-1})
Amprolium*	20	18.7	107.2	27.0	33.8	20	19.5	90.8	24.3	33.9
Decoquinate	20	20.4	102.0	23.5	27.9	20	5.3	104.9	22.2	24.9
Diclazuril	2	26.1	104.5	2.63	3.55	5	6.6	98.5	5.7	6.7
Halofuginone	6	3.1	100.3	6.19	6.64	1	11.7	106.1	1.3	1.6
Lasalocid	150	21.4	105.4	203	277	1	15.4	119.6	1.3	1.7
Maduramicin	12	14.3	112.0	15.8	22.1	2	12.1	103.4	2.3	2.7
Monensin	2	15.2	101.8	2.60	3.09	2	13.3	106.7	2.7	3.7
Narasin	2	17.5	109.2	2.48	3.25	1	16.9	104.1	1.4	1.9
Nicarbazin	300	10.0	94.9	347	406	5	12.4	97.1	5.7	7.6
Robenidne	25	13.6	104.3	32.9	41.1	5	9.0	97.0	5.8	7.2
Salinomicin	3	12.7	101.1	4.08	5.60	2	19.4	104.5	2.8	3.8
Semduramicin	2	17.9	107.6	2.87	3.95	2	15.3	107.8	2.7	3.3
Toltruzil*	25	12.1	99.8	31.5	40.2	25	14.5	103.7	35.5	49.3
Toltrazuril sulfoxide*	25	14.1	92.1	31.4	39.4	25	15.3	97.2	34.1	45.3
Toltrazuril sulfone*	25	14.1	92.8	29.4	36.7	25	21.2	99.4	34.8	46.8
Arprinocid*	5	13.5	110.6	6.32	8.28	5	7.6	99.3	5.79	7.45
Nequinate*	5	15.1	95.1	5.72	7.06	5	13.5	110.6	6.3	8.3
Ethopabat*	5	9.5	99.5	6.05	7.19	5	8.4	101.9	6.1	7.1
Clazuril*	5	18.7	91.6	5.69	7.10	5	18.7	105.5	6.0	7.8
Clopidol*	10	17.8	110.3	14.6	19.4	10	16.1	104.8	12.9	17.1

** Coccidiostats without regulation - target levels for validation selected by authors.*

In conclusion, a sensitive and reliable method for simultaneous determination of twenty coccidiostats in wide range of food products was developed. The validation of the developed method proofed its fitness for confirmatory analysis. Presented method can be used for routine analysis of residues of coccidiostats in all matrices in scope of the method.

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THE OCCURRENCE OF CHLORAMPHENICOL RESIDUES IN TISSUES OF CHICKENS EXPOSED TO LOW DIETARY CONCENTRATIONS OF THE CHLORAMPHENICOL

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Abstract

Recently, data regarding possible natural occurrence of chloramphenicol in plant materials were presented. The potential for contaminated feed of plant origin to cause residues of this banned compound in chicken tissues was investigated. The chloramphenicol substance was added to common chicken feed at the concentrations of 0, 10, 50 and 200 micrograms of chloramphenicol per kilogram of feed. The final concentrations of chloramphenicol in feed were verified by LC-MS/MS method. Four separated groups of broiler chickens (eight animals in each group) were fed all their life (35 days) with this contaminated feed. They were allowed *ad libitum* access to this feed and fresh water. After slaughtering the chickens, the residues in muscle and liver tissues were determined using GC/MS-NCI method. No residues were detected in tissues of animals from groups fed by feed containing 0, 10 and 50 μg kg^{-1}. Low chloramphenicol residual concentrations were observed in a few of muscle samples obtained from group of chickens fed with feed containing chloramphenicol in added concentration 200 μg kg^{-1}. No residues were detected in the remaining samples of this group.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic prohibited for use in food-producing animals for its harmful effects independent of its concentration. Simultaneously, being a natural substance produced by bacteria *Streptomyces venezuelae*, the occurrence of CAP was demonstrated in various materials of plant origin (Berendsen *et al.*, 2010; Berendsen *et al.*, 2013; Nordkvist, 2013; Stolker *et al.*, 2012). CAP content determined in plants varied in concentrations from 0.1 μg kg^{-1} till several hundreds of μg kg^{-1}. Under the terms of official control, it was suggested that some of unexplained non-compliant findings within the residue monitoring programs may originate from natural occurrence of CAP. Nevertheless, no residues were detected in chicken tissues obtained from animals exposed to low concentrations of CAP in drinking water (Ramos and Rey-Maquiera, 2010).

Reference Point for Action (RPA) according to Regulation (EC) No 470/2009 for chloramphenicol is 0.3 μg kg^{-1} for all matrices.

The aim of this study was to observe CAP residues in edible chicken tissues obtained from the animals exposed to all-life diet simulating the possible natural concentrations in plant feeding.

Experimental

Chloramphenicol-contaminated feed

Milled, broiler grower feed was intentionally contaminated with chloramphenicol substance to target concentrations of 10, 50 and 200 μg kg^{-1}. Plain, untreated feed was used as the zero concentration level for the control purposes.

Preparation of contaminated feed. The drug was incorporated into the feed in gradual steps. Crystals of chloramphenicol substance were pulverized in ceramic mortar, 15.0 mg were weighed and mixed with 150 g of wheat flour in glass round-bottom flask by rotation for 60 min. A portion of 150 g of this flour mixture was blended with 600 g of milled feed on the knife mill Grindomix GM 200, Retsch (2 x 1 minute at maximum speed) in three identical partitions, which were combined in one plastic bag (mixture A).

Next blending step is described in the Table 1. Ten portions of each of the mixtures B, C and D were prepared by mixing of corresponding amount of mixture A with 200 g of plain milled feed on the knife mill Grindomix GM 200, Retsch (1 min at maximum speed). Each portion was placed into the plastic bag.

Table 1. Preparation of the mixtures B, C and D.

Target CAP concentration in feed	Mixture	Amount of mixture A	Amount of milled feed
10 μg kg^{-1}	B	2.5 g	200 g
50 μg kg^{-1}	C	12.5 g	200 g
200 μg kg^{-1}	D	50 g	200 g

The final blending step was performed on small conical spiral feed mixer. The content of each plastic bag with the mixtures B, C and D were subsequently incorporated into the 5 kg-batches of plain milled feed. The mixing was conducted for 20 min for

each batch. Five batches of the same concentration were joined in one plastic box. Two boxes of broiler feed (*i.e.* 50 kg) for each target concentration of 0, 10, 50 and 200 μg kg^{-1} were prepared.

Analytical verification of CAP concentration in feed. The true concentrations of CAP in contaminated feed were verified by LC-MS/MS determination of CAP in randomly taken feed samples from each batch of final broiler feed. The sample preparation consisted of liq/liq extraction and SPE purification.

The internal standard CAP-d5 at the concentration 5 μg kg^{-1} was added to a 1-g portion of crushed feed sample. Hereafter, 6 mL of the solution acetonitrile / 4% NaCl (aq) (1/1) (v/v) was added, and the sample was thoroughly homogenized with Ultra Turrax disperser and centrifuged at 3,000 x g for 5 min (at room temperature). The supernatant was treated with 2 x 3 mL of hexane (discarded) and 2 x 3 mL of ethyl acetate (collected and evaporated at 50°C). The dry residue was dissolved in 2 mL of 5% acetonitrile in water using an ultrasonic bath.

The SPE-C18 cartridge (1 g, 6 mL tubes) was conditioned with 10 mL of methanol and 20 mL of deionised water. Then the dissolved sample extract was put onto the cartridge. The column was subsequently washed with 5 mL of 5% acetonitrile in water, 5 mL of 20 % acetonitrile in water and the analyte was eluted with 5 mL of 40% acetonitrile in water. The eluate was extracted with 2 x 3 mL of ethyl acetate. The separated organic layers were combined and evaporated to dryness. The sample was dissolved in 0.2 mL of 20% aqueous solution of methanol and filtered through a syringe filter (RC, 0.2 μm) to the insert of chromatographic vial.

The chromatographic analysis was performed using Agilent Technologies equipment LC 1200 coupled with Triple Quad 6460 detector. The chromatographic column ZORBAX SB-C18 (2.1 x 50 mm, 1.8 μm) and pre-column ZORBAX SB-C18 (2.1 x 15 mm, 1.8 μm) were used, the column temperature was 45°C and the column flow was 0.4 mL min^{-1}. The injection volume was 20 μL. The mobile phase consisted of water and methanol, the gradient used is described in the Table 2. The approximate retention time of chloramphenicol was 3.5 min.

Table 2. Mobile phase gradient for LC-MS/MS method

Time	Methanol %	Water %
0 min	20	80
5 min	60	40
6 min	100	0

The detection conditions were as follows: ion source ESI-, drying gas temperature 325°C, drying gas flow 10 L min^{-1}, capillary voltage 3,500 V, fragmentor 100 V, collision energy 10 V, dwell time 200 ms. The ion transitions used are stated in the Table 3.

Table 3. Ion transitions for chloramphenicol measurement

Analyte	Precursor ion *m/z*	Product ion *m/z*
CAP (quantifier)	321	152
CAP (qualifier)	321	257
CAP-d5 (internal standard)	326	157

The method described was fully validated according to Commission Decision 2002/657/EC. The decision limit CCα was obtained at 0.5 μg kg^{-1}, the detection capability CCβ was 1 μg kg^{-1}. The matrix-spiked calibration (prior to the extraction) with eight levels of concentrations in the range 0 – 300 μg kg^{-1} was used for the determination of CAP content in feed samples.

Animal study

A number of 36 one-day-old broiler chickens were purchased from a standard commercial hatchery. Four animals were immediately sacrificed and their muscle tissue was tested for CAP residues. Remaining 32 animals were segregated into four fully isolated boxes, eight animals in each box. The chickens in each box were separately provided with previously prepared contaminated feed with CAP concentrations of 0, 10, 50 and 200 μg kg^{-1} and with fresh water on the *ad libitum* basis for 35 days. All the animals were sacrificed in that time considering this period as ordinary for reaching market weight for broilers.

Analysis of tissues

The samples of muscle and liver tissue were separately collected from the sacrificed animals and stored frozen at -20°C. The muscle tissue from both breasts, both legs and liver tissue of each individual were separately minced thoroughly prior to GC/MS-NCI analysis.

Analytical procedure for determination of CAP residues in tissues. The sample preparation consisted of liq/liq extraction and SPE purification. The internal standard CAP-d5 at the concentration 0.5 μg kg^{-1} was added to a 2-g portion of minced tissue sample. Hereafter, 6 mL of the solution acetonitrile / 2% NaCl (aq) (1/1) (v/v) was added, and the sample was thoroughly homogenized with Ultra Turrax disperser and centrifuged at 3,000 x g for 5 min (at room temperature). The supernatant was treated with 2 x 3 mL of hexane (discarded) and 2 x 3 mL of ethyl acetate (collected and evaporated at 50°C). The dry residue was dissolved in 2 mL of 5% acetonitrile in water using an ultrasonic bath.

The SPE-C18 cartridge (1 g, 6 mL tubes) was conditioned with 10 mL of methanol and 20 mL of deionised water. Then the dissolved sample extract was put onto the cartridge. The column was subsequently washed with 5 mL of 5% acetonitrile in water, 5 mL of 20 % acetonitrile in water and the analyte was eluted with 5 mL of 40% acetonitrile in water. The eluate was extracted with 2 x 3 mL of ethyl acetate. The separated organic layers were combined and evaporated to dryness.

The evaporated sample was transferred by tert-butyl methyl ether to the derivatisation vial. The solvent was evaporated under a nitrogen stream and derivatisation reagent (50 μL of BSTFA with 1% TMCS) was added. The sample was treated at 75°C for 45 min. The rest of the reagent after derivatisation was evaporated under a stream of nitrogen, and the dry residue was dissolved in 50 μL of anhydrous toluene for chromatographic analysis.

The chromatographic analysis was performed using Agilent equipment GC 6890 with a cool on-column injector and MSD 5973N detector in NCI mode. The chromatographic column was HP-1MS, length 30 m, I.D. 0.25 mm, film thickness 0.25 μm, carrier gas flow rate 1.2 mL min^{-1}. Ions *m/z* 466,468, 376, 378 for CAP and *m/z* 471 for internal standard were monitored in SIM mode.

The method described was fully validated for both muscle and liver tissue according to Commission Decision 2002/657/EC. The decision limit CCα was obtained at 0.05 μg kg^{-1}, the detection capability CCβ was 0.06 μg kg^{-1}. The matrix-spiked calibration (prior to the extraction) with six levels of concentrations in the range 0 – 2 μg kg^{-1} was used for the determination of CAP residues in muscle and liver samples.

Results

Chloramphenicol-contaminated feed

The results of CAP determination in feed samples of each batch are presented in the Table 4.

No residues were detected in samples of untreated feed (CCα = 0.5 μg kg^{-1}).

Table 4. Results of feed sample analyses

Target concentration	Measured concentration (average)	Standard deviation
0 μg kg^{-1}	not detected	-
10 μg kg^{-1}	8.3 μg kg^{-1}	3.2
50 μg kg^{-1}	49.5 μg kg^{-1}	14.3
200 μg kg^{-1}	223 μg kg^{-1}	40.5

Residues in tissues

The individual results of CAP determination in muscle tissue (breast and legs separately) and liver tissue are presented in Table 5.

No residues were detected in liver tissue samples of animals from all feeding groups and in muscle tissues of animals from groups fed by feed containing 0, 10 and 50 μg kg^{-1}. Low chloramphenicol residual concentrations were observed in muscle samples of four animals from group of chickens fed with feed containing chloramphenicol in added concentration 200 μg kg^{1}. All these concentrations were lower than RPA for CAP (0.3 μg kg^{-1}), whereas the concentration found in leg muscle were slightly higher than breast muscle concentrations. No residues were detected in the remaining six animals of this group.

Table 5. Results of tissue sample analyses

CAP concentration in feed	Animal number	Breast tissue	Leg tissue	Liver
0 μg kg^{-1}	1	not detected	not detected	not detected
	2	not detected	not detected	not detected
	3	not detected	not detected	not detected
	4	not detected	not detected	not detected
	5	not detected	not detected	not detected
	6	not detected	not detected	not detected
	7	not detected	not detected	not detected
	8	not detected	not detected	not detected
10 μg kg^{-1}	1	not detected	not detected	not detected
	2	not detected	not detected	not detected
	3	not detected	not detected	not detected
	4	not detected	not detected	not detected
	5	not detected	not detected	not detected
	6	not detected	not detected	not detected
	7	not detected	not detected	not detected
	8	not detected	not detected	not detected
50 μg kg^{-1}	1	not detected	not detected	not detected
	2	not detected	not detected	not detected
	3	not detected	not detected	not detected
	4	not detected	not detected	not detected
	5	not detected	not detected	not detected
	6	not detected	not detected	not detected
	7	not detected	not detected	not detected
	8	not detected	not detected	not detected
200 μg kg^{-1}	1	not detected	not detected	not detected
	2	0.06 μg kg^{-1}	0.11 μg kg^{-1}	not detected
	3	not detected	not detected	not detected
	4	not detected	0.14 μg kg^{-1}	not detected
	5	0.07 μg kg^{-1}	0.15 μg kg^{-1}	not detected
	6	not detected	not detected	not detected
	7	not detected	not detected	not detected
	8	0.16 μg kg^{-1}	0.18 μg kg^{-1}	not detected

Discussion and conclusions

Four groups of chicken broilers were fed all their life (35 days) with milled feed intentionally contaminated with CAP at the four different levels simulating the concentrations which were detected in materials of plant origin. The CAP concentrations found in straw (Berendsen *et al.*, 2013; Nordkvist, 2013; Stolker *et al.*, 2012) were in majority below 10 μg kg^{-1}, altogether lower than 50 μg kg^{-1}. No residues were detected in tissues obtained from chicken animals fed with these CAP concentrations. Based on this observation it can be concluded that any detection of CAP residues in chicken tissue (muscle or liver) cannot be legitimized by the reference to natural occurrence of CAP. Also the absence of detectable residues in most of samples obtained from group of animals fed with feed contaminated at the concentration 200 μg kg^{-1} shows very quick depletion of this low CAP amount intake. A few detected concentrations were below the established RPA for chloramphenicol.

In comparison, detectable concentration of CAP residues in chicken muscle tissues were found 35 days after the last a kg^{-1}dministration of hypothetic therapeutic dose of chloramphenicol to one-day-old chicken (Rejtharova *et al.,* 2010). A dual depletion mechanism is presumed, different for acute therapeutic treatment with high concentration and for low concentration chronic exposure. Any CAP concentration confirmed in edible chicken tissues indicates the illegal treatment of food animals with prohibited substance and should be followed by the official investigation.

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THE DETERMINATION OF TESTOSTERONE ESTERS AND ESTRADIOL ESTERS IN *BOVINE* AND *PORCINE* BLOOD SERUM

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Abstract
A monitoring of steroid esters in blood serum is desirable for revealing of possible illegal use of natural hormones as growth promoters. A method for determination of testosterone propionate, testosterone benzoate, testosterone isocaproate, testosterone decanoate and estradiol benzoate in *bovine* and *porcine* blood serum was developed. The procedure consists of extraction on HybridSPE-Phospholipid columns, clean-up on HLB SPE columns and LC MS/MS measurement. This method has been validated according to CD 2002/657/EC. Decision limits for all analytes were observed in the range 5 – 30 pg mL^{-1}. The method described is considerably robust and can be applied both for screening and confirmatory analyses in routine residue monitoring.

Introduction
Anabolic steroids are forbidden for use in food-producing animals in the European Union. The steroids are applied in the form of esters to the animals in cases of illegal use. The steroid esters cannot occur naturally in animal tissues. Analytical detection of intact steroid esters in blood serum can be used as an official proof of illegal treatment of animals with these growth promoters. In our laboratory the sensitive method for determination of anabolic steroid esters in blood serum, concretely testosterone propionate, testosterone benzoate, testosterone isocaproate, testosterone decanoate and estradiol benzoate, was developed. The detailed method description and obtained validation parameters are presented.

The method described was verified by analyses of blood serum samples obtained from pigs treated with anabolic hormones in preliminary residual animal study.

Materials and Methods
The reference standards of analytes were supplied by Sigma-Aldrich (estradiol benzoate, testosterone benzoate and propionate) and by EDQM (testosterone isocaproate and decanoate). Testosterone propionate-d3, benzoate-d3 and decanoate-d3 were used as the internal standards, all supplied by Bank of reference standards RIVM/RIKILT. Substances were dissolved in methanol and diluted to low concentration working solutions. All solvents used were obtained from Merck and they were of SupraSolv quality. The Hybrid SPE-Phospholipid columns (product no. 55267-U) and Select HLB SPE tubes (product no. 54183-U) were purchased from Supelco (a member of the Sigma-Aldrich Group).

Primary extraction
A volume of 1.0 mL of blood serum was instilled to a centrifugation tube. The sample was spiked with internal standards working solution (at the final concentration in serum 100 pg mL^{-1}) and eventually fortified with esters standards working solution. 3 mL of acetonitrile with 1 % formic acid were added and the sample was thoroughly homogenized using vortex mixer and afterwards centrifuged at 5000 x g, 10 min at the room temperature. The clear supernatant was transferred on dry HybridSPE-Phospholipid column and the eluate was collected. The column was washed with 1 mL of methanol which was also collected. A combined acetonitrile and methanol extract was evaporated to dryness at 40°C under the gentle stream of nitrogen.

Extract clean-up on HLB SP columns

Column preparation*. Column was conditioned with 6 mL of methanol followed by 6 mL of water.*

Sample clean-up. The extract from the primary extraction was dissolved in 2 mL of 50 % methanol in water and it was applied on the column. The evaporation flask was washed with additional 1 mL 50 % methanol, which was also applied on the column. The column was washed with 3 mL 80 % methanol in water and the analytes were eluted with 4 mL of solution methanol/dichloromethane (7/3) to the clean evaporation flask and all solvents were evaporated to dryness at 40 °C under the gentle stream of nitrogen. The residues in the flask were dissolved in 0.5 mL of 75 % acetonitrile with water by using ultrasonic bath and filtered through a filter (Hydrophilic PTFE, 0.2 µm) to the chromatographic vial.

Measurement
The chromatographic analysis was performed by using Agilent Technologies equipment LC 1260 Infinity coupled with Triple Quad 6495 detector. The chromatographic column Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 µm) and pre-column Poroshell

EC-C18 (2.1 x 5 mm, 2.7 µm) were used. The column temperature was 45 °C and the mobile phase flow was 0.3 mL min^{-1}. The sample was injected in the volume of 20 µL. The mobile phase consisted of acetonitrile with 0.05 % formic acid (A) and 50 % acetonitrile with water with 0.05 % formic acid (B). The gradient used in this method is described in the Table 1. The detection conditions were as follows: ion source AJS ESI+, drying gas temperature 325 °C, drying gas flow 10 $L.min^{-1}$, capillary voltage 4000 V. The ion transition and retention times of all esters are stated in the Table 2.

Table 1. Mobile phase gradient.

Time	(A) %	(B) %
0 min	50	50
10 min	100	0
11 min	100	0

Table 2. MRM transitions and retention times for esters.

Analyte	Precursor ion *m/z*	Product ion *m/z*	Fragmentor (V)	Collision energy (eV)	Retention time (min.)
estradiol benzoate[a]	377	105	380	18	3.2
testosterone benzoate	393	271	380	14	4.6
testosterone benzoate – d3	396	105	380	24	4.6
testosterone decanoate	443	271	380	16	12.4
testosterone decanoate – d3	446	274	380	16	12.4
testosterone isocaproate[a]	387	271	380	16	6.4
testosterone propionate	345	271	380	14	3.0
testosterone propionate – d3	348	274	380	14	3.0

[a] *Testosterone benzoate – d3 was used as the internal standard for the analytes estradiol benzoate and testosterone isocaproate.*

Animal study

In the informative animal study, nine pigs were treated with an injection product containing testosterone propionate, testosterone isocaproate and testosterone decanoate. 3-months-old animals were injected with 21 mg of testosterone propionate, 42 mg of testosterone isocaproate and 70 mg of testosterone decanoate by a single application.

Blood serum samples were obtained one day after dosage from 9 treated and 4 control animals. One blood serum sample was taken from the treated animal 1 month after hormones application.

Results and Discussion

The method was validated according to CD 2002/657/EC.

The linearity was observed by measuring a blank blood serum samples and samples fortified at different concentration levels (0, 10, 20, 50, 100, 200 pg mL^{-1}). The relative response was plotted against the entered concentration, the curve was constructed and the linear regression was applied. The correlation and regression coefficients are described in the Table 3. The reproducibility was obtained by the measurement of the set of 6 samples, prepared at the different days, fortified at the same concentration level (50 pg mL^{-1}). The relative standard deviations for all analytes are also stated in the Table 3.

Table 3. Linearity and reproducibility.

Analyte	Slope	Intercept	Regression coefficient	RSD %
estradiol benzoate	6.497	0.338	0.9981	22.2
testosterone benzoate	1.681	0.045	0.9677	21
testosterone decanoate	14.234	0.154	0.9975	2.9
testosterone isocaproate	11.308	0.148	0.9975	17.6
testosterone propionate	13.598	0.169	0.9869	5.3

Blank samples and samples fortified at 6 different concentration levels were prepared repeatedly (eleven times) on different days from blood serum samples obtained from various animals for determination of the within-laboratory reproducibility. From the Student's t value at the 99% confidence level (2.326), the standard deviation of the intercept and the regression coefficients, the decision limit and the detection capabilities were established. Regression coefficient, standard deviations of

the intercepts, decision limits (CCα) and detection capabilities (CCβ) obtained are stated in the Table 4. The procedure was verified both for *bovine* and *porcine* blood serum.

Table 4. Results of within-laboratory reproducibility (66 observations).

Analyte	Slope	Intercept	Standard deviation of the intercept	CCα (pg.mL^{-1})	CCβ (pg.mL^{-1})
estradiol benzoate	6.391	0.283	0.0406	14.8	25.2
testosterone benzoate	1.639	0.039	0.0071	10.1	17.2
testosterone decanoate	15.106	0.145	0.0432	6.6	11.3
testosterone isocaproate	4.720	0.119	0.0546	26.9	45.9
testosterone propionate	14.686	0.155	0.0325	5.1	8.7

Analyses of samples from animal study

A number of 14 samples obtained in the informative animal study were analysed by the method described. The results are presented in the Table 5.

Table 5. Concentrations of steroid esters in blood serum samples (pg mL^{-1}).

Samples	Testosterone decanoate	Testosterone isocaproate[a]	Testosterone propionate
Control animals (4 samples)	not detected	not detected	not detected
Treated animals – 1 day (9 samples)	45 - 440	55 - 291	79 - 212
Treated animal – 1 month (1 sample)	10.1	detected, not confirmed	10.3

[a] *Testosterone propionate – d3 was used as the internal standard for testosterone isocaproate*

Conclusions

The validation according to CD 2002/657/EC verified the suitability of the method for the screening and confirmatory purposes for monitoring of esters residues. The method described provides sufficient sensitivity and robustness for routine laboratory control of *bovine* and *porcine* blood serum samples (all decision limits and detection capabilities are far below 0.1 ng mL^{-1}). The sample preparation procedure allows a high throughput of handled samples at low laboratory material cost.

Acknowledgements

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SCREENING METHOD FOR THE IDENTIFICATION OF BANNED COMPOUNDS IN URINE

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Abstract

A very effective analytical procedure for qualitative analysis of chloramphenicol, chlorpromazine, colchicine, dapson, dimetridazole, metronidazole, ronidazole, furazolidone, furaltadone, nitrofurantoin and nitrofurazone in urine was developed. During method development different sample extraction and clean-up procedures were tested. The final procedure involved enzymatic hydrolysis followed by a chemical hydrolysis/derivatisation extraction procedure with a QUECHERS clean-up step. The compounds of interest were detected using an LC-MS/MS equipped with a ESI, operating in positive and negative multiple reaction monitoring (MRM). The developed method is validated according Commission Decision 2002/657/EC.

Introduction

After the positive findings of banned antibiotics in 2013 and 2014 in The Netherlands the need for a screening method that can determine all banned veterinary drugs mentioned in Table 2 of EC 37/2010 became urgent. Within the Dutch National Residue Control Plan a large variety of compounds is already monitored in several matrices including the banned compounds. However, in most cases methods are focussing on one compound (*i.e*. chloramphenicol) or a group of compounds (*i.e*. nitrofurans) in tissues, milk and eggs. To determine in an early stage whether a banned compound has been used, monitoring and investigating non-invasive materials is necessary.

The research presented focusses on the development of a monitoring method for the screening of banned compounds mentioned in Table 2 of EU 37/2010 in urine. The benefits of analysis in urine are the possibility to detect compounds in an early stadium and the method is non-invasive. The compounds of interest are chloramphenicol, chlorpromazine, colchicine, dapson, dimetridazole, metronidazole, ronidazole, furazolidone, furaltadone, nitrofurantoin and nitrofurazone. The developed multi-method is able to screen all named compounds using one sample preparation procedure and LC-MS/MS analysis. The main challenge of method development was to combine chloramphenicol and the nitrofurans in one single method. Chloramphenicol occurs as a glucuronide in urine and has to be hydrolysed enzymatically before mass spectrometry analysis (Berendsen *et al.*, 2011). Nitrofurans are metabolized in a living organism resulting in protein bound and free metabolites. For analysis it is necessary to hydrolyse the protein bound metabolites followed by derivatisation of all present metabolites resulting in nitrophenyl derivatives.

The final sample preparation consists of a enzymatic and chemical hydrolysis extraction followed by derivatisation of the nitrofuran compounds and a QUECHERS clean-up step.

Materials and Methods

Materials

Ultra LC-MS grade water, methanol and acetonitrile (Actu-all chemicals, Oss, The Netherlands), formic acid 98-100% LC-MS grade, ammonia solution 25%, HCL solution 32%, acetic acid, trinatriumphosphate.dodecahydrate, sodiumhydroxide (Merck, Darmstadt, Germany), 2-nitrobenzaldehyde (Sigma-Aldrich, Saint Louis, MI, USA), β-glucuronidase/arylsulfatase (VWR, Radnor, Pennsylvania, United States) were used. QUECHERS Extract Pouches and AOAC dispersive SPE 15 Ml kit were obtained from Agilent technologies. Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18.2MΩ cm^{-1} (Millipore, Billerica,MA, USA). The reference standard of chlorpromazine, colchicine, dapson, semicarbazide.HCL (SEM), dimetridazole, ronodazole, metronidazole, metronidazole-d_4, 1-amino-imidazolidin-2,4-dione-[2,4,5-^{13}C] (AHD-$^{13}C_3$) were obtained from Sigma-Aldrich (Saint Louis, MI, USA), Chlorpromazine-d_6 HCL, 3-amino-2oxazolidinon (AOZ), 3-amino-2oxazolidinon-d_4 (AOZ-d_4), 5-methylmorfolino-3-amino-2-oxazolidinon (AMOZ), 5-methylmorfolino-3-amino-2-oxazolidinon-d_5 (AMOZ-d_5), 1-Amino-hydantoine (AHD) hydrochloride, semicarbazide Hydrochloride ($^{15}N_2$,^{13}C-SEM), chloramphenicol-d_5, dimetridazole-d_3, ronidazole-d_3, 3-(Nitrophenyl)methylene)-amino-2-oxazolidinon (NPAOZ), 5-Methylmorfolino-3-(nitrophenyl)methylene-3-amino-2-oxazolidinon (NPAMOZ), 1-(Nitrophenyl)methylene)-amino-hydantoine (NPAHD), Nitrophenylmethylene-semicarbazide (NPSEM) were obtained from Witega (Berlin, Germany), dapson-d_8, CAP-glucuronide were obtained from Toronto research chemicals (Toronto, ON, Canada) and 3,5-dinitro-N'-nitrophenyl)methylene]salicylhydrazide (NPDSH) was synthesised at RIKILT (Wageningen, The Netherlands).

Sample preparation

The required amount of urine was transferred into a polypropylene centrifuge tube. After pH setting, enzymatic hydrolysis was carried out using β-glucuronidase/arylsulfatase for about an hour at 50°C. After enzymatic hydrolysis, chemical hydrolysis and derivatisation is being performed overnight at 37°C using a HCL and NBA (2-nitrobenzaldehyde) solution). Consequently, sample clean-up was performed using acetonitrile and a QUECHERS kit followed by dispersive SPE. Prior to LC-MS/MS analysis the sample was filtered using a Whatman Rezist filter and transferred into a LC-MS vial.

Compound structures

Figure 1 shows molecular structures of the selected compounds (Table 2 of EC 37/2010).

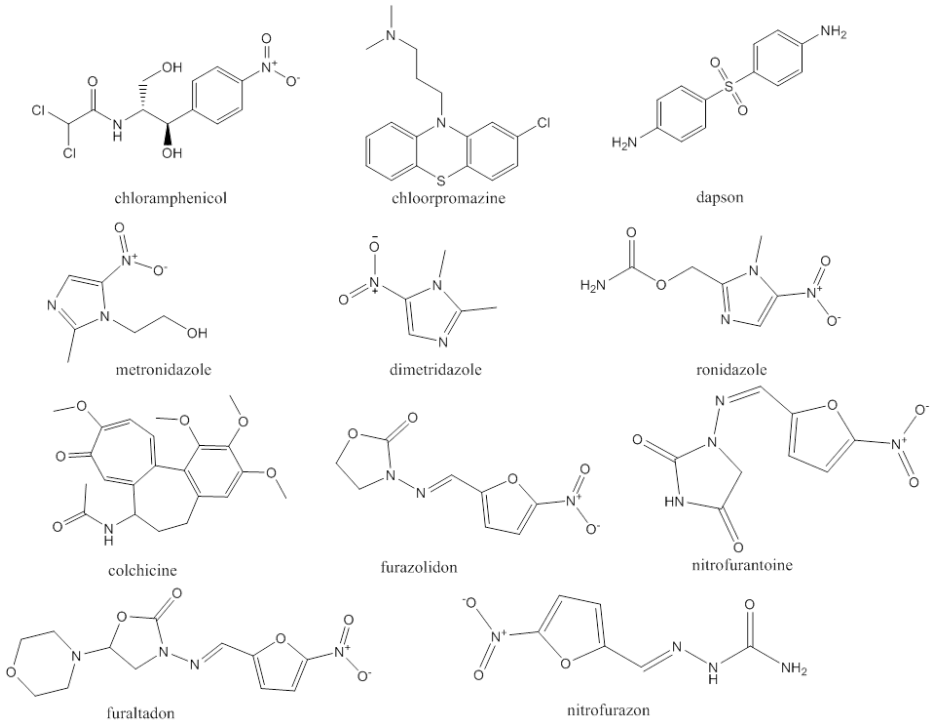

Figure 1. Molecular structures of the selected compounds (Table 2 of EC 37/2010): chloramphenicol, chlorpromazine, dapson, metronidazole, dimetridazole, ronidazole, colchicine, furazolidone, furaltadone, nitrofurantoin and nitrofurazone

LC-MS/MS analysis

The LC system consisted of a Waters (Milford, MA, USA) model Acquity equipped with a HSS T3 C18 column of 2.1 x 100mm, 1.8µm, placed in a column oven at 35°C and an injection volume of 10 µL. The gradient was (solvent A, 1 M ammonium formate and 0,16% formic acid; solvent B, 1 M ammonium formate and 0,16% formic acid in MeOH): 0-1.0 min, linear increase to 50% B; 5-8.5 min, at 50% B; 8.5-10.0 min, linear increase to 95% B; 10.0-10.5 min, linear decrease to 0% B; 10.5-17.0 min, at 0% B; operating at a flow of 0.4 mL min^{-1}. Detection was carried out by LC–MS/MS using an AB Sciex (Ramingham, MA, USA) Q-Trap 6500 mass spectrometer in the positive and negative electrospray ionisation (ESI) mode. The operating parameters were: Curtain gas 40 L h^{-1}, Ion spray voltage 4,500 V, Temperature: 400 °C, Ion source gas 50 $Lh^{-}1$ and Ion source 50 $Lh^{-}1$. The antibiotics were fragmented using collision induced dissociation (N_2) and the scheduled Selected Reaction Monitoring (SRM) transitions are presented in Table 1. Data was processed using Multiquant software V2.1.1 (AB Sciex). The nitrofuran compounds (furazolidone, furaltadone, nitrofurantoin and nitrofurazone) were hydrolysed, resulting in the marker metabolites (AOZ, AMOZ, AHD and SEM) and derivatisation of these markers resulted in nitrophenyl derivatives (NPAOZ, NPAMOZ, NPAHD and NPSEM)(see Figure 2 for the corresponding structures). The nitrophenyl derivatives were measured using this LC-MS/MS analysis.

Table 1. SRM transitions of the forbidden compounds and their internal standards

Component	Precursor ion (*m/z*)	Product ion (*m/z*)	DP	CE (eV)	CXP
Chlorpromazine	319.0	86.0	60	25	12
		58.0	60	67	8
Chlorpromazine-d_6	325.0	92.0	60	25	12
	325.0	64.0	60	61	10
Colchicine	400.0	357.9	51	31	12
		152.0	51	117	10
		309.9	51	35	10
		164.9	51	101	10
Dapson	249.1	155.8	46	19	10
		107.8	46	29	12
		91.8	46	29	12
Dapson-d_8	257.0	160.0	46	20	10
Dimetridazole	142.1	97.7	26	13	12
		56.8	26	27	8
		95.8	26	21	14
		80.1	26	35	12
Dimetridazole-d_3	145.0	99.0	26	13	12
Metronidazole	172.1	127.9	26	19	16
		81.9	26	33	12
		130.8	26	19	14
Metronidazole-d_4	176.0	127.9	26	19	16
Ronidazole	201.0	132.9	26	25	14
		139.94	26	15	10
		64.8	26	47	8
Ronidazole-d_3	204.1	143.0	26	15	10
NPAHD	249.0	134.0	60	17	4
		104.0	60	27	4
		51.0	60	57	4
		178.0	60	30	10
NPAHD- $^{13}C_3$	252.2	134.1	60	12	12
NPAMOZ	335.3	262.3	60	15	10
		291.3	60	15	13
NPAMOZ-d_5	340.4	296.3	60	15	13
NPAOZ		104.1	41	17	20
	236.2	134.1	41	17	20
NPAOZ-d_5	240.2	134.1	80	17	12
NPSEM	209.2	134.1	60	34	12
		166.2	60	13	12
		192.2	60	15	12
NPSEM-$^{15}N_2.^{13}C$	212.2	168.2	60	10	12
Chloramphenicol	321	152.0	-60	-25	-12
		194.0	-60	-15	-12
Chloramphenicol-d_5	325.9	157.0	-60	-12	-12
		199.0	-60	-22	-12

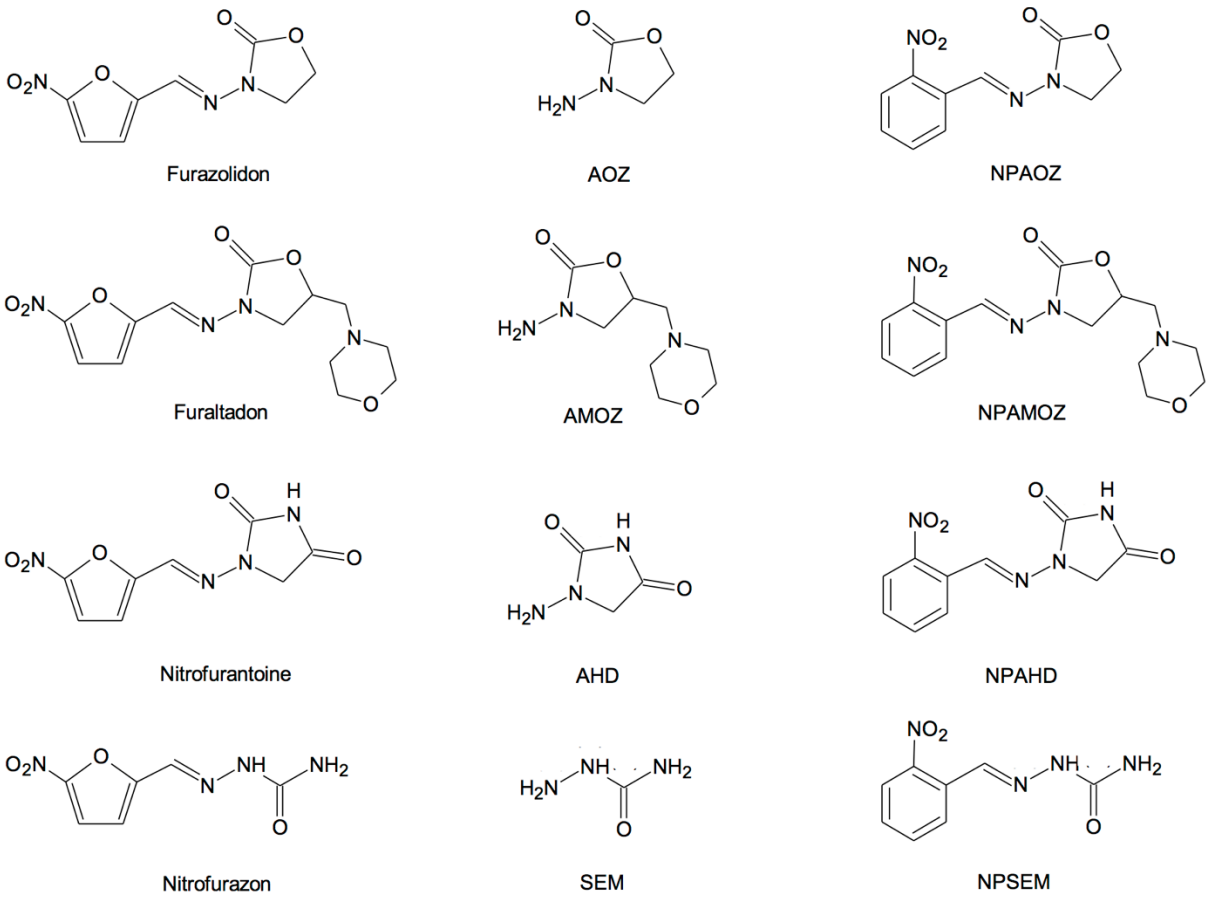

Figure 2. Chemical structures of nitrofurans (furazolidone, furaltadone, nitrofurantoin and nitrofurazone), corresponding marker metabolites (AOZ, AMOZ, AHD and SEM) and nitrophenyl derivatives (NPAOZ, NPAMOZ, NPAHD and NPSEM) of the marker metabolites.

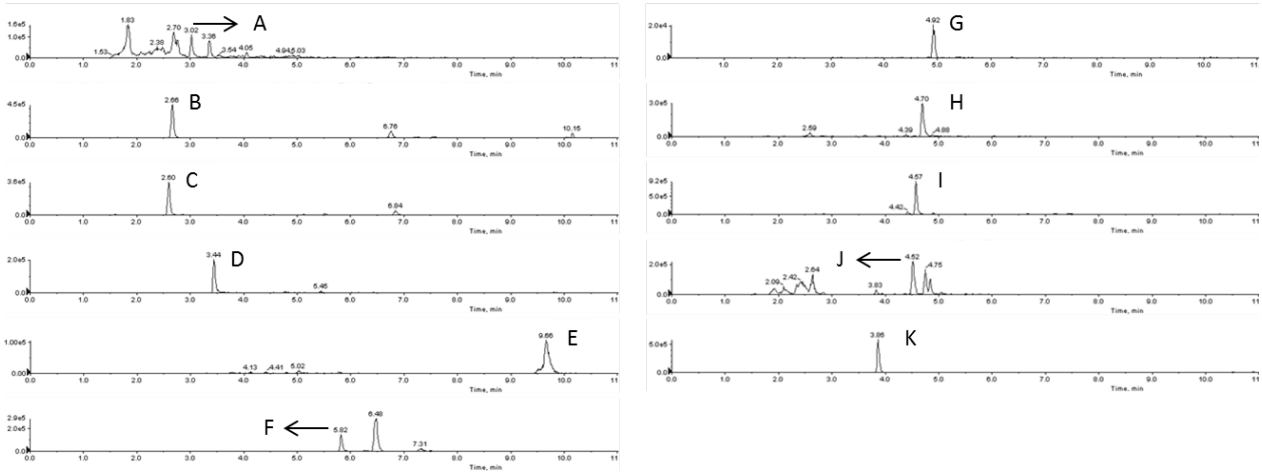

Figure 3. chromatogram of all compounds. A: dimetridazole 95.8 m/z, B: metronidazole 127.9 m/z, C: ronidazole 139.9 m/z, D: dapson 155,8 m/z, E: chlorpromazine 58.0 m/z, F: colchicine 357.9 m/z, G: chloramphenicol 152.0 m/z, H: NPSEM 166.2 m/z, I: NPAOZ 134.1 m/z, J: NPAHD 134.0 m/z, K: NPAMOZ 291.3 m/z.

Results and discussion

During method development it was observed that it is possible to combine the enzymatic and chemical hydrolysis in one method. The enzymatic hydrolysis has to be performed prior to the chemical hydrolysis because of the stability of the used enzymes. The developed method has been tested using a blank urine samples fortified with the compounds of interest at concentrations ranging from 0.15 to 1.0 µg L^{-1} for chloramphenicol and 0.5 - 2.0 µg L^{-1} for the other compounds. Results show that qualitative analysis is possible at 0.3 µg L^{-1} for chloramphenicol and for the other compounds at 1.0 µg L^{-1}. A chromatogram of a blank urine sample fortified with all compounds at a concentration of 1.0 µg L^{-1} is given in Figure 3. Momentarily the method is validated according CD 2002/657/EC.

Conclusions

The aim of the research was to develop one generic method for quantification of all banned compounds listed in Table 2 of EC 37/2010. The fact that chloramphenicol occurs as a glucuronide in urine and has to be hydrolysed enzymatically combined with the fact that the nitrofurans have to be hydrolysed to their marker metabolites followed by derivatisation to yield nitrophenyl derivatives, was the main challenge.

It can be concluded that the combination of two different hydrolysis steps in one method is possible. yielding qualification of all compounds in one single method. The method is able to detect chloramphenicol at a level of 0.15 µg L^{-1} and for chlorpromazine, colchicine, dapson, dimetridazole, metronidazole, ronidazole, furazolidone, furaltadone, nitrofurantoin and nitrofurazone at 0.5 µg L^{-1}.

Acknowledgements

This research was funded by the Dutch Ministry of Economic Affairs under its statutory tasks.

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EFFECT OF DIET RICH IN CRUCIFEROUS PLANTS TO THE PRESENCE OF ENDOGENOUS THIOURACIL IN URINE AND MILK OF CATTLE

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Abstract

The aim of the study was to determine the effect of a diet rich in rapeseed meal on thiouracil formation in the urine and milk of dairy cows. Two-week feeding dairy cows with feed containing 30% of rapeseed meal did not cause a permanent increase in concentration of endogenous thiouracil in urine and milk samples and it should not be the cause of noncompliant results. The process of increase and decrease of the concentration of thiouracil in urine samples in different animals is individual and has the cyclical nature. The highest determined concentration of natural thiouracil in urine was 3.61 µg L^{-1}. It was found that endogenous thiouracil occurs in two tautomeric forms. Few days storage of urine samples in the frozen state, affects the stability of the natural thiouracil, whereas acidic environment of the samples (pH 2) improves the stability of the compound and its isomer, which are stable even after two months of storage at the temperature below -18°C. Two-week feeding cows rapeseed enriched feed up to the maximum allowed dose, does not cause an increase of thiouracil concentration in samples of milk, serum, and faeces above decision limits.

Introduction

Thyreostatic drugs are a complex group of substances that have different chemical structures. Their common feature is inhibition of synthesis of thyroid hormones, which promotes increases in the live weight of animals prior to slaughter. Therefore, the thyreostats can be used for anabolic purposes in fattening of animals, in particular of cattle. The weight gain mainly results from an increased filling of the gastro-intestinal tract and augmented water retention within edible tissue (Vanden Bussche *et al.,* 2009). Therefore, the meat derived from treated animals is of lower quality. Moreover, it may contain residues harmful to human health, because some compounds of this group possess carcinogenic and teratogenic properties. For this reason, the use of thyreostatic drugs for animal fattening purposes has been banned in the European Union since 1981 (Council Directive 81/602/EEC), and that means zero tolerance for residues.

In a document published in 2007, European Reference Laboratories established the "recommended concentration" at 10 µg L^{-1} (kg^{-1}) for these compounds. The introduction of a very sensitive method in the control of thyreostats in samples of animal origin led to the detection of the presence of thiouracil in *bovine* and *porcine* urine in some European countries. According to 2013 EFSA report (EFSA Report, 2013) it is the most frequently reported compound from group A.

The presence of thiouracil in urine of animals may result from the illegal administration of this compound or the use of cruciferous plants in animal feeding. The particular reports accessible in the literature confirm that TU can occur endogenously in some animal species as a consequence of diet containing cruciferous plants *(Brassicaceae, Cruciferae)* (Pinel *et al*., 2006) including rape, which is widely used in Poland in feed for livestock. Properties of this plant, such as high protein content, low price and availability, make its share in feeding the animals, including dairy cattle, significant.

Research conducted in Poland in 2010-2011 have shown that in 12.9% of the *bovine* urine samples thiouracil was present above the decision limit of 0.91 µg L^{-1}. In fact, 2.5% of the samples were over the recommended concentration of 10 µg L^{-1} (CRL Guidance Paper, 2007). The presence of thiouracil above CCα (0.63 µg L^{-1}) but below 10 µg L^{-1} have also been found in samples of raw milk and milk powder. Taking into account the natural occurrence of thiouracil in *bovine* urine, it is justified to take action to verify whether this carcinogenic compound is present in other biological matrices including milk, which is the main raw material used in the manufacture of foods intended for children. Therefore, studies were undertaken to determine the effect of rapeseed meal rich diets on the concentration of thiouracil in urine and milk of dairy cows. Stability studies on natural thiouracil in urine samples at different storage conditions were also performed.

Materials and Methods

Reagents and chemicals

Methanol (HPLC isocratic grade), *n*-hexane (ultra-resi analysed), acetonitrile (LC-MS reagent), and sodium hydroxide were purchased from Mall Baker (Deventer, The Netherlands). Analytical grade reagents such as diethyl ether, acetic acid (99.5%), *ortho*-phosphoric acid (85% purity), boric acid, and concentrated hydrochloric acid were obtained from POCH (Poland). Water was purified using a Milli-Q system (Millipore, USA). Derivatisation reagent 3-iodobenzyl bromide (3IBBr) was provided by Sigma-Aldrich (Germany). Britton-Robinson buffer (0.4 M), pH 8.0 was prepared by dissolving boric acid (24.73 g) in warm

water (800 mL), then adding *ortho*-phosphoric acid (26.7 mL), acetic acid (23 mL), 50% sodium hydroxide solution, and diluting with water to 1,000 mL. A 50% sodium hydroxide solution in water was prepared by dissolving the solid substance (500 g) in water (500 mL). A 0.1% acetic acid solution was prepared by mixing acetic acid (0.25 mL) with water (249.75 mL). Solution of 3IBBr was drawn up by dissolving reagent (10 mg) in methanol (4 mL). The injection solvent comprised of acetonitrile and 0.1% acetic acid solution (25:75, v/v).

Standards of thyreostats: 2-thiouracil (TU) and internal standard (IS) 5,6-dimethyl-2-thiouracil (DMTU) were obtained from Sigma-Aldrich (Germany). The standards were stored in accordance with the manufacturer's recommendation. Primary standard stock solutions of each compound were prepared in methanol at a concentration of 1 mg mL^{-1}. Intermediate standards solutions were prepared by further dilution of stock solution with methanol to obtain final concentrations of 100 µg mL^{-1}; 10 µg mL^{-1} and 1 µg mL^{-1}.

Dairy cows experiment

The experiment conducted on six dairy cows. Three animals of the control group received standard feed intended for dairy cows containing up to 11% rapeseed meal, based on the concentrates. The other three cows during 2 weeks were fed feed enriched with rapeseed meal in the maximum permissible amount of 30%. During the experiment, twice a day - in the morning and evening, the samples of urine and milk were collected. Urine samples were collected non-invasively; on the last day of the experiment also the blood and faeces samples have been taken. The material was analyzed on the day of collection or after freezing (temp <-18°C) within a maximum of 4 days.

Stability studies of the natural thiouracil in urine samples

Urine samples were stored at the following temperature conditions: 2 - 8°C - 3 days; <-18°C - 10 days, 30 days. Additionally, the influence of acidification of urine samples after collection on stability of thiouracil was investigated.

Sample preparation and LC-MS/MS analysis

The analytical protocols describing the preparation of urine samples and milk samples, and LC-MS/MS analysis have been published (Wozniak *et al*., 2011; 2014). In brief, TU was extracted from urine samples with diethyl ether after derivatization with 3-iodobenzylbromide in basic medium, and detected and identified by means of LC-ESI (+)-MS/MS. Thiouracil from the milk samples was extracted with methanol after the precipitation of the protein and then the same protocol as for urine samples was used. For the analysis of serum and faeces samples, the method designed for urine samples was applied, after the introduction of the necessary modifications.

Results and discussion

In the control group of animals, thiouracil concentration in urine of cows does not exceed the decision limit of 0.91 µg L^{-1}. After two weeks feeding cows with feed containing 30% rapeseed meal, no significant increase of thiouracil concentration in the urine of the animals was observed. In the first week of administration, an increase in thiouracil concentration was observed in samples taken in the evening, which returned to baseline in samples taken in next day morning. The process of increasing and decreasing thiouracil concentrations in the urine samples was specific for each animal; there were no regularities between the duration of the experiment and the level of concentration.

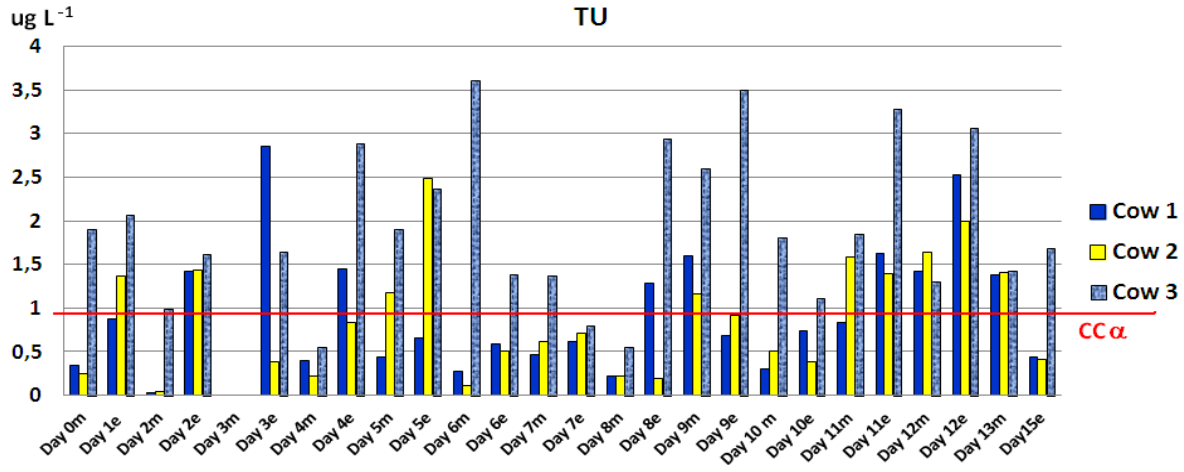

Figure 1. The concentrations of thiouracil in urine samples taken from the three cows, in the subsequent days of the experiment.

In Figure 1, concentrations of thiouracil in urine of individual animals during the experiment are presented. It can be stated that the increase and decrease of the concentration was cyclic. The highest concentration of thiouracil was recorded after the fifth day of administration of rapeseed meal- enriched feed (3.61 µg L^{-1}). After analysis of the chromatograms of all samples, it was found that TU was always accompanied by an unidentified peak with a retention time shorter by 0.5 min, having the same transition as thiouracil and the same relative intensity of ions (Figure 2).

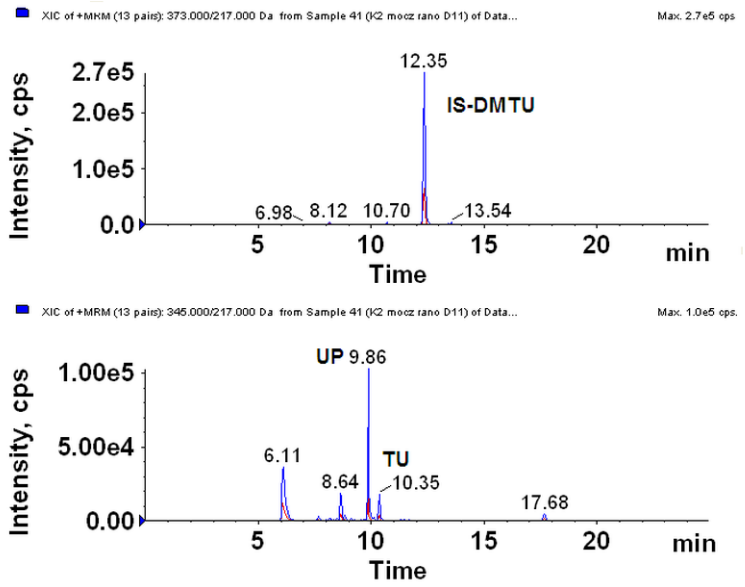

Figure 2. LC-MS/MS chromatograms of urine sample containing confirmed 1.58 µg L^{-1} thiouracil and 6.92 µg L^{-1} of an unidentified substance (expressed as TU).

The concentration of the compound corresponding to this peak expressed as TU concentration is subject to the same manner of fluctuations as thiouracil (Figure 3). The level of this compound is about 3 times higher than TU. According to the literature, thiouracil may be present in the six tautomeric forms (Khvorostov 2006). Because the unidentified compound meets the confirming criteria for thiouracil, required for LC-MS/MS methods according to the Commission Decision 2002/657 /EC, with the exception of retention time, it can be assumed that this is an isomer of TU. Additionally, analysis of samples on the QTRAP mass spectrometer in EPI scan mode confirmed this assumption. Coefficients fit (Fit, RevFit) of spectrum of unidentified peak to spectrum of thiouracil exceeded the value of 0.7.

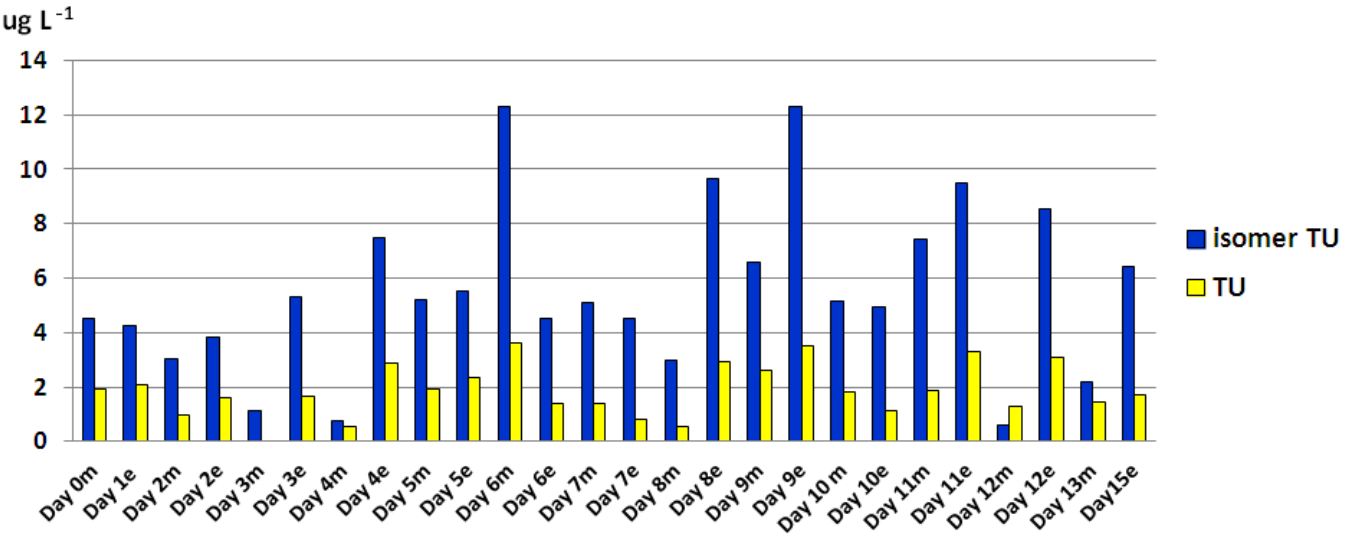

Figure 3. The concentration of TU and TU-isomer in the urine samples from cow No. 3 in the experiment.

Because so far, there are no data on the stability of the endogenous thiouracil in urine samples, studies were undertaken to verify how the conditions of storage of samples after collection, affect the result of the analysis (Table 1).

Table 1. Effect of storage conditions on thiouracil concentration in urine samples.

Sample number	Analysis directly after collection	4 °C 3 days	<-18 °C 10/30 days	pH2; <-18 °C 10/30/50 days
TU (µg L^{-1})				
C1	< CCα	1.28	< CCα/< CCα	1.71/1.10/1.58
C2	1.58	1.42	< CCα/< CCα	1.92/1.75/2.54
C3	1.85	1.32	< CCα/< CCα	2.49/1.97/3.53
Isomer TU expressed as TU (µg L^{-1})				
C1	4.09	< CCα	< CCα/< CCα	5.35/9.98/5.26
C2	6.92	< CCα	< CCα/< CCα	6.24/10.80/6.87
C3	7.41	< CCα	3.60/< CCα	8.98/13.50/11.20

Table 1 shows that the three-day storage of urine samples in a refrigerator (2 - 8 °C) prior to analysis does not affect the test results of natural thiouracil, while it significantly reduces the concentration of the isomer. After 10 days of storage in the freezer, the concentration of thiouracil may fall to below the decision limit of the method. Similar results were obtained for isomer TU, despite the much higher value of the initial concentrations. Literature data and our own experience show that thiouracil is non-stable and retains durability in strongly acidic conditions (pH 1-2) (Vanden Bussche *et al.,* 2012). Therefore, in some countries, urine samples are acidified and stabilized with EDTA after arrival at the laboratory. The impact of urine sample acidification on the result of endogenous thiouracil concentration is presented in Table 2.

Table 2. Effect of storage conditions on the thiouracil concentration in urine samples.

Number of samples	<-18° C 3 days	pH2; <-18 °C 3 days	<-18° C 3 days	pH2; <-18 °C 3 days
TU (µg L^{-1})			Isomer TU expressed as TU (µg L^{-1})	
15	1.68±0.8	3.12±0.54	3.92±3.25	8.50±3.22

Table 2 shows that after three days storage of urine samples in a freezer (<-18 ° C), the concentration of thiouracil in the acidified urine samples was two times higher than in the same samples at physiological pH (6.5-7.5). The results of these studies confirm the instability of the natural thiouracil in the matrix and the need to conduct the pre-treatment of pH upon sampling.

In the milk samples collected during the experiment, there was no presence of thiouracil above decision limit of 0.63 µg L^{-1}. Additionally, 50 samples of milk collected from dairy cows originating from individual owners were examined. Thiouracil was not detected above CCα in any of these samples. The results are not consistent with the results obtained in studies with commercial samples, of which more than 20% contain TU above the CCα of the method used.

Conclusions

Feeding dairy cows with feed containing 30% of rapeseed meal did not cause a permanent rise in concentration of endogenous thiouracil in urine. It is therefore not the reason of non-compliant results. The highest detected concentration of thiouracil was 3.61 µg L^{-1}. Two-week feeding dairy cows rapeseed enriched feed up to the maximum allowed dose did not cause of formation of thiouracil in samples of milk above concentration of 0.63 µg L^{-1} either. In serum and faeces samples, no concentrations above the respective detection limits was observed. The stability studies showed that the three-day storage of urine samples at refrigerator temperature did not alter the concentration of thiouracil, whereas freezing of the samples longer than 10 days significantly reduced the level of this compound. Acidification of urine samples (pH 2) improves the stability of endogenous thiouracil which is stable after 2 months of storage at a temperature lower than -18°C. Because thiouracil concentrations detected in urine samples tested in the frame of the national control program often exceed 3 µg L^{-1}, and occasionally even 10 µg L^{-1}, it can be concluded that the feeding of animals with feed containing rapeseed meal is only one of the factors affecting the formation of endogenous thiouracil.

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TRUE NON-TARGETED ANALYSIS OF ANTIMICROBIAL ACTIVE COMPOUNDS USING BIO-ASSAY DIRECTED SCREENING FOLLOWED BY LC-HRMS

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Abstract

A true non-targeted analysis method of antimicrobial active compounds using Bio-assay directed screening and LC-HRMS analysis is reported. Suspect samples were extracted using a generic protocol and fractionated on two different liquid chromatography fractionation systems with different chromatographic conditions (A and B). The behaviour of the active compound under these different conditions yields information about the physico-chemical properties of the compound and introduces variations in co-eluting compounds in the fractions which were essential for data interpretation and evaluation. The fractions containing the active compound(s) of both systems A and B were selected based on a microbiological effect-based bio-assay. The selected fractions A and B were analysed using liquid chromatography combined with high resolution mass spectrometry. Peak-picking was carried out automatically by identifying all compounds present in both fraction A and B. With this, the data was severely cleaned without increasing the risk of false negative results. The method was assessed using three relevant applications. In all cases, the compounds causing microbiological inhibition were successfully identified and confirmed.

Introduction

Antibiotics are widely used in animal husbandry to treat and prevent bacterial infections, and to promote growth (Zhou *et al.*, 2013). Antibiotics are commonly administered to animals through feed and water or by injection (Cromwell *et al.*, 2002). Antibiotic use in animal husbandry has tremendous effects on the environment and antibiotic resistance development (Kümmerer *et al.*, 2003; Nõlvak *et al.*, 2013; Zhu *et al.*, 2013; Barra Caracciolo *et al.*, 2015). The exposure to pharmaceuticals can have tremendous impact on the microbial populations in the environment. For antimicrobial pharmaceuticals (anti-viral, bacterial, fungal), a major concern is the selection, enrichment and spread of resistant micro-organisms. The compounds compromise drugs of importance in human and animal medicine. Recently, it was acknowledged that responsible use and thus decrease of the antibiotic use is of critical importance. In the Netherlands policies were made to address this matter (Gerards *et al.*, 2011). To enforce these policies, monitoring antibiotics in, among others, feeding stuffs are inevitable to ensure compliance with legislation and high quality and safety for both livestock and consumers.

A frequently used strategy for monitoring antibiotics consists of a fast effect-based microbiological screening method followed by targeted confirmation of the suspect samples using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). However, if a suspect sample in the microbiological screening cannot be confirmed by the targeted LC-MS/MS analysis, a non-targeted analysis is needed to identify the unknown antimicrobial compound (Borràs *et al.*, 2011). Recent instrumental advances lead to the development of high resolution mass spectrometry (HRMS) capable of determining molecular masses with high mass resolution and mass accuracy, enabling full scan non-targeted analysis (Nielen *et al.*, 2007; De Brabander *et al.*, 2009).

In this paper we present a non-targeted analysis method using a microbiological effect-based screening followed by LC-HRMS aiming for the detection and confirmation of unknown antimicrobial compounds. The challenge was to reduce the tremendous amount of data to a small selection preserving the power of a non-targeted approach. Specific attention is paid to sample preparation, data reduction and data evaluation. The result is a broadly applicable non-targeted method that yields a limited selection of 'tentative candidates' which can be further elucidated by their fragmentation pattern or using a database. We demonstrate that this procedure is capable for identification of unknown microbial growth inhibitors in feed, which is considered to be one of the most challenging matrices.

Materials and Methods

Materials

Ultra LC-MS grade water, methanol and acetonitrile (Actu-all chemicals, Oss, The Netherlands), formic acid 98-100% LC-MS grade, ammonia solution 25% and ammonium formate (Merck, Darmstadt, Germany) were used. Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18.2 MΩ cm^{-1} (Millipore, Billerica,MA, USA). PEG average molecular weight 200, 300 and 600 were obtained from Sigma-Aldrich (Saint Louis, MI, USA). PEG average weight 1,000 and 1,500 were obtained from Acros Organics (Geel, Belgium). The Exactive Orbitrap calibration solvent positive ESI and negative ESI were obtained from ThermoFisher Scientific (San Jose, CA, USA). External calibration standard: PEG standard of 50 µg L^{-1}. Didecyldimethylammonium chloride (DDAC) was obtained from Dr. Ehrenstorfer LGC (Teddington, United Kingdom)

Approach

Figure 1 shows a flowchart of the different steps within the method for the identification of unknowns. After sample preparation, the sample was fractionated under different chromatographic conditions. Next, a microbiological screening assay was used to select fractions containing anti-microbial active compounds, followed by HRMS analysis of these fractions and data processing using MetAlign® software. After generating a tentative candidate list, the most important and most relevant compounds were selected based on the molecular formula, fragmentation patterns, obtained physico-chemical information and literature.

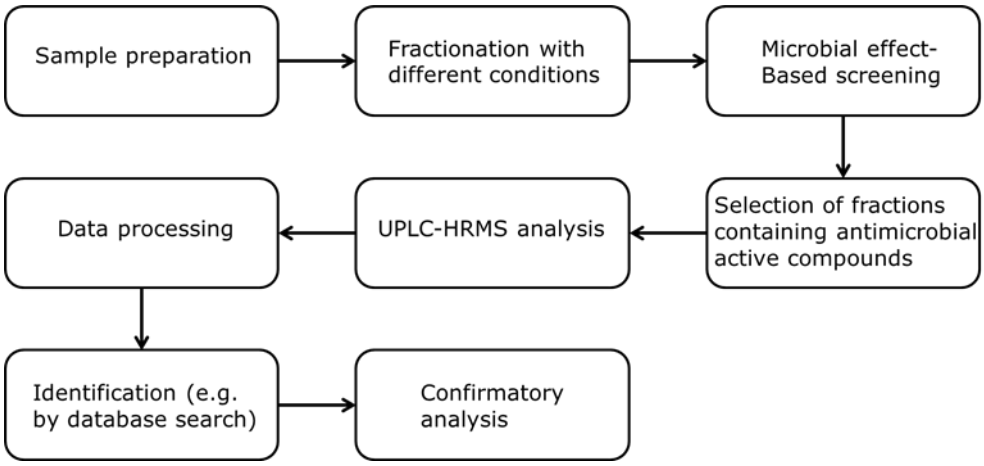

Figure 1. Flowchart of the different steps within the new unknown identification method.

Sample preparation and fractionation

The required amount of animal feed was transferred into a polypropylene centrifuge tube and extraction solution was added. Subsequently, the tube was shaken and centrifuged. The upper layer was transferred to a 12 mL polypropylene tube. The extract was diluted with extraction solution and divided in different glass recovery vials and fractionated.

The fractionation system consisted of an Agilent 1200 series vacuum degasser, binary solvent pump, standard micro and preparative auto sampler, thermostatic column compartment, variable wavelength detector and a preparative scale fraction collector. Each sample was fractionated using a C18 analytical column and two different fractionation procedures. Fractionation procedure A applies acidic solvents and fractionation procedure B applies slightly alkaline solvents. The whole method takes 30 min in which a gradient from 0% till 100% organic solvent is applied including conditioning. During the first 25 min of a run, every 0.5 min a new fraction was collected, yielding 50 fractions for each run. In total, one sample extract is injected ten times to be able to increase the total volume per fraction. MeOH was added to each collected fraction followed by evaporation until dry. Finally, the residue was dissolved in water/MeOH.

Effect based screening

The microbial growth inhibition test was performed initially as described in Pikkemaat *et al.* (2008) with some modifications.

The presence of growth inhibiting compounds becomes visible as a clear zone (absence of bacterial growth) around the sample hole. In case multiple sequential sample holes show inhibition, the fraction in the hole with the largest inhibition zone is selected. The fraction that contains the active compound obtained from system A is called fraction A and the one obtained from system B is called fraction B. Note that the microbiological active compound can show a different retention time in both fractionation systems due to the different pH conditions. This results in different fractions showing microbial growth inhibition in the effect-based screening.

LC-HRMS analysis

The LC system used for HRMS analysis consisted of a Thermo Fisher Scientific (Dionex) (San Jose, CA, USA) Ultimate 3000 system with RS pump, RS auto-sampler and RS column compartment. The analytical column used was a C18 analytical column. The used solvents were, solvent A, water with ammonium formate and formic acid; solvent B, acetonitrile and water with ammonium formate and formic acid. The whole chromatographic method took 16 min with a gradient from 0% B till 100% B with conditioning step and a flow of 0.4 mL min^{-1}. The injection volume was 5 µL and a column temperature of 40°C. Before injection the final extract was diluted by adding a solution of H_2O/MeOH.

Detection was carried out using a Thermo Scientific Q-Exactive Orbitrap MS operating with a heated electrospray ionization source in positive and negative mode. The instrument was calibrated using a pierce LTQ Velos ESI Positive and negative calibration solution. In final confirmation, targeted MS2 is applied.

Method testing

The non-targeted method for the identification of unknown antimicrobial compounds is tested using three samples. Two are test samples that were prepared by addition of a known antimicrobial compound to a blank feed sample. The added compounds were only known by the expert that spiked the samples and were unknown to the analyst. Different types of feed and spike levels were used. The third sample involves a truly unknown spice mixture used as addition for animal feed to improve growth of the animal. This sample clearly showed microbial growth inhibition within the screening and in which no compound was confirmed using targeted mass spectrometry analysis.

Results and discussion

Method development resulted in a standard approach for non-targeted analysis using a microbiological effect-based screening followed by LC-HRMS aiming for the detection and confirmation of unknown antimicrobial compounds. Figure 2 shows a flowchart of the standard approach.

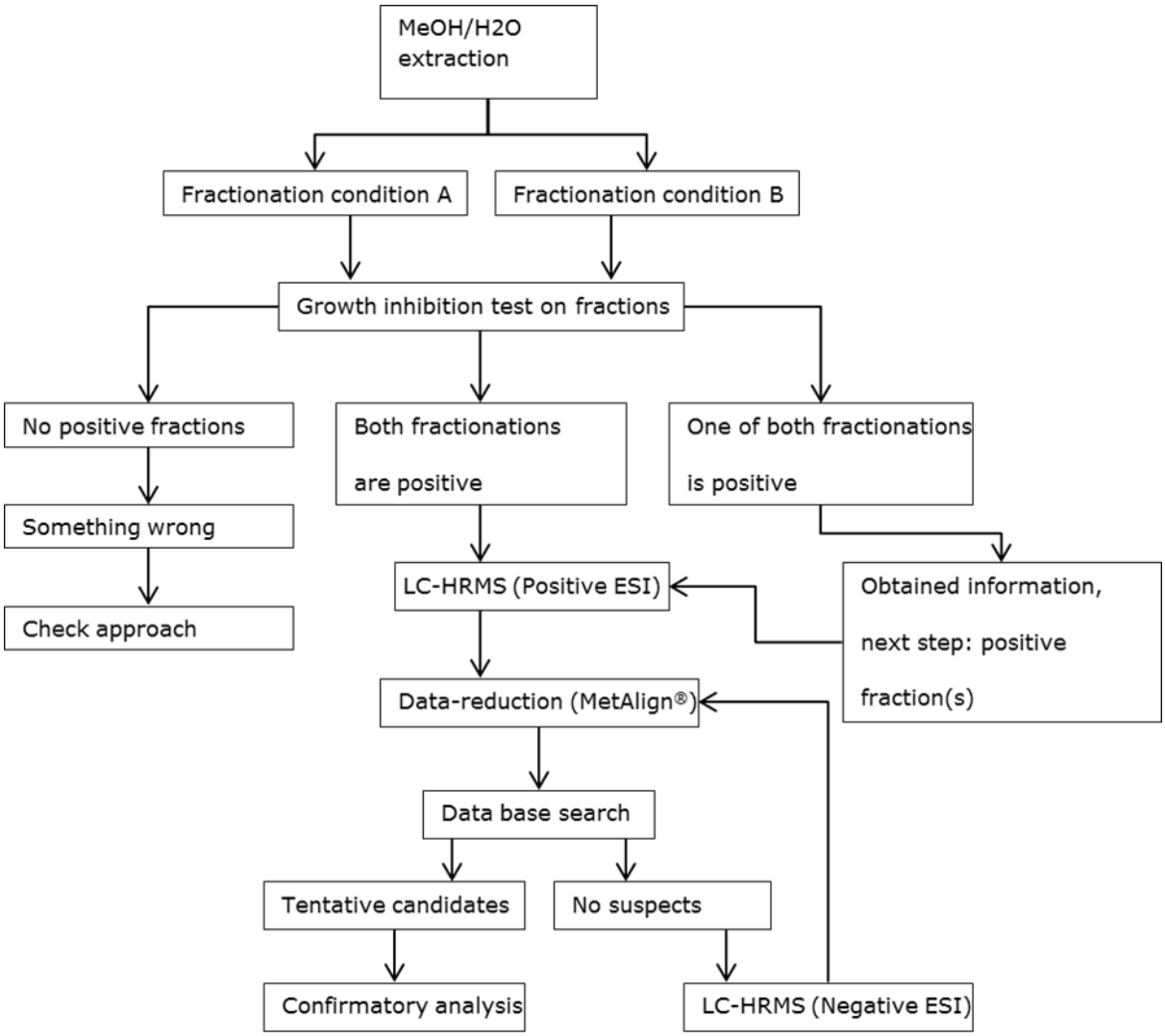

Figure 2. Flowchart of the standard approach.

After sample extraction, fractionation was achieved using two different systems. Fractions originating from both systems were screened using the microbiological screening assay. Depending on the results, different steps could be taken. If there were no antimicrobial active fractions found, it could be concluded that something is wrong with the extraction or fractionation. For example, it could be that the fractionation was not carried out correctly or that the active compound was somehow degraded or too far diluted. Another possibility was that only one fraction shows growth inhibition. A possible explanation is that the active compound is degraded under the applied pH conditions in one of the two systems.

Further processing aims for the antimicrobial active fractions A and B only. The information on retention time obtained from the fractionation can be used as input for the identification process, for instance if the unknown compound is neutral, a weak acid or a weak base. MetAlign® is applied to yield a list of 'tentative candidates'. Next, possible identities of the unknown

compound are selected by the expert and confirmatory analysis was carried out using commercially available compounds. LC-MS/MS was also carried out when 'tentative candidates' were not commercially available to gain more structure-related information. When no 'tentative candidates' remain, HRMS analysis using negative ionisation mode was carried out following the described data evaluation procedure. HRMS analysis using positive ionisation mode was carried out first because the majority of small organic compounds can be ionised in positive ionisation mode.

All three samples used for method testing were correctly identified and confirmed using the developed standard approach. Results were published elsewhere.

Conclusions

It is concluded that the combination of an effect-based bio-assay, HRMS analysis and intelligent data processing by matching two different LC fractions is a strong tool in the identification of unknown bacterial growth inhibitors in feed. As feed is considered a difficult matrix, it is expected that this procedure can be applied to other matrices, including food products as well. Of prime importance is to use a generic extraction so the probability of effectively extracting the unknown compound is increased. The use of fractionation in combination with an effect based analysis demonstrated to be mandatory to effectively select the relevant chromatographic signal(s). Furthermore, the use of two fractionation systems, running at different pH, in combination with the peak matching procedure, proved to be of a unique approach to achieve data reduction, while limiting the probability of a false negative. Finally, the availability of fit-for-purpose databases, preferably containing only compounds having antimicrobial properties, is of great importance to effectively search for the unknown active compound. It remains a challenge to identify the compound of interest, depending on the type and concentration of the compound, and the complexity of the matrix. For this, expert knowledge remains of high importance.

Acknowledgements

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RESIDUES OF VETERINARY MEDICINAL PRODUCTS IN FOODS OF ANIMAL ORIGIN IN THE EU RASFF (1979-2014)

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Abstract

The Rapid Alert System for Food and Feed (RASFF) of the European Union (EU) was initiated in 1979 and has been in operation until today. Data originating from the RASFF Portal were used to describe the history (from 1979 until 31/12/2014) of transgressions on residues of veterinary medicinal products reported in foods of animal origin in alert and border rejection notifications. Over the last 35 years, 407 alert and 257 border rejection notifications have been submitted incriminating the top-five (in terms of citation frequency) food product categories, which are foods of animal origin, namely crustaceans, meat, fish, poultry and honey and royal jelly. Combinations of these food product categories with a certain residue of a veterinary medicinal product that appear more often in the RASFF are: i) crustaceans and products thereof with nitrofuran (metabolite) (n=76), crustaceans and products thereof with chloramphenicol (n=42), honey and royal jelly with chloramphenicol (n=39), fish and fish products with malachite green (n=38), meat and meat products (other than poultry) with phenylbutazone (n=24) for alert and ii) crustaceans and products thereof with nitrofuran (metabolite) (n=112), meat and meat products (other than poultry) with ivermectin (n=43), fish and fish products with nitrofuran (metabolite) (n=23), crustaceans and products thereof with chloramphenicol (n=12) for border rejection notifications. The EU RASFF member states that have contributed the most alert and border rejection notifications were Germany, Belgium, Great Britain and Belgium, Great Britain, Spain, respectively. Regarding Third Countries, the most often cited as countries of origin in border rejection notifications were India, Brazil, Bangladesh, China and Vietnam.

Introduction

The European Union (EU) is the world's biggest importer and exporter of foodstuffs, and as such it is deemed necessary to possess the communication channels with its own Member States (MSs) but also with other (third) countries worldwide. The facilitation of such communication concerning food related incidents of veterinary public health concern is well implemented by the Rapid Alert System for Food and Feed (RASFF) which has been in existence since 1979.

Its legal basis is Regulation 178/2002 and within its framework, alert and border rejection notifications play a major role in the protection of the consumers. Alert notifications are sent when a food presenting a serious health risk is on the market and when rapid action is required. Border rejection notifications concern food consignments that were rejected at the external borders of the EU RASFF MSs, when a health risk has been found. Both types of notifications have been legislated in Commission Regulation 16/2011. The mode of action for the RASFF is based on controls enforced by each member to identify hazards in food, then to submit the alert notification, which aims at giving all RASFF members the information to confirm whether the product in question is on their market, so that they can also take the necessary measures. The border rejection notifications are sent to all EU RASFF MSs border inspection posts (BIPs) in order to reinforce controls and to ensure that the rejected product does not re-enter the EU via other BIPs.

The members of the RASFF network consist of the EU-28 Member States, the European Commission, EFSA, Norway, Liechtenstein, Iceland and Switzerland. The RASFF portal features an interactive searchable online RASFF database, which allows for public access to summary information about all the transmitted RASFF notifications.

The aim of this paper was to highlight the incidence of specific residues of veterinary medicinal products in the most often cited foods of animal origin that have triggered alert and border rejection notifications over the last 35 years (1979-2014) as recorded in the EU RASFF Portal. Furthermore, an attempt was also made to follow the yearly trend on those types of notifications *vis-a-vis* the specific food product categories with the actual hazards appearing with the highest frequency. Finally, the EU RASFF MSs with the greatest numbers of submissions to the system as notifying countries and as countries of origin, together with the third countries' transgression history have been examined in this paper.

Materials and methods

The data that were used in this paper were retrieved from the EU RASFF Portal: https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchForm&cleanSearch=1 (accessed on 24/02/2016). All alert and border rejection notifications in food, for various product categories (meat, crustaceans, fish, honey and royal jelly, poultry) and various subjects/hazards, namely residues of veterinary medicinal products (RVMP) (*e.g*. chloramphenicol, nitrofuran, malachite green, phenylbutazone, ivermectin etc.) from 1979 until 31/12/2014, were selected and were inserted into Excel files, and different combinations of parameters were studied thoroughly. Also, alert and border rejection notifications concerning the five most cited

foods of animal origin, submitted by all EU RASFF MS when the latter were notifying countries (NC) but also when they were countries of origin (CO), were examined. Finally, Third Countries (TCs) were assessed as countries of origin over the last 35 years.

Results

Specific foods of animal origin most frequently cited in alert and border rejection notifications for RVMP in the EU RASFF (1979-2014)

The data shown in Table 1, clearly indicate that the top-five food product categories (all foods of animal origin) were responsible for just over 85% (346/407) and 97% (257/263) of alert and border rejection notifications, respectively.

Table 1. Alert and border rejection notifications in various foods of animal origin from RASFF (1979-2014).

Food product categories of animal origin	Alert notifications	Border rejection notifications
Crustaceans and products thereof	117	144
Meat and meat products (other than poultry)	70	60
Fish and fish products	56	32
Poultry meat and poultry meat products	41	2
Honey and royal jelly	62	19
Sum of top-five food product categories	346	257

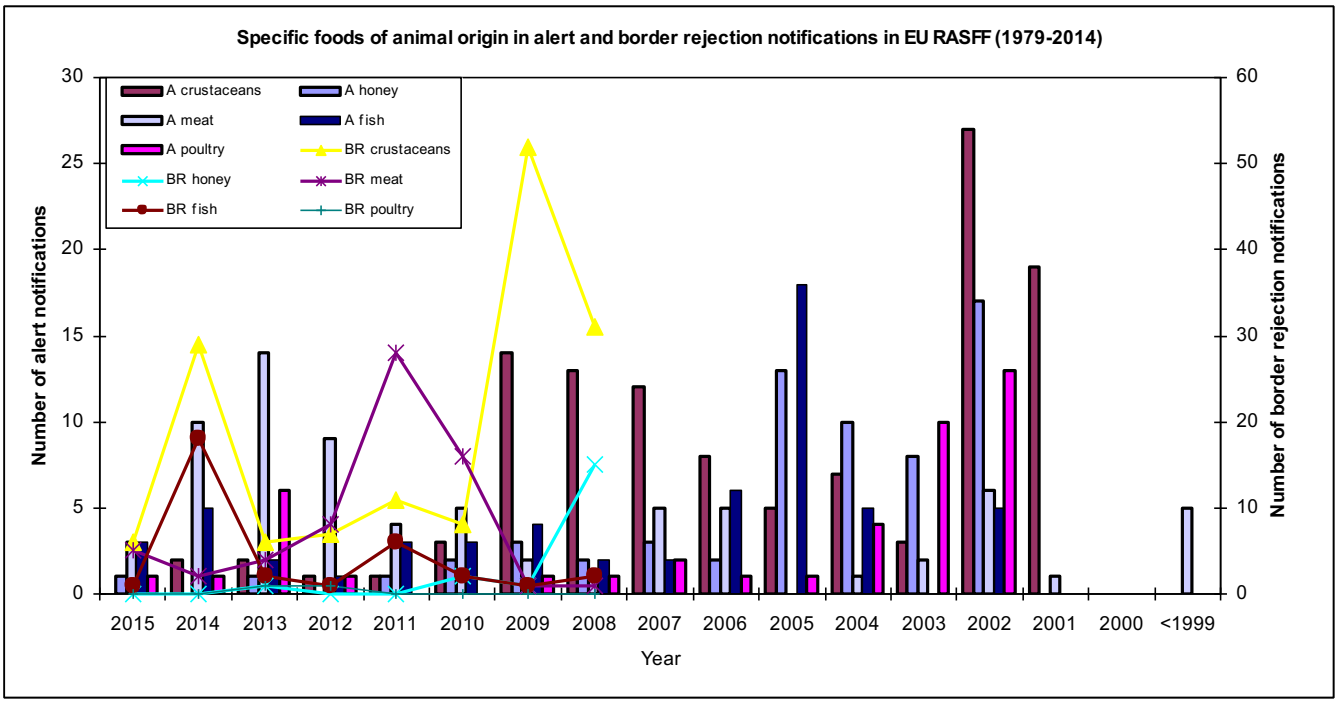

Figure 1. Yearly breakdown of alert and border rejection notifications for selected foods of animal origin for RVMP (RASFF 1979-2014).

This finding was further highlighted with a more in-depth and targeted analysis, in order to show the yearly fluctuations of both types of notifications in the top-five most cited food product categories (Figure 1).

Specific hazards most frequently cited in the top-five most cited foods of animal origin in alert and border rejection notifications for RVMP in the EU RASFF (1979-2014)

The most frequently cited residues of veterinary medicinal products in the RASFF alert and border rejection notifications were the following: i) prohibited substance chloramphenicol, ii) prohibited substance nitrofuran (metabolite), iii) unauthorised substance malachite green, iv) unauthorised substance in meat phenylbutazone v) unauthorized substance in meat ivermectin, vi) unauthorized substances in honey erythromycin and streptomycin and vii) numerous substances with levels above the MRL (*e.g.* amoxicillin, oxytetracycline, doxycycline, sulfadiazine, enrofloxacin, sulfadimethoxine etc.).

Combinations of specific hazards and specific foods of animal origin most frequently cited in alert and border rejection notifications for RVMP in the EU RASFF (1979-2014)

In Figure 2, a complete yearly overview of the most frequently identified specific hazards/RVMP (residues of a specific pharmacologically active substance) cited in the top-five most often incriminated foods of animal origin examined in this paper is depicted. The yearly mode of presentation of the alert and border rejection notifications submitted in the EU RASFF over the last 35 years, allows for the visualization of the overall trend of occurrence of chloramphenicol, nitrofuran (metabolite) malachite green, phenylbutazone and ivermectin residues.

Crustaceans and products thereof. In alert notifications (n=117) the specific hazards/RVMP most frequently implicated were nitrofuran (metabolite) and chloramphenicol. Four EU RASFF MSs have submitted the most alert notifications, namely Belgium, Germany, Great Britain, The Netherlands, which collectively amount to 80.3% of all alert notifications. The year that alert notifications peaked was 2002 (n=27) owing to notifications for nitrofuran-containing samples from Thailand (12) and chloramphenicol-containing samples (5) from Vietnam.

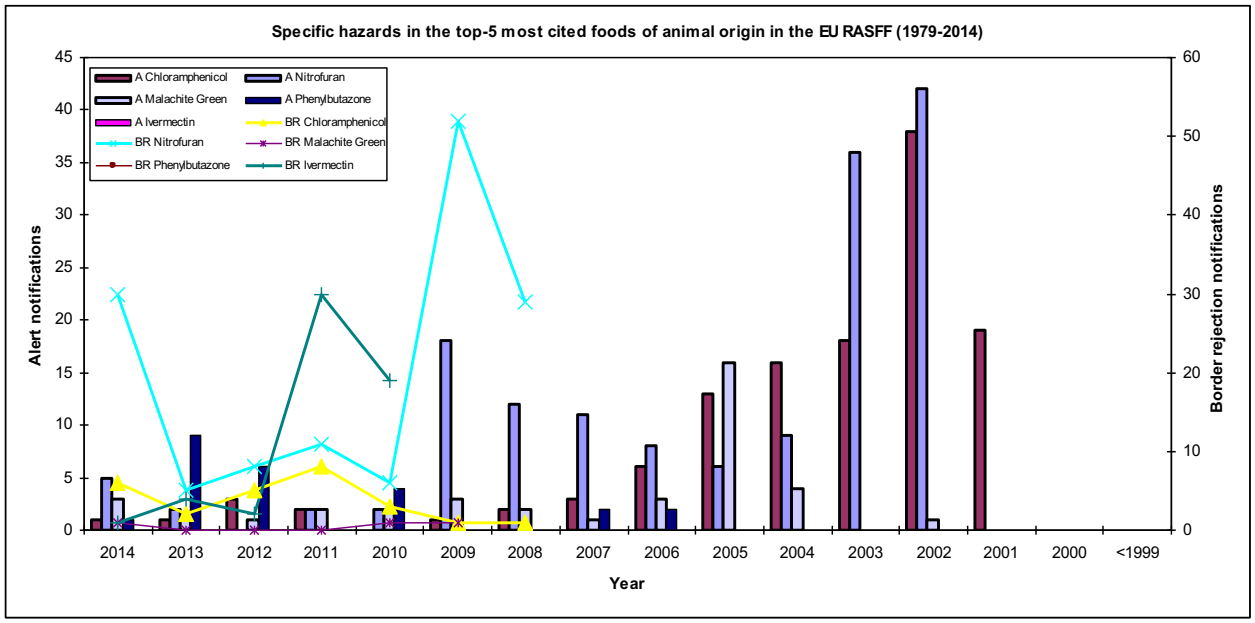

Figure 2. Yearly breakdown of alert and border rejection notifications submitted in the EU RASFF (1979-2014) for the top-five most frequently cited foods of animal origin containing the most often cited RVMP.

In border rejection notifications (n=144) the specific hazards most frequently implicated were nitrofuran (metabolite) and chloramphenicol but also (oxy)tetracycline, amounting to a total of 95.83% of all such notifications. The EU RASFF MSs that submitted the most border rejection notifications are Belgium, Germany, Great Britain, Italy which amount to 86.80% of all such notifications. Third countries that have most frequently been notified against (as countries of origin) were India (n=64), Bangladesh (n=46), Vietnam (n=13), China (n=10) which account for 92.4% of all such notifications. It was 2009 that the border rejection notifications peaked owing to nitrofuran-containing samples (51) mainly from Bangladesh (n=31) but also from India (n=16).

Meat and meat products (other than poultry). Regarding alert notifications (n=70) the hazards most frequently implicated were: unauthorized substance phenylbutazone (n=24) and to a lesser extent chloramphenicol and nitrofuran (metabolite). It is worth mentioning that 17 out of 24 alert notifications incriminating phenylbutazone in horse meat were notified by Great Britain (as a notifying country). Other hazards that have triggered alert notifications were hormones, sulphonamides, metronidazole, (oxy)tetracycline etc. The year that alert notifications peaked was 2013 (n=14) owing to the presence of the unauthorised substance phenylbutazone, and in three of those, adulteration (presence of horse DNA) was also found, the latter being a food fraud issue. The EU RASFF MS that appear to have submitted the most alert notifications in the RASFF Portal are in descending order the following: Great Britain, Belgium, Germany which amount to 60% of all alert notifications for meat and meat products (other than poultry).

Concerning border rejection notifications (n=60) the single hazard most frequently implicated was ivermectin responsible for 71.7% of all such notifications and to a lesser extent chloramphenicol and nitrofuran. Regarding the presence of unauthorized substance ivermectin, all 43 notifications implicated Brazil as the country of origin with notifying countries such as Great Britain and Belgium being the ones that submitted the most border rejection notifications. The peak year for border rejection notifications in meat, was 2011 (n=28) because 25 samples from Brazil were found to contain ivermectin.

Honey and royal jelly. In alert notifications (n=62) the hazards most frequently implicated were mainly chloramphenicol and with a much lesser frequency, streptomycin and nitrofuran (metabolite), which together accounted for 88.7% of all notifications. The EU RASFF MSs that appear to have submitted the most alert notifications are Germany, Belgium, Italy, Great Britain, which amount to 62.9% of all alert notifications for "honey and royal jelly". The countries of origin that appear to have been notified against, most frequently were China for the presence of chloramphenicol in 19 out of 20 notifications. The main reason for the peak noticed in 2002 (n=17) was the presence of chloramphenicol from China, that was mainly detected by Belgium.

In border rejection notifications (n=19) the specific hazards most frequently found were erythromycin and oxytetracycline (both unauthorised substances for honey), amounting to a total of 73.7% of all such notifications. Other hazards incriminated in such notifications were streptomycin, lincomycin, ciprofloxacin, sulphamethazine and sulphadimidine. The single EU RASFF MS that submitted the most notification was Spain which amount to 73.7% of all such notifications. China with thirteen notifications implicating it as the country of origin was followed by Argentina, Turkey and Israel. The year that border rejection notifications peaked was 2008 (n=15), owing to the presence of erythromycin (unauthorized substance) in honey from China (n=9).

Fish and fish products. In alert notifications (n=56) the hazards most frequently implicated were mainly the unauthorized substance malachite green and to a much lesser extent the prohibited substance nitrofuran (metabolite), together accounting for 80.4% of all such notifications. The EU RASFF MSs that submitted the most alert notifications over the last 35 years are: Germany and Great Britain which amount to 66.1% of all alert notifications. The year that alert notifications in fish peaked was 2005 (n=18), owing to the presence of malachite green (n=16) originating from Vietnam (5), China (3) etc.

In border rejection notifications (n=32) the hazards most frequently implicated were basically nitrofuran (metabolite) and to a much lesser extent malachite green, together accounting for 81.2% of all such notifications. Interestingly, 22 out of 23 transgressions with nitrofuran metabolite originated from Vietnam. The highest number of border rejection notifications was submitted by Spain (n=15), whereas the country that was notified most frequently as the country of origin was Vietnam (n=28). The year that border rejection notifications in meat peaked was 2014 (n=18), 16 of which originated from Vietnam and contained the prohibited substance nitrofuran (metabolite).

Poultry meat and poultry meat products. In alert notifications (n=41) the hazards most frequently implicated were mainly the prohibited substance nitrofuran (metabolite) and chloramphenicol, accounting for 73.17% of all alert notifications. The year that alert notifications peaked was 2002 (n=13) all of which were found to contain the prohibited substance nitrofuran (metabolite) and originated from Brazil (n=5) and Thailand (n=8). Only two border rejection notifications were recorded in the RASFF Portal for poultry meat.

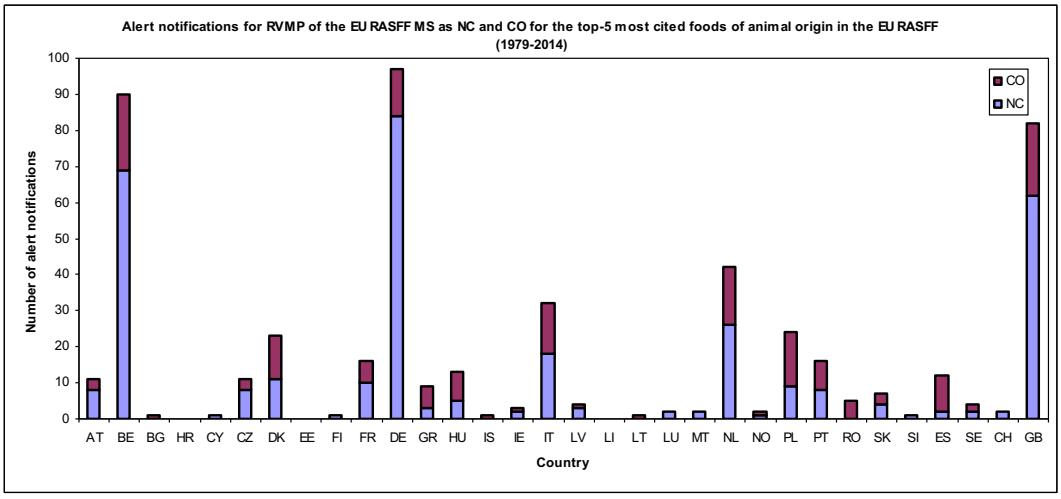

Figure 3. Alert notifications notified in the EU RASFF concerning all the RASFF MSs as notifying countries and as countries of origin for the top-5 most often cited food products of animal origin in the EU RASFF (1979-2014).

EU RASFF Member States as notifying countries (NC) and countries of origin CO) in alert and border rejection notifications in the EU RASFF (1979-2014)

The number of alert notifications issued against all the EU RASFF member states collectively (as countries of origin) were almost half of those that were notified by them as notifying countries, over the last 35 years in the EU RASFF for the top-5 most cited foods of animal origin examined in this paper (Figure 3). The MSs that notified the most alert notifications were Germany, Belgium and Great Britain. The EU RASFF MSs that were cited against (as countries of origin) most often were Belgium, Great Britain and The Netherlands.

Border rejection notifications concerning the top-5 most cited Third Countries (TC) as countries of origin (CO) in the EU RASFF (1979-2014)

Border rejection notifications cited against India (as a country of origin) were all submitted for crustaceans (n=64) and mainly involved the presence of the prohibited substance nitrofuran (metabolite) in 57/64 notifications, whereas for Bangladesh it was the absolute total of such notifications (46/46) that was found to contain the aforementioned hazard. Brazil (n=48) was mostly notified against for meat, containing, ivermectin (43/47), a substance unauthorized in beef. Vietnam (n=41) was a rather different case since predominantly fish were notified against, owing to the presence of the prohibited substance nitrofuran (metabolite) (22/28), but followed by crustaceans with (oxy)tetracycline (10/13) being the hazard contained. Even more diversified in terms of food product categories incriminated for containing hazards, was the case of China (n=38), where in 8/11, 10/13 and 4/10 such notifications in meat, honey and royal jelly and crustaceans chloramphenicol and erythromycin was detected, respectively. The aggregate number of border rejection notifications issued against the above-mentioned five Third Countries (as countries of origin) represented just over 92% of all border rejections filled over 35 years against the top-5 most cited food product categories under examination in this paper.

Discussion

In the 407 alert notifications submitted over the last 35 years in the RASFF the top-five food products implicated most frequently (all of which are foods of animal origin) are crustaceans, meat, honey and royal jelly, fish and poultry. The combinations of food product categories with specific hazards (residues of veterinary products), most predominantly appearing are crustaceans with nitrofuran (metabolite) and chloramphenicol, honey and chloramphenicol, fish and malachite green, meat with phenylbutazone and poultry with nitrofuran (metabolite). In crustaceans and honey, alert notifications recorded after 2010 and 2006 were less than 4 per year, respectively, whereas for fish and poultry were less than 7 after 2006 and 2004, respectively. In the case of meat until 2012 the yearly count of alert notifications was less than 10, but a surge in 2013 and 2014 occurred owing to the presence of the unauthorized substance phenylbutazone and the presence of horse DNA (a food fraud issue).

Three EU RASFF Member States were responsible for submitting just over half (52.8%) of all alert notifications for residues of medicinal products in the top-five food product categories under examination in this paper.

In the 257 border rejection notifications submitted over the last 35 years in the RASFF the top-five food products implicated the most (all of which are foods of animal origin) are crustaceans, meat, fish, honey and poultry. The combinations of food product categories with specific hazards (residues of veterinary products), mainly incriminated are crustaceans with nitrofuran (metabolite), meat with ivermectin, fish with nitrofuran (metabolite) and honey with erythromycin.

In honey, the yearly count of border rejection notifications recorded from 2009 until 2014 were less than three, in meat from 2012 until 2014 less than 9, and in fish till 2013 less than 7 per year, whereas for crustaceans from 2010 until 2013 it ranged from 6-11, but in 2014 a sharp rise was witnessed due to the presence of nitrofuran (metabolite) mainly, but also oxytetracycline. A similar rise was noted for fish in 2014 principally because of the presence of nitrofuran (metabolite). The third countries (TCs) responsible for the greatest numbers of such notifications were India, Brazil, Bangladesh, Vietnam and China, together amounting to just over two-thirds (67.1%) of all such notifications submitted to the RASFF.

Conclusions

Clearly, a closer more focused approach to the historic traces of food contamination with residues of veterinary products would help both the EU RASFF MSs and the TCs to minimize their transgression activity for the future, via the coherent implementation of the existing EU food legislation. The Food and Veterinary Office (FVO) of the EU could additionally support the countries' efforts to protect public health by securing that they offer safe foods to the global community.

Novel analytical methodologies validated for multi-class veterinary drug residues, according to EU standards (Commission Decision 2002/657), coupled with the continuous advancement in analytical instrumentation, certainly show the way forward. Risk analysis of pharmacologically active substances in foods of animal origin could help to clarify the actual threat to human health and efforts should be made to mitigate their harmful effects.

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DETERMINATION OF FENBENDAZOLE RESIDUES IN FERMENTED DAIRY PRODUCTS BY ION-PAIR LIQUID CHROMATOGRAPHY

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Abstract

A simple, rapid and sensitive liquid chromatographic method that allows for the quantitative determination of fenbendazole residues in fermented dairy products is described. Samples were extracted with a mixture of acetonitrile-phosphoric acid and the extracts were defatted with hexane to be further partitioned into ethyl acetate. The organic layer was evaporated to dryness and the residue was reconstituted in mobile phase. Separation of fenbendazole and its sulphoxide, sulphone, and *p*-hydroxylated metabolites was carried out isocratically with a mobile phase containing both positively and negatively charged pairing ions. Overall recoveries ranged from 79.8 to 88.8%, while precision data, based on within and between days variations, suggested an overall relative standard deviation in the range of 6.3 to 11.0%. The detection and quantification limits were lower than 9 and 21 μg kg^{-1}, respectively. The method has been successfully applied to quantitate fenbendazole residues in Feta cheese and yoghurt made from spiked and incurred *ovine* milk.

Introduction

Fenbendazole, a broad spectrum benzimidazole anthelminthic, has been widely used against all classes of helminths that commonly infect animals. Following oral administration to cattle and sheep, fenbendazole is slowly absorbed from the gut and rapidly transformed to a range of metabolites, with fenbendazole sulphoxide (oxfendazole) and fenbendazole sulphone (oxfendazole sulphone) being the major ones (FAO/WHO, 1991). Therefore, its administration in these animals may result in the presence of remarkable concentrations of fenbendazole residues in foodstuffs of animal origin, of which milk and dairy products are most important because of their daily consumption.

The presence of fenbendazole residues in dairy products is potentially harmful for the consumers, although fenbendazole is not harmful *per se*. One of its metabolites, oxfendazole, has been proven responsible for teratogenic and embryotoxic effects in laboratory animals when administrated during the primary stages of pregnancy (FAO/WHO, 1991). To protect consumers' health from the presence of harmful concentrations of fenbendazole residues in marketed milk, a maximum residue level (MRL) of 10 μg kg^{-1} for the marker residue of fenbendazole (sum of extractable residues which may be oxidized to oxfendazole sulphone) has been established by the European Union (European Commission, 2009).

To ensure the safety of the marketed milk regarding the presence of violating residues, several analytical methods have been reported and comprehensively reviewed over the past decades (Botsoglou and Fletouris, 2001; Danaher *et al*., 2007). A literature search shows that liquid chromatography (LC) with ultraviolet (Boontongto *et al*., 2014; Chen *et al*., 2010; Fletouris *et al*., 1994; Fletouris *et al*., 1996a; Fletouris *et al*., 1996b; Santaladchaiyakit and Srijaranai, 2012, 2014; Wang *et al*., 2014), and mass spectrometric detection (Aguilera-Luiz *et al*., 2008; Hu *et al*., 2010; Jedziniak *et al*., 2009; Martínez-Villalba *et al*., 2013; Wang *et al*., 2016) have been proven to be the most powerful tools and thus the most widely used for the analysis of fenbendazole residues.

At present, no analytical methodology is available for the extraction, separation, and quantification of fenbendazole and its sulphoxide, sulphone and hydroxylated metabolites in fermented dairy products. The recently published multi-residue LC-MS/MS method (Gomez-Perez *et al*., 2013) for the analysis of veterinary drug residues in cheese does not cover the EU prerequisite to consider fenbendazole sulphone and *p*-hydroxyfenbendazole in the determination. The objective of this study was to develop and validate, according to Commission Decision 2002/657/EC (European Commission, 2002), a simple, rapid, sensitive and cost-effective LC method for the accurate and precise determination of the marker residue of fenbendazole in fermented dairy products. The method is aimed to be applied to quantitate fenbendazole and its metabolites in Feta cheese and yoghurt made from spiked and incurred *ovine* milk.

Materials and Methods

Chemicals and equipment

LC-grade acetonitrile and reagent-grade ethyl acetate, hexane, phosphoric acid, disodium hydrogen phosphate, octanesulphonate sodium salt, and tetrabutylammonium hydrogen sulphate were obtained from Merck (Darmstadt, Germany). Oxfendazole, *p*-hydroxyfenbendazole, fenbendazole sulphone, and fenbendazole reference standards were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Liquid-chromatography was carried out using a model UFLC Shimadzu system (Shimadzu Corp., Kyoto, Japan) equipped with a LC-20AD pump, a DGU-20A5 on-line degassing unit, a SIL-20AD automatic sample injector, a CTO-20A column oven and a SPD-M20A photo-diode array detector. LC solution software (Shimadzu) was utilized to control the system. Analyses were performed on a reversed-phase Nucleosil 100-5 C_{18}, 5 µm material in Macherey-Nagel (Düren, Germany) analytical (250 x 4.6 mm i.d.) and guard (10 x 4.6 mm i.d.) columns.

A Milli-Q purification system (Millipore, Bedford, MA, USA) was used for ultra-purification of tap water, and an Ultra-Turrax (Staufen, Germany) high-speed blender, a G-560E vortex mixer (Scientific Industries, Bohemia, NY, USA), a KUBOTA-7780 centrifuge (KUBOTA Corporation, Tokyo, Japan), and an evaporation unit (ReactiTherm, Pierce Chem., Rockford, IL, USA) were used for sample preparation.

Sample extraction and clean-up

A 1.5-g sample (yoghurt or grated cheese) was transferred into a 15-mL graduated centrifuge tube to which the extraction solution (mixture of acetonitrile:1 M phosphoric acid, 9:1) was added to a final volume of 7 mL. The content was homogenised for 30 s using an Ultra-Turrax and centrifuged for 1 min at 4,000 *g*. A 3-mL aliquot of the clear supernatant was transferred into another 15-mL tube and 2 mL of 0.1 M phosphate buffer, pH 11, were also added. After vortex mixing for 10 s, the extract was defatted by partitioning with 5 mL of hexane under high speed vortex mixing for 30 s, and centrifuged as described above. The top hexane layer was discarded and following the addition of 5 mL ethyl acetate, vortex mixing for 1 min and centrifugation, the top organic layer separated was transferred into another 15-mL tube to be further purified by vortex mixing (1 min) and centrifugation after the addition of 3 mL of 0.1 M phosphate buffer, pH 11. The supernatant ethyl acetate layer was transferred into another tube and evaporated to dryness at 50^{o}C under nitrogen. The remaining residue was dissolved in 300 µL of mobile phase and a 50-µL aliquot was submitted to LC analysis.

LC conditions

Separation of fenbendazole and its sulphoxide, sulphone, and *p*-hydroxy metabolites was carried out isocratically using an acetonitrile–0.01 M phosphoric acid (33:67, v/v) mobile phase containing 4.0 mM octanesulphonate sodium salt and 5 mM tetrabutylammonium hydrogen sulphate. Following its preparation, the mobile phase was filtered by passing through a 0.2 µm Nylon-66 filter (Anachem, Luton, UK). The mobile phase was delivered in the system at a rate of 1 mL min^{-1}.

The Nucleosil 100-5 C_{18} analytical and guard columns were equilibrated with the mobile phase each time before use and kept thermostated at 55°C during runs. Detection was performed at 290 nm using a diode-array detector.

Method validation

Validation was performed according to the Commission of the European Communities guidelines (European Commission, 2002) using blank cheese and yoghurt samples. The specificity, linearity, sensitivity, accuracy, precision, applicability, and stability of the analytes were the criteria used to evaluate the developed method.

Results and discussion

Sample extraction and clean-up

Fenbendazole and its metabolites are weakly basic hydrophobic compounds with appreciable solubility in polar organic solvents such as ethyl acetate and dichloromethane. Initial experiments on sample extraction and clean-up using procedures previously developed in our laboratory for milk analysis (Fletouris *et al*., 1994, 1996a, 1996b) failed to suggest a universal analytical scheme for milk, yoghurt and cheese samples. In contrast to milk analysis (Fletouris *et al*., 1996a), the ethyl acetate extracts of yoghurt and cheese contained too many matrix-interfering compounds. Furthermore, fenbendazole, unlike its metabolites, was poorly partitioned into ethyl acetate or dichloromethane, which indicated substantial binding of this compound to yoghurt and cheese proteins. In an effort to disrupt these interactions and recover fenbendazole from milk samples, acetonitrile was investigated as a potential extraction solvent (Fletouris *et al*., 1994, 1996b). Although successful for milk analysis, this effort was not effective in yoghurt and especially cheese analysis, probably due to their high protein content. A thorough investigation of the extraction efficiency of acetonitrile showed that its efficiency was better than that of ethyl acetate, but the fenbendazole losses due to protein binding could not be eliminated unless acidic conditions were applied (pH<4.0). Best recovery of all analytes and effective precipitation of matrix proteins was achieved only when a mixture of acetonitrile-1 M phosphoric acid (9:1, v/v), was used as the extraction solvent.

To purify the aqueous acetonitrile extract prior to injection, a series of consecutive liquid-liquid partitioning clean-up steps was evaluated. As co-extracted lipids interfered with the analysis, especially when cheese extracts were concentrated to a small volume, a defatting procedure was assessed. It was found that addition of a pH 11 phosphate buffer, followed by hexane washing of the aqueous acetonitrile extract, efficiently removed co-extracted lipids without affecting the test analytes. Further purification of the aqueous acetonitrile extract could be made possible by partitioning with ethyl acetate. By this treatment, partitioning of fenbendazole and its metabolites into ethyl acetate was quantitative, whereas some hydrophilic

constituents could be efficiently removed. Additional purification could be attained by washing the organic phase with a pH 11 phosphate buffer in order to remove a number of remaining weakly-acidic compounds. This simple and cost-effective extraction procedure resulted in quite clean extracts without causing any significant adverse effect on the recoveries of fenbendazole and its metabolites. Applying this procedure for the analysis of a series of various blank yoghurt and cheese samples provided valuable information concerning the cleanliness of the extracts and the presence of interfering peaks.

Liquid chromatography

To achieve baseline separation of benzimidazoles on reversed-phase columns, gradient elution has been mainly used (Aguilera-Luiz *et al*., 2008; Boontongto *et al*., 2014; Chen *et al*., 2010; Gomez-Perez *et al*., 2013; Hu *et al*., 2010; Jedziniak *et al*., 2009; Martínez-Villalba *et al*., 2013; Santaladchaiyakit and Srijaranai, 2012; Santaladchaiyakit and Srijaranai, 2014; Wang *et al*., 2014; Wang *et al*., 2016). Although gradient elution has proved to be quite useful for the liquid chromatographic determination of benzimidazole residues in milk, an isocratic separation would be most valuable for routine determination in terms of repeatability in retention times, peak areas and peak heights or linearity of the calibration curves. In this respect, the results of a previous study (Botsoglou *et al*., 1997), concerning the retention profile of fenbendazole and its metabolites in ion-pair liquid chromatography, were evaluated. According to this study, baseline separation could be obtained when the mobile phase contained 5.0 mM tetrabutylammonium and 1.0-4.0 mM octanesulfonate pairing ions. Nevertheless, the best resolution from matrix interferences was achieved when the mobile phase contained 5.0 mM tetrabutylammonium and 4.0 mM octanesulfonate pairing ions and delivered isocratically to the stationary phase. Under these conditions, oxfendazole eluted at 5.4 min, *p*-hydroxyfenbendazole at 7.0 min, fenbendazole sulphone at 8.5 min and fenbendazole at 17.9 min (Figure 1).

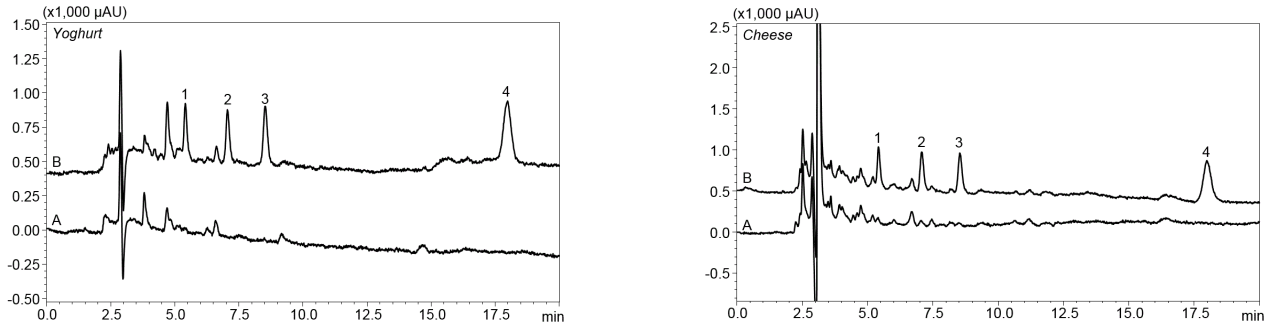

Figure 1. Typical chromatograms of blank (A) and fortified (B) with oxfendazole (1), p-hydroxyfenbendazole (2), fenbendazole sulphone (3) and fenbendazole (4) in sheep yoghurt and Feta cheese samples.

Calibration curves based on seven data points with three replicates at each point could be described by the following equations: y = 4.1147 + 308.78x, r^2=0.9999, for oxfendazole, y = 7.4809 + 168.19x, r^2=0.9997, for *p*-hydroxyfenbendazole, y = 6.5712 + 243.2x, r^2=0.9998, for fenbendazole sulphone and y = -12.101 + 91.316x, r^2=0.9998, for fenbendazole, where y represents the peak height in µAU and x is the quantity in ng of each analyte per 50 µL injected.

The efficiency of the ion-pair liquid chromatographic system along with the cleanliness of the extracts enabled detection and quantification limits in the range of 3.0 to 8.7 and 9.9 to 20.9 µg kg^{-1}, respectively, for all analytes in both yoghurt and cheese samples.

Method validation

The precision of the automatic sample injector was evaluated for all four analytes by injecting two mixed working standard solutions ten times each, on a single occasion. For each working standard solution, the relative standard deviation of the peak height was calculated. The results showed that the injection system was adequately precise, with relative standard deviations of the peak heights lower than 1.8% for all four analytes.

The specificity of the new method was also evaluated to ensure that there was no interference with the test analytes from matrix co-extractives. Chromatograms obtained from blank yoghurt and cheese extracts, and from reagent blanks showed that the peaks attributable to test analytes were resolved sufficiently from other peaks (baseline resolution) allowing reliable quantification (Figure 1).

The accuracy of the method was evaluated by spiking blank yoghurt and cheese samples with oxfendazole, *p*-hydroxyfenbendazole, fenbendazole sulphone and fenbendazole at five fortification levels and analysing six replicates per level. Least squares and regression analysis of the data obtained by analysing the fortified yoghurt and cheese samples showed that the relationship between the amounts added and the amounts found, could be adequately described by linear regressions. The

practically zero intercept values and the excellent linearity of the regression lines permit the estimation of the overall recovery of the method on the basis of the slope of the corresponding regression line. Overall recoveries of all analytes ranged between 80.5 and 88.8% for yoghurt, and 79.8 and 87.6% for cheese (Table 1).

The precision of the method was evaluated by spiking blank yoghurt and cheese samples with oxfendazole, *p*-hydroxyfenbendazole, fenbendazole sulphone and fenbendazole at three fortification levels for each compound, and analysing six replicates, on three different days under within-laboratory reproducibility conditions. The results showed that overall precision (RSD%) for all analytes was lower than 11% for both products (Table 1).

Table 1. Linearity, accuracy and precision data for the determination of fenbendazole residues in yoghurt and Feta cheese made from ovine milk.

Dairy product	Residue	Fortification range[a] (μg kg^{-1})	Correlation coefficient (r^2)	Overall recovery ± SD (%)	Overall RSD (%)
Sheep yoghurt	Oxfendazole	10.1 – 100.8	0.9924	80.5 ± 8.1	6.3
	p-Hydroxyfenbendazole	10.1 – 100.8	0.9923	84.7 ± 9.1	9.0
	Fenbendazole sulphone	9.9 – 98.9	0.9915	88.8 ± 8.2	7.4
	Fenbendazole	20.9 – 104.5	0.9848	80.6 ± 7.7	11.0
Feta cheese	Oxfendazole	10.1 – 100.8	0.9906	82.7 ± 9.0	7.6
	p-Hydroxyfenbendazole	10.1 – 100.8	0.9956	79.8 ± 9.8	8.1
	Fenbendazole sulphone	9.9 – 98.9	0.9889	87.6 ± 7.0	6.5
	Fenbendazole	20.9 – 104.5	0.9920	81.5 ± 6.4	8.1

[a] *Five fortification levels with six replicates per level.*

Storage stability

In total, four experiments were carried out to investigate the stability of oxfendazole, *p*-hydroxyfenbendazole, fenbendazole sulphone and fenbendazole in solution (n=10) at three temperatures (25°C in presence of light or dark, 4°C, -30°C). Moreover, six experiments were performed to determine the stability of the analytes in incurred and fortified yoghurt and cheese samples, and in final yoghurt and cheese extracts (solutions ready for LC analysis).

The results indicated that oxfendazole and fenbendazole sulphone were stable in the mobile phase for at least 36 weeks at all three temperatures (25°C, 4°C, -30°C) examined. As regards the stability of *p*-hydroxyfenbendazole and fenbendazole, the results indicated that the two analytes were quite stable for 24 weeks at all three temperatures, but a reduction of about 9% and 6% was noted for both analytes after 36 weeks of storage at 25°C, in the light and in the dark, respectively. However, standard solutions of these two analytes in mobile phase were stable for at least 36 weeks when stored at 4°C and -30°C.

Regarding the stability of the analytes in the different matrices, the results indicated that the test compounds were stable during storage of fortified and incurred yoghurt and cheese samples (n=10) at -80°C for at least 12 months. With regard to the stability of the analytes in the final yoghurt and cheese extracts (n=10), the results indicated no significant change in the concentration of all four analytes, during storage at 25°C for 7 days.

Applicability

In order to validate the method with real samples, a trial was carried out to quantitate fenbendazole residues in Feta cheese that was prepared from *ovine* milk spiked with oxfendazole, *p*-hydroxyfenbendazole, fenbendazole sulphone and fenbendazole. The analysis results showed that all four analytes were present in the cheese curd at concentrations that corresponded to 52-63% of the initial quantity of each analyte added to milk.

The method was also successfully applied to quantitate fenbendazole residues in Feta cheese made from incurred *ovine* milk. The results indicated that the only residues found in the cheese curd were oxfendazole, fenbendazole sulphone and fenbendazole. The method was further used to quantitate fenbendazole residues in yoghurt made from the above mentioned incurred *ovine* milk.

Moreover, the applicability of the method was also tested in a variety of other marketed fermented dairy products, namely, Kaseri, Telemes, Graviera, Kefalotyri, Edam, Camembert and Blue cheese, and different types of yoghurt made from *bovine* or *ovine* milk. Because of the good analytical characteristics of the method, no matrix interferences were observed and the recovery values were well within those already stated in the accuracy evaluation experiments. Mould-ripened cheeses, however, such as Blue cheese and Camembert, could not be analysed due to significant interference with oxfendazole and *p*-hydroxyfenbendazole from matrix co-extractives.

Conclusions

The developed method requires only a very small sample mass, and offers considerable savings in terms of solvent requirements, costly materials, sample manipulation, and analysis time. In addition, the method has satisfactory analytical characteristics with regard to recovery, sensitivity, selectivity, and repeatability. Sample throughput (extraction/clean-up/LC determination) is 15 samples in a total time of about 8 h by a single analyst. The method utilizes commercially available reagents and equipment and is designed to be performed safely by trained analysts. Owing to these advantages, the method might be considered suitable for the routine monitoring of fenbendazole residues in fermented dairy products.

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ANALYSIS OF ISOMERIC PYRROLIZIDINE ALKALOIDS BY ONLINE MULTIPLE HEART-CUTTING 2D-LC QTOF-MS

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Abstract
Pyrrolizidine alkaloids (PAs) are an important class of secondary plant metabolites. Different PAs have different toxicities, but it is difficult to determine the total toxicity of an extract as not all isomeric PAs can be separated in one LC run. The goal of this research is to increase the separation of 4 groups of isomeric pyrollizidine alkaloids (PAs) which have similar MS fragmentation patterns. Seven different stationary phases and two different mobile phases were used to characterise the elution behaviour of the isomeric PAs in 1D-LC. After the selection of the stationary and mobile phases, different parameters like temperature and flow rate were optimized in 1D-LC. Subsequently, 2D-LC runs were performed in multiple heart-cutting mode. The best separation was achieved by the use of a Zorbax UPLC SB-Aq column at pH 3 in the first dimension and an Acquity UPLC BEHC18 column at pH 8 in the second dimension. This resulted in the separation of all PAs in one run with help of an online multiple heart-cutting 2D-LC QToF-MS method.

Introduction
The use of 2D-LC based methods for food safety applications could be interesting, as this method could allow for the analyses of complex mixtures at low concentrations in a single run. Within RIKILT several 2D-LC applications are currently developed, such as the analysis of multiple classes of residues (antibiotics and growth-promotors) or isomeric compounds. Here, an example of the separation of isomeric pyrrolizidine alkaloids (PAs) is given.

PAs are an important class of secondary plant metabolites (Figure 1). Their assumed function is to defend the plant against insect attacks and from being consumed by mammalian herbivores. Consumption of plant material containing PAs (*e.g.* tea, honey) can result in toxic effects. It is known that different PAs could have different toxicities (EFSA 2011), but it is difficult to determine the total toxicity of an extract as not all isomeric PAs can be separated in a single LC run. Currently, PAs are analysed using either high pH (De Nijs *et al*. 2014) or low pH (Colegate *et al*. 2014), but none of the methods can separate all PAs (Figure 1). For example, compounds 7, 8 and 9 could only be separated at high pH, while compound 1 and 3 can only be separated at low pH. The goal of this research is to increase the separation of isomeric PAs, which have similar MS fragmentation patterns, using a 2D-LC QToF-MS. The use of a 2D-LC QToF-MS could allow for the separation of all compounds using different stationary and mobile phases in the two dimensions.

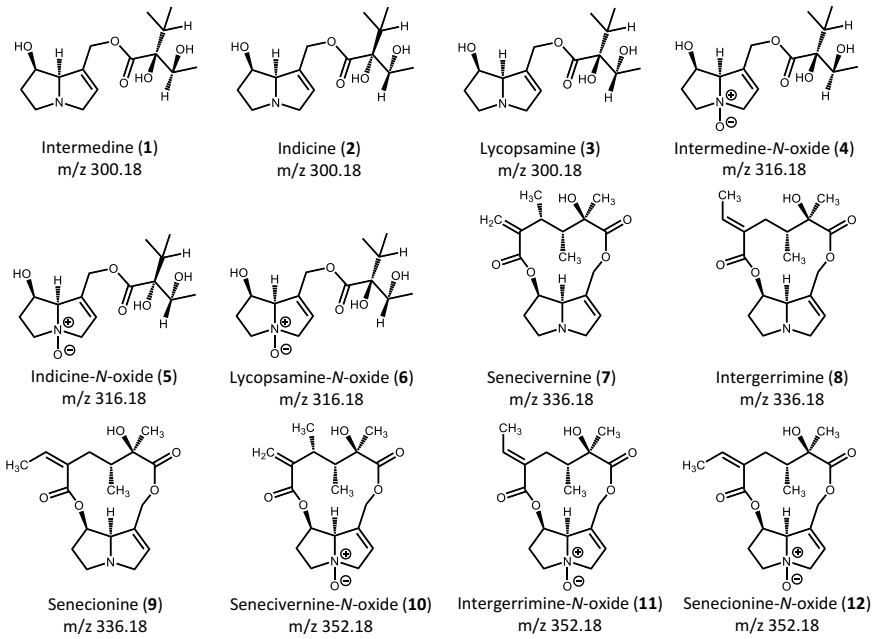

Figure 1. Structures of pyrrolizidine alkaloids.

In this study, we used a commercial available 2D-LC system, a 2D-LC QToF-MS system, consisting of a pump, column and DAD-detector in both dimensions (Figure 2). It is estimated that extracts, *e.g.* food products, contain 8,000 to 30,000 compounds. The number of compounds which can be separated in one run, is called peak capacity. The theoretical peak capacity can be calculated according to $n = 1 + (t_G / w_{ave})$ where t_G represents the gradient time and w_{ave} the average peak width at baseline (Neue *et al.,* 2005). A peak capacity between 50-300 is typical for an 1D-LC run, which will not be sufficient to separate all compounds in one run. Hyphenation with a MS will increase the peak capacity significantly, however co-eluting isomers with similar fragmentation patterns cannot be distinguished. 2D-LC can be used to increase the peak capacity and this will result in wider range of compounds which can be detected in one run. In case of full orthogonality and no dilution effects, the peak capacities of both dimensions can be multiplied ($n_{total} = {}^1n \times {}^2n$) where ${}^1n$ is the peak capacity of the first dimension and ${}^2n$ the peak capacity of the second dimension (Vivoi-Truyols *et al*., 2010). In practice the peak capacity will be lower since true orthogonality is never achieved. However, the peak capacity with a 2D-LC run is significantly increased over a 1D-LC run.

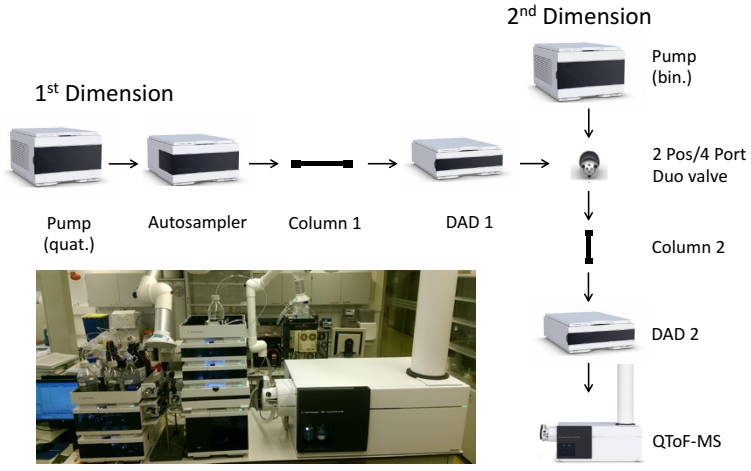

Figure 2. (Schematic) Set-up of an online 2D-LC QToF-MS system.

The effluent of the first dimension can be transferred to the second dimension in two different ways: comprehensive (Figure 3A) and multiple heart-cutting (Figure 3B). In comprehensive mode all effluent of the first column will be injected on the second column and will be analysed in the second dimension with very fast gradients (Frank, 2012). In this way, the run time of the second dimension is equal to the collection time of the first dimension. In multiple heart-cutting mode only parts of the effluent of the first column will be injected to the second column. Typically, the run time of the second dimension is longer than the collection time of the first dimension.

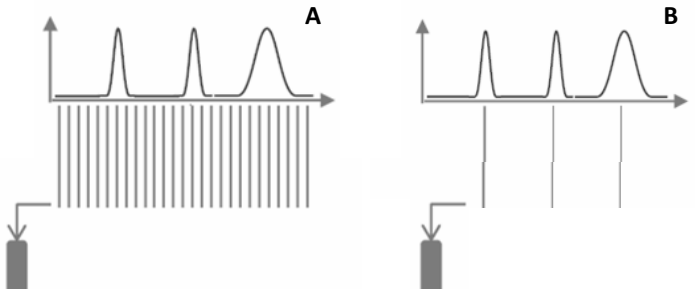

Figure 3. Schematic overview of the effluent going from the first to the second dimension in comprehensive (A) and multiple heart-cutting (B) modes.

Materials and Methods

Materials

Formic acid (FA), water and acetonitrile (ACN), all ULC/MS grades, were purchased from Aktu-All (Oss, The Netherlands). Ammonia formate (ULC/MS grade) and ammonia hydroxide (>25%; ULC/MS grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Analytical standards of lycopsamine, lycopsamine *N*-oxide, intermedine, intermedine *N*-oxide, senecionine,

senecionine *N*-oxide, senecivernine, senecivernine *N*-oxide, indicine, indicine *N*-oxide, intergerrimine, intergerrimine *N*-oxide were purchased from Phytolab (Vestenbergsgreuth, Germany).

Standard solutions
Stock solutions of 100 µg mL^{-1} pure standards were prepared in methanol. The different stock solutions were combined into a mixed standard solution of 0.1 µg mL^{-1} in water. The solutions were stored at 5°C until use.

Online 2D-UHPLC QToF-MS
Samples were analysed on an Agilent 1290 infinity 2D-LC system (Waldbronn, Germany) equipped with a quaternary pump in the first dimension and a binary pump in the second dimension, two DAD detectors, sample manager and column oven compartment. Standard solutions (2 µL) were injected on a first dimension Zorbax UPLC SB-Aq column (100 x 2.1 mm, 1.8 µm particle size; Agilent, Waldbronn, Germany). Water acidified with 0.02% (v/v) FA pH 3, eluent A, and ACN acidified with 0.02% (v/v) FA pH 3, eluent B, were used as eluents. The flow rate was 200 µL min^{-1}, the column temperature was controlled at 25˚C. The following elution profile was used: 1-20 min, linear gradient from 5-70% B; 21-22 min linear gradient from 70-5% B, 22-33 min, isocratic on 5% B. The interface valve to connect the LC systems contains a multiple heart-cut module with twelve 60 µL loops in multiple heart-cutting mode or 2 sample loops of 80 µL in comprehensive mode. In multiple heart-cutting mode, loops were filled for 100% and different time segments were collected. Compounds with *m/z* 316 were collected between 2.80 and 4.35 min, compounds with *m/z* 336 were collected between 5.70 and 7.25 min and compounds with *m/z* 352 were collected between 6.84 and 8.39 min. Afterwards the samples were injected on a second dimension Acquity UPLC BEHC18 column (100 x 2.1 mm, 1.7 µm particle size; Waters, Milford, MA, USA). 50 mM ammonium formate in water adjusted to pH 8 with ammonia hydroxide, eluent A2, and ACN, eluent B2, were used as eluents in this dimension. The flow rate was 500 µL min^{-1} and the column temperature was controlled at 40°C. The following elution pattern was used in the multiple heart-cut mode: 1-7.3 min, linear gradient from 8-31% B; 7.3-7.95 min, isocratic on 8% B.

Mass spectrometric (MS) data were obtained by analysing the samples on an Agilent 6540 quadrupole time-of-flight (QToF) (Waldbronn, Germany). The 2D-UHPLC system was connected via an electrospray interface (ESI). Data were collected over a scan range of 100-1000 amu in positive ionisation mode. The MS parameters were set as follows: nebulizer gas pressure at 45 psi, sheath gas temperature of 350°C, sheath gas flow of 11 L min^{-1}, drying gas flow rate of 16 L min^{-1}, capillary voltage of 3,500 V, fragmentor voltage of 360 V, acquisition rate of 5 spectra s^{-1}. A reference solution was used for mass-axis calibration prior to analysis, width of precursor ion was set as narrow (1.3 Hz). Analysis of MS data was performed with MassHunter B.07.00.

Results and Discussion
Seven different columns (100 x 2.1 mm 1.7 µm) with different hydrophobicity of their stationary phase were selected and two different mobile phases were used (pH 3 and pH 8) to characterise the elution behaviour of the isomeric PAs (Figure 1) in 1D-LC. Separation of compound 4-6 and 7-9 was achieved with an Acquity UPLC BEHC18 column at pH 8 (chromatographic resolution > 0.93) and separation of compound 1-3 was achieved with an Zorbax UPLC SB-Aq column at pH 3 (chromatographic resolution >0.84). With the Acquity UPLC BEHC18 column (pH 8) compound 11 could be separated from the co-eluting compounds 10 and 12 (chromatographic resolution = 1.67), while the Zorbax UPLC SB-Aq column (pH 3) could separate compound 10 from the co-eluting compounds 11 and 12 (chromatographic resolution = 0.89). Both columns interacted differently with the compounds at different pH in 1D-LC, so in theory it should be possible to separate all isomeric compounds with these columns at different pH in one 2D-LC run.

Optimisation of the flow rate, temperature and the influence of the injection solvent was performed in 1D-LC prior to 2D-LC runs. The first parameter to take into account is the injection solvent, since the first dimension effluent will be injected on the second column. Compounds solubilized in different ACN concentration (0, 50, 100% v/v) were injected on both columns and it was shown that separation was still achieved when the compounds were solubilized in 50% ACN with the Acquity UPLC BEHC18 column, while all compounds eluted immediately from the Zorbax UPLC SB-Aq column. For this reason, the Zorbax UPLC SB-Aq was chosen as first dimension column and the Acquity UPLC BEHC18 as second dimension column. Afterwards, optimization of the flow rate and temperature was performed in order to obtain the best separation. A relative high flow rate in the second dimension is preferred, as it will give higher peak capacities with similar chromatographic resolution when small column particles (~1.8 µm) are used. In the first dimension a relative low flow rate is preferred to be able to park all peaks from the first dimension. Too high flow rates in the first dimension will fill the loops too fast and as a consequence not all peaks from the first dimension can be parked and afterwards analysed in the second dimension. Finally, the temperature was adjusted to obtain the best separation in both dimensions. It was, for example, shown that compounds 1-3 gave better separation at low temperatures, while compound 10 and 11 gave better separation at high temperature on the Zorbax UPLC SB-Aq column at pH 3.

Following the optimization steps, 2D-LC runs were performed in multiple heart-cutting mode. Only compounds which could not be separated in the first dimension were parked and transferred to the second dimension (Figure 4A). For the different

compounds, different segment (collection) times were used. In Figure 4B it can be seen that the co-eluting compounds 4+5 can be separated in the second dimension (chromatographic resolution = 1.00). This is also the case for co-eluting compounds 7-9 (chromatographic resolution > 1.7). Compounds 10-12 could be partly separated in the first dimension and partly separated in the second dimension. Taken these result together it can be concluded that all PAs could be separated in one run with help of an online multiple heart-cutting 2D-LC QToF-MS method.

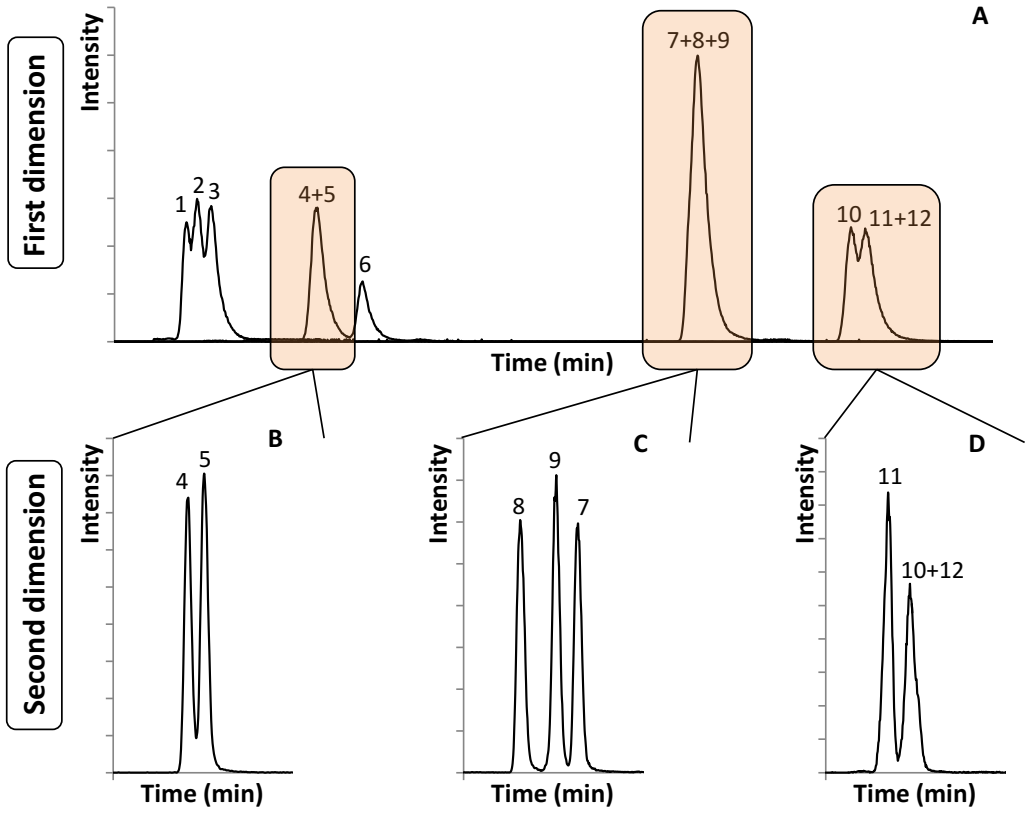

Figure 4. MS profile of the PAs in the low pH first dimension (A) and in the time segments in the high pH second dimension. Segment times: 3.25-3.55 min (B), 6.32-6.62 min (C), 7.77-8.07 min (D). Peak numbers refer to compounds in Figure 1.

Conclusions

All PAs could be separated in one run with help of an online multiple heart-cutting 2D-LC QToF-MS method. Compounds 1-3 could be separated in the first dimension, compounds 10-12 could be partly separated in the first dimension and partly separated in the second dimension and compounds 4-5 and 7-9 could be separated in the second dimension. This separation was achieved by the use of a Zorbax UPLC SB-Aq column at pH 3 in the first dimension and an Acquity UPLC BEHC18 column at pH 8 in the second column. The next step is to test this method in practice by analysis of isomeric PAs in food products.

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QTRAP® LC-MS/MS METHOD FOR DETERMINATION OF ESTRADIOL IN *BOVINE* SERUM WITH 4-(DIMETHYLAMINO) BENZOYL CHLORIDE DERIVATIZATION

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Abstract

A QTRAP®LC-MS/MS analytical method for the determination of natural steroid hormones 17α/β-estradiol in *bovine* serum well below recommended concentration (RC) for 17β-estradiol at 0.1 µg L^{-1} was developed. The procedure covered extraction with a mixture of tert-butyl methyl and petroleum ethers (30:70, v/v) followed by derivatisation with 4-(dimethylamino) benzoyl chloride. An Inertsil®ODS-3 analytical column (150 x 2.1 mm, 3 µm) maintained at a temperature of 30°C was used. The mobile phase consisted of acetonitrile/0.01% formic acid mixture (85:15, v/v) and was pumped isocratically at a flow rate of 300 µL min^{-1}. The QTRAP5500 instrument was operated in the positive electrospray ionization mode and controlled by Analyst software. Three transitions in the multiple reactions monitoring mode were monitored. The method was validated in accordance with the Commission Decision 2002/657/EC performance criteria. The apparent recoveries for 17α/β-estradiol were in the range from 85% to 108%. The repeatability was less than 5% and reproducibility did not exceed the limit of 5%. The linearity was satisfactory with correlation coefficients in excess of 0.98. The decision limits (CCα) and the detection capabilities (CCβ) ranged from 0.02 µg L^{-1} to 0.04 µg L^{-1} and from 0.03 µg L^{-1} to 0.06 µg L^{-1}, respectively, and were lower than RC.

Introduction

Steroids hormones are the largest group of anabolic hormones including estrogens, androgens and gestagens, which all are sex hormones produced in relevant endocrine organs in living organisms. Sex hormones have found practical application in veterinary medicine in cattle especially in those individuals both males and females with low levels of endogenous hormones in order to compensate that.

In Poland and other EU countries, administration of hormones to food-producing animals for growth stimulation purposes is banned according to Council Directive 96/22/EC (Council Directive 96/22/EC, 1996). But in some regions outside the EU, *e.g*. Canada, Australia and the USA, certain groups of hormones are authorized for the fattening of animals. Estradiol, testosterone, progesterone, and their esters and derivatives belong to this group of allowed hormones. They are usually given as an implants.

According to the International Agency for Research on Cancer, many of the compounds including 17β-estradiol, which is of Group 1A of carcinogenic compounds for humans, have also toxic properties (IARC). In connection with the prohibition of anabolic hormones as well as consumer health protection from potentially harmful residues, it is necessary to control the hormones residues in samples of animal origin according to Council Directive 96/23/EC. The testing for natural hormones, estradiol and testosterone, is usually carried out in serum or plasma, because there are no established criteria for the concentrations of them in the urine due to the large individual variation of the endogenous hormone, which depends on the age and gender of the animal. There are no maximum residue levels established for the banned compounds either. Maximum permitted concentration in the serum of cattle under the age of eighteen months proposed for 17β-estradiol is 0.1 µg L^{-1} (CRL Guidance Paper, 2007).

The methods used for the detection of residues of the banned compounds should meet the performance requirements set out in Commission Decision 2002/657/EC. Chromatographic techniques especially liquid-chromatography with tandem mass-spectrometry play the most important role in analysis of residues of anabolic hormones. However, the possibility of using liquid-chromatography mass-spectrometry for the detection of estrogenic steroid hormones is often limited due to the weak ionization sensitivity of them in the ESI source. Chemical derivatisation by adding a molecule that improves ionization, enhances the signal. A wide variety of reagents are available for the derivatisation of anabolic steroids. The most frequently used are dansyl chloride, picolinic acid, hydroxylamine and methoxyamine (Keski-Rahkonen *et al*., 2015; Santa *et al*., 2007; Yang *et al*., AN 611).

The aim of the study was to develop a simple method for the determination of 17α/β-estradiol in *bovine* serum by liquid-chromatography mass-spectrometry using a new derivatisation reagent 4-(dimethylamino)benzoyl chloride (DMABC), and the evaluation of its usefulness to enhance ionization. The method was optimized for derivatisation, chromatographic separation and mass-spectrometric detection of the derivatives. The method was validated and allows the determination of 17α/β-estradiol significantly below the currently maximum permitted concentrations.

Materials and Methods

Reagents and chemicals

Tert-butyl methyl ether (for residues, Baker), petroleum ether (fraction 60°C, for residues, Baker), acetonitrile (LC grade, Baker), methanol (LC grade, Baker), ethanol (HPLC or analytical grade, Baker, POCH); derivatisation reagent: 4-(Dimethylamino)benzoyl chloride (HPLC grade, Sigma Aldrich); formic acid (analytical grade, POCH), acetic acid (analytical grade, POCH), ammonium formate (HPLC grade, Sigma Aldrich); water acquired with a Milli-Q apparatus (Millipore, USA) were used.

The extraction mixture consisted of tert-butyl methyl ether and petroleum ether (30/70, v/v). Solution of 0.01% formic acid was prepared by mixing of 100 µL formic acid with 999.9 mL water. A 2.5 mg mL^{-1} DMABC solution was prepared by dissolving 5 mg of the substance in 2 mL acetone. The mobile LC-MS/MS phase was a mixture of acetonitrile and 0.01% formic acid (85:15, v/v). Standards of 17α-estradiol and 17β-estradiol and the internal standard 17β-estradiol-d3 were purchased from RIKILT - Institute of Food Safety (The Netherlands). All standards were kept at 2 to 8°C according to the recommendations of the certificates. Primary standard stock solutions at 10 µg L^{-1} were prepared from ampoules by dissolving the content of 0.1 mg standard in methanol. Primary standard solutions were kept frozen not longer than one year. Working standard solutions were obtained by tenfold dilution of primary standard solutions to the concentrations of 0.1 µg L^{-1} and 0.01 µg L^{-1} in methanol. Working standard solutions were stored at 2-8 °C in the dark not longer than 6 months.

Sample preparation

Frozen *bovine* serum was thawed at room temperature, stirred, filtered through a filter intended for biological samples and subsequently 5 mL was placed in a tube for centrifugation. To the sample 10 mL of tert-butyl methyl ether/petroleum ether mixture (30:70, v/v) and 5 µL of internal standard at a concentration of 0.1 µg mL^{-1} to obtain the final concentration of 0.1 µg L^{-1} were added. The tube was closed with a stopper and the content of the tube was shaken using Vortex stirrer for about 3 min. The samples were centrifuged at 7,728 *g* for 10 min at -15°C and then put in a freezer below -18°C for 1.5-2 h. The cold ether layer was decanted into glass tubes, which were placed in a heating block maintained at 60°C ± 2°C. The solvent was evaporated under the gentle stream of nitrogen. The dry residue was dissolved in 200 µL ethanol and transferred into another glass tube intended for derivatisation. The ethanol was evaporated to dryness under conditions described above.

For the derivatisation, 200 µL DMABC solution in acetone was added to the dry residue and mixed thoroughly using Vortex stirrer. The derivatisation reaction was carried out in a heating block at a temperature of 60°C (±2 °C) for 6 min, whereupon the volatile components were completely evaporated in the gas stream. After this step the residue was dissolved in 200 µL mobile phase consisting of acetonitrile and 0.01% formic acid (85:15, v/v). The whole was mixed, transferred into glass vials with inserts. Vials were placed in a autosampler and LC-MS/MS run program was started.

LC-MS/MS measurement

Chromatographic separation of 17α-estradiol and 17β-estradiol was achieved using a 1200 HPLC binary pump system (Agilent Technologies, Waldbronn, Germany) on an Inertsil® ODS-3 analytical column (150 mm x 2.1 mm, 3 µm) (GL Sciences Inc., Tokyo, Japan) with an octadecyl guard cartridge (4 mm x 2 mm) (Phenomenex, Torrance, CA, USA). The mobile phase consisting of acetonitrile/0.01% formic acid (85:15, v/v) was pumped in an isocratic mode at the total flow rate of 300 µL min^{-1}. The column was maintained at a constant temperature of 30°C. The volume of sample injected was 25 µL.

The HPLC system was coupled to Linear Ion Trap Quadrupole QTRAP5500 mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada) equipped with an electrospray ionization (ESI) Turbo Spray source operating in positive mode and controlled by Analyst software (version 1.5.1). The temperature of the source was set at 500°C, nitrogen was used as curtain gas (35 psi), air was used as nebulizer gas (40 psi) and auxiliary gas (40 psi) nitrogen was also used as collision gas and set at medium position. The TurboIon-Spray source was operated with the capillary voltage set at 4,500 V. The data was acquired in multiple reactions monitoring MRM mode. The direct infusion of standard solution of each analyte to make a selection and tuning of characteristic MRM transitions as well as optimize MS/MS collision energies was performed.

Validation

The method developed was submitted to a validation process in accordance with Commission Decision 2002/657/EC requirements. For the analysis of the factorial effect ResVal software was applied (CRL Laboratory, RIKILT, The Netherlands).

The validation parameters: linearity, specificity, repeatability, reproducibility, recovery, decision limits, detection capabilities of the method were evaluated. Blank serum samples were spiked with 17α/β-estradiol to obtain 0.05, 0.10 and 0.15 µg L^{-1}. In addition, spiking levels 0, 0.2 and 0.5 µg L^{-1} were introduced. A set of 21 samples was analysed thrice on three separate days. The validation process included also the analysis of ten blank serum samples to check signal specificity when analysing simultaneously ten serum samples fortified at 0.1 µg L^{-1}.

Results and Discussion

First, the conditions of the derivatisation reaction and the chromatographic separation were optimised. For this purpose, LC-MS/MS chromatograms of standard solutions of 10 ng compounds, which were intact or derivatised, were recorded. The results for the 17α- and 17β-estradiol standards with and without derivatisation are presented in Figure 1a and 1b.

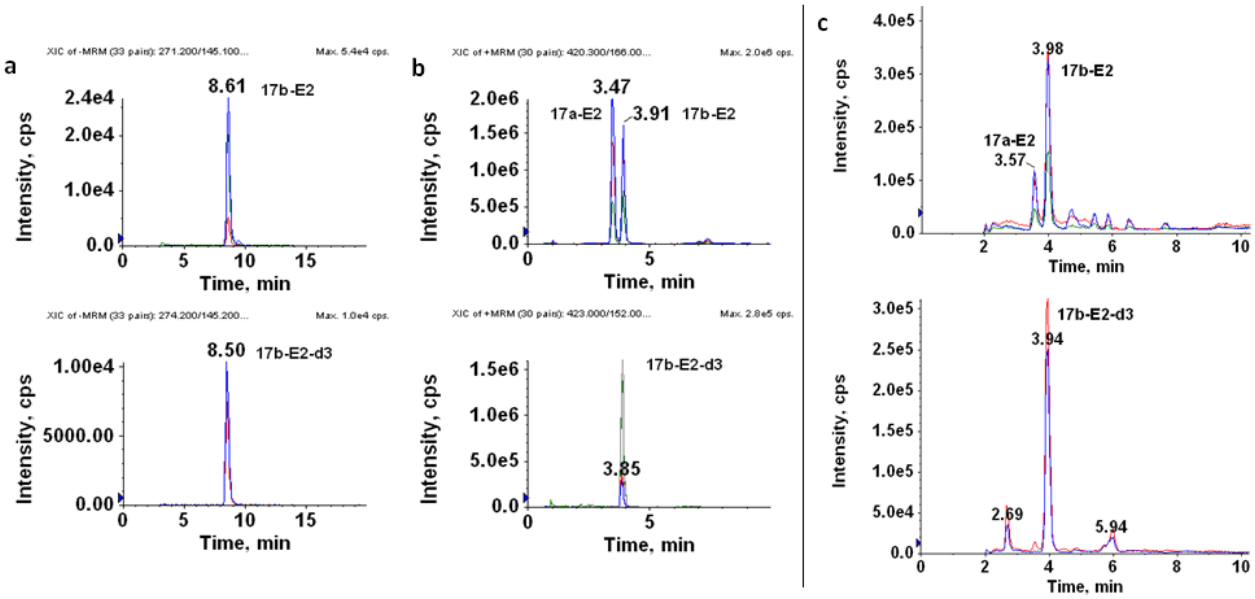

Figure 1. LC-MS/MS chromatograms of: a) standards of 17α- and 17β-estradiol (without derivatisation), b) the standards after derivatisation with DMABC/acetone, and c) a sample of bovine *serum spiked with 17α- and 17β-estradiol at 0.05μg L^{-1}.*

During the studies on the selection of the appropriate solvent in the mixture for derivatisation, it was concluded that the most intense signals for 17α/17β-estradiol derivatives were obtained when the derivatisation reaction with 4-(dimethylamino)benzoyl chloride was carried out in either acetone or acetonitrile. Only weak signals were obtained when methanol was used (Figure 2).

Subsequently the impact of the amount of DMABC solution in acetone on the efficacy of the derivatisation reaction was checked. On guidance of MS-signals, it was concluded that the derivatisation reaction should be performed using the following optimised conditions: at 60°C for six min using 200 μL of a 2.5 mg mL^{-1} DMABC solution.

Next, the impact of mobile phase modifiers on the intensity of the 17α/β-estradiol derivatives signals was investigated. The best ionization and signal intensities were obtained for acetonitrile in combination with formic acid and ammonium formate, while proper ionization was observed for acetic acid. The methanol mobile phase gives slightly lower chromatographic signals for the DMABC derivatives than acetonitrile as the mobile phase (Figure 3).

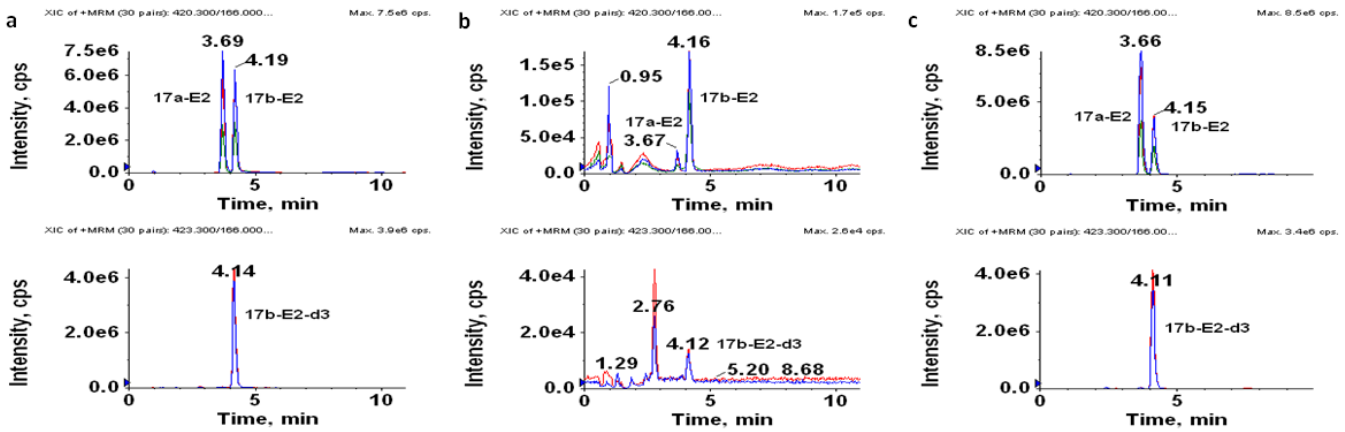

Figure 2.LC-MS/MS chromatograms showing the effect of the solvent used in the DMABC derivatisation: a) DMABC/acetone, b) DMABC/methanol, and c) DMABC/acetonitrile.

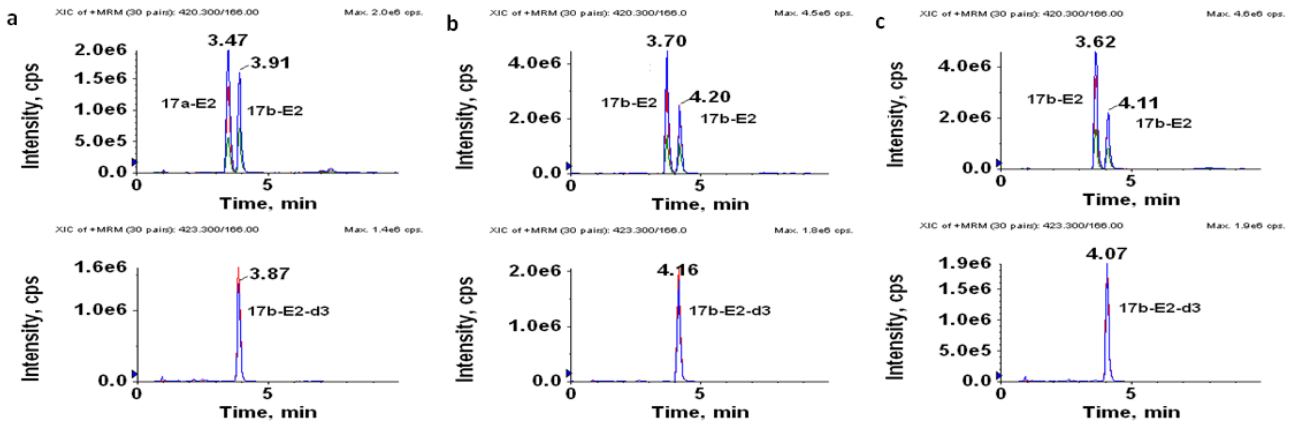

Figure 3. LC-MS/MS chromatograms showing the the effect of solvent modifiers and mobile phase on the signal intensities of 17α- and 17β-estradiol DMABC derivatives: a) acetonitrile/acetic acid (85:15, v/v), b) acetonitrile/ammonium formate (85:15, v/v), and c) acetonitrile/formic acid (85:15, v/v).

A satisfactory separation and detection of of 17α-estradiol and 17β-estradiol was obtained with the proposed analytical conditions. Three MRM transitions for 17α-estradiol and 17β-estradiol DMABC derivatives and two transitions for 17β-estradiol-d3 were monitored. Optimal values for MS parameters for the monitored, characteristic transitions are listed in Table 1.

Table 1. LC-MS/MS ion acquisition parameter (MRM) mode used for identification of 17α-estradiol, 17β-estradiol and 17β-estradiol-d3

Compound	MRM (*m/z*)	CE (eV)	DP (V)	EP (V)	CXP (V)
17α-estradiol	**420.3>166.0**	35	200	10	15
	420.3>151.0	70	200	10	15
	420.3>134.0	95	200	10	15
17β-estradiol	**420.3>166.0**	35	200	10	15
	420.3>151.0	70	200	10	15
	420.3>134.0	95	200	10	15
17β-estradiol-d3	423.3>166.0	38	150	10	15
	423.3>151.0	71	150	10	15

[a] *Transitions in bold were used for quantification.*

The linearity of the LC-MS/MS method was determined by a four order concentration range of the standards in mobile phase corresponding to the following 17α/β-estradiol concentrations in a sample: 0, 5 10^{-3}, 0.01, 0.02, 0.05, 0.10; 0.20; 0.50 and 1.0 µg L^{-1}. The calibration curves were established using blank samples fortified from 0.05 to 0.5 µg L^{-1}. The regression parameters of standard and calibration curves were satisfactory and the correlation coefficients were greater than 0.98.

The specificity of the method was demonstrated by analysis of blank serum samples. No interferences in the range of the retention times of 17α-estradiol and 17β-estradiol were found. The validation results of determination of 17α-estradiol and 17β-estradiol at 0.1 µg L^{-1} are presented in Table 2.

The recovery of 17α-estradiol and 17β-estradiol from *bovine* serum ranged from 85.1% to 108.2%. Acceptable and satisfactory values for repeatability and within-lab reproducibility were obtained. The calculated values of decision limits (CCα) and detection capabilities (CCβ) were a few fold lower than the recommended concentration of 0.1 µg L^{-1}. An example of an LC-MS/MS chromatogram of a serum sample fortified with 17α-estradiol and 17β-estradiol at 0.05 µg L^{-1} is shown in Figure 1c.

Table 2. Method performance at 0.1 µg L^{-1} concentration level in serum

Compound	Recovery (%)	Repeatability (RSD, %)	Within-lab reproducibility (RSD, %)	Measurement uncertainty at 0.1 µg L^{-1} (U)	CCα (µg L^{-1})	CCβ (µg L^{-1})
17α-estradiol	85.5	1.4	1.8	37.5	0.02	0.03
17β-estradiol	99.6	2.9	3.2	30.9	0.04	0.06

Conclusions

The application of 4-(dimethylamino)benzoyl chloride reagent for the derivatisation carried out under optimal conditions greatly improved the ionization of 17α/β-estradiol. It has been found that the intensity of the estradiol signal increased about one hundred times as compared to the chromatographic signal of that compound without a derivatisation (Figure 1.). The LC-MS/MS method was validated in accordance with the requirements of the Commission Decision 2002/657/EC and revealed decision limits of 0.02 μg L^{-}1 for 17α-estradiol and 0.05 μg L^{-1} for 17β-estradiol, which are well below the current maximum permitted concentration.

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DETERMINATION OF ANTIBIOTIC RESIDUES IN HONEY

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Abstract
Within the framework of the Dutch National Residue Control Plan, a large variety of antibiotics has to be monitored in honey. Conventionally, in tissues, most antibiotics are screened using microbial bioassays. However, these assays are not applicable to honey. To combine screening, quantification and confirmation, a multi-method was developed for the determination of 50 antimicrobials, including chloramphenicol, dapson, trimethoprim, aminoglycosides, tetracyclines, macrolides, quinolones and sulphonamides using UHPLC-MS/MS.
After hydrolysis using trifluoroacetic acid (to hydrolyse the sulphonamides bound to sugars), an extraction with McIlvain buffer was carried out. After centrifugation, each sample extract was separated into two fractions. The first fraction was analysed only for aminoglycosides, while the second fraction was used for the analysis of chloramphenicol, dapson, trimethoprim, tetracyclines, macrolides, quinolones and sulphonamides. The fraction for aminoglycosides was applied to an Oasis MCX cartridge, and the aminoglycosides were eluted with an acetonitrile/ammonium formate (1 M)/formic acid (2 M) solution. Subsequently, this eluate was diluted with acetonitrile and measured with UHPLC-MS/MS in HILIC mode. For the second fraction, a SPE clean-up (Strata-X RP) was applied. The compounds of interest were eluted from the Strata-X cartridge using methanol. Subsequently, the organic solvent was evaporated. For the evaporation a "keeper" solvent dimethyl sulfoxide was applied. The analysis of antimicrobials in the second fraction was done with UHPLC-MS/MS in reversed-phase mode. The method has been developed and validated in collaboration with the Netherlands Food and Consumer Product Safety Authority using a criteria-based validation approach according to Commission Decision 657/2002/EC.

Introduction
Within the framework of the Dutch National Residue Control Plan, a large variety of antibiotics has to be monitored in honey. In the beekeeping sector antibiotics can be used for preventive or therapeutic reasons, for instance to fight foulbrood.

Method development was based on a fully validated standard operation procedure optimized for the analysis of antibiotics in faeces. This procedure has been customized for the matrix honey. One of the challenges is the sugar bound sulphonamides, for which reason a hydrolysis step using trifluoroacetic acid was introduced to the method. Because of the difference in polarity of the antibiotics it is necessary to split the extract during the sample preparation *e.g.* one aliquot to be used for aminoglycosides and another aliquot to be used for the other compounds. This resulted in two different clean-up procedures per sample extract and thus 2 measurements for each sample. The method was validated, using a criteria based validation approach according to decision 657/2002/EC.

Materials and Methods

Samples
Blank samples were tested and provided by the Netherlands Food and Consumer Product Safety Authority.

Reagents and solutions
HPLC grade methanol and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands), 25% ammonia, ammonium formate, formic acid, disodium hydrogenphosphate dihydrate, Na_2EDTA, potassium dihydrogenphosphate, sodium hydroxide from Merck (Darmstadt, Germany), trifluoroacetic acid from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was prepared using a Milli-Q system (18 MQ cm^{-1})(Millipore, Billerica, MA, USA). EDTA-McIlvain buffer was prepared by dissolving 74.4 g Na_2EDTA in 500 mL citric acid solution (0.1 M, 21.0 g citric acid monohydrate), 280 mL phosphate buffer (0.2 M, 35.6 g $Na_2HPO_4*2H_2O$) and water was added till a volume of 2.0 litre. The pH was measured and if necessary corrected using citric acid solution or phosphate buffer until pH 4.0 was obtained. Water was added to adjust the volume to 2.0 L.

Reference standards
The following reference standards were obtained from Sigma-Aldrich:
Chloramphenicol, oxytetracycline, tetracycline, chlortetracycline, demeclocycline doxycycline, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamoxole, sulfadimidine, sulfamethizole, dapson, sulfamethoxypyridazine, sulfamonomethoxine, sulfachloropyridazine, sulfadoxine, sulfamethoxazole, sulfisoxazole, sulfadimethoxine, sulfaquinoxaline, marbofloxacin, norfloxacin, ciprofloxacin, enrofloxacin, danofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid, flumequine, dihydrostreptomycin, streptomycin, tilmicosin, tiamulin, erythromycin, tylosin, valnemulin, josamycin, lincomycin and spiramycin.

The following reference standards were obtained from Witega:
Chloramphenicol-d_5, ciprofloxacin-d_8, difloxacin-d_3, enrofloxacin-d_5, flumequine-$^{13}C_3$, nalidixic acid-d_5, norfloxacin-d_5, oxolinic acid-d_5, sarafloxacin-d_8, spiramycin-d_3, sulfadimethoxine-d_6, sulfadimidine-$^{13}C_6$, sulfadoxine-d_3, sulfamerazine-$^{13}C_6$, sulfamethizole-$^{13}C_6$, sulfamethoxazole-d_4, sulfamethoxypyridazine-d_3, sulfapyridine-$^{13}C_6$, sulfaquinoxaline-$^{13}C_6$, sulfathiazole-$^{13}C_6$ and sulfisoxazole-$^{13}C_6$.

The following reference standards were obtained from TRC:
Danofloxacin-d_3, dapsone-d_8, erythromycin-^{13}C-d_3, lincomycin-d_3, sulfachloorpyridazine-$^{13}C_6$ and sulfadiazine-d_4.

Methyldihydrostreptomycin was synthesized in house, trimethoprim was obtained from Fluka and four macrolides were obtained elsewhere: tulathromycin from Pfizer (New York, NY, USA), tylvalosin from Eco Animal Health (London, UK), gamithromycin-d_4 from Santa Cruz Biotechnology and pirlimycin from Pharmacia and Upjohn Co. (Bridgewater, NJ, USA).

Stock solution preparation
Stock solutions were prepared by accurately weighing 3.00 to 6.00 mg (± 0.02 mg) of reference standard. After correction for purity and counter-ions present, the reference standards were dissolved in solvent (on a weight basis) to obtain the required concentration. Separate stock solutions in methanol were prepared at a concentration of 100 mg L^{-1} of tetracyclines and at a concentration of 1,000 mg L^{-1} for sulfonamides. Separate stock solutions of 1,000 mg L^{-1} were prepared in acetonitrile for all macrolides and in water for the aminoglycosides. Separate stock solutions of quinolones were prepared by dissolving the analyte in 2 M ammonia using sonification (30 min) to obtain a concentration of 5,000 mg L^{-1} after which the solution was diluted to 100 mg L^{-1} with methanol.

Sample preparation
Two grams (2.00 ± 0.05 g) of honey samples were weighed in a 50-mL polypropylene tube and for the matrix-matched standard samples a blank honey was weighed in quintuple. Trifluoroacetic acid (2 mL, 0.25% v/v) was used to hydrolyse the samples for 30 min at 40°C. After extraction with 10 mL McIlvain buffer every extract was split into two fractions. One fraction was used for the determination of the aminoglycosides (polar compounds) and the other fraction was used for the other compounds.

Clean-up of honey samples for the analysis of aminoglycosides
For the determination of the aminoglycosides the extract was applied to an Oasis 3cc MCX (Waters) SPE cartridge and washed with 6 mL 20% acetonitrile / ammonium acetate (10 mM). The cartridges were dried by using vacuum. The aminoglycosides were eluted using a 20% acetonitrile/ammonium formate (1 M)/formic acid (2 M) solution. A 250 µL aliquot of the eluate was transferred into an LC-MS/MS sample vial and diluted with 250 µL acetonitrile to a 60% organic solution.

Clean-up of honey samples for the analysis of chloramphenicol, dapson, trimethoprim, tetracyclines, macrolides, quinolones and sulphonamides
For the determination of tetracyclines, macrolides, quinolones, sulfonamides, trimethoprim, dapson and chloramphenicol, the extract was applied to a Strata X-RP (Phenomenex). After washing using 5 mL water and drying using vacuum the compounds of interest were eluted with 5 mL methanol in a test tube containing DMSO as a “keeper” solvent. The methanol was evaporated using a gentle nitrogen stream at 40°C. The residues were dissolved in 20% methanol and transferred into an LC-MS/MS sample vial.

UHPLC-MS/MS analysis
Two UHPLC-MS/MS systems were used for the determination of antimicrobials in honey. A combination of a Sciex QTRAP 5500 with a Shimadzu UHPLC system was used consisting of a column oven including a 6-way column switch, 2 binary pumps and an auto sampler (all Nexera X2). The second UHPLC-MS/MS system was a Sciex QTRAP 6500 with a Shimadzu UHPLC system. This LC system consists of two binary pumps, an autosampler (all Nexera X2) and a column oven (CTO-20AC).

The aminoglycosides separation was carried out using a Waters Acquity UPLC BEH HILIC 1.7 µm, 2.1 × 50 mm and a 0.2 M ammoniumformate / acetonitrile gradient containing 0.1 M formic acid. The separation of other antimicrobials was carried out using a Waters Acquity UPLC HSS T3 1.8 µm, 2.1 × 100 mm column, and a water/methanol gradient containing 0.1% formic acid and 2 mM ammonium formate.

The SRM transitions are given in Tables 1 to 5.

Table 1. SRM transitions sulfonamides, dapson and trimethoprim.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
Sulfadiazine	251	156	Sulfamethoxypyridazine	281	156
	251	92		281	108
Sulfadiazine-d4	255	160	Sulfamethoxypyridazine-d_3	284	156
Sulfacetamide	215	156	Trimethoprim	291	123
	215	92		291	230
Sulfapyridine	250	156	Trimethoprim-d_9	300	123
	250	92	Sulfamethoxazole	254	156
Sulfapyridine-$^{13}C_6$	256	162		254	92
Sulfathiazole	256	156	Sulfamethoxazole-d_4	258	160
	256	92	Sulfisoxazole	268	156
Sulfathiazole-$^{13}C_6$	262	161.9		268	113
Sulfamerazine	265	156	Sulfisoxazole-$^{13}C_6$	274	162
	265	172	Sulfamonomethoxine	281	156
Sulfamerazine-$^{13}C_6$	271	98		281	92
Dapson	249	156	Sulfachloropyridazine	285	156
	249	108		285	92
Dapsone-d_8	257	160	Sulfachloorpyridazine-$^{13}C_6$	291	162
Sulfamoxole	268	156	Sulfadoxine	311	156
	268	92		311	108
Sulfamethizole	271	156	Sulfadoxine-d_3	314	108
	271	92	Sulfaquinoxaline	301	156
Sulfamethizole-$^{13}C_6$	277	162		301	92
Sulfadimidine	279	186	Sulfaquinoxaline-$^{13}C_6$	307	162
	279	124	Sulfadimethoxine	311	156
Sulfadimidine-$^{13}C_6$	285	186		311	92
			Sulfadimethoxine-d_6	317	162
Sulfaphenazole	315	156			
	315	108			

Table 2. SRM transitions quinolones.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
Norfloxacin	320	276	Difloxacin	400	299
	320	302		400	356
Norfloxacin-d_5	325	231	Difloxacin-d_3	403	359
Ciprofloxacin	332	288	Nalidixicacid	233	159
	332	231		233	187
Ciprofloxacin-d_8	340	235	Nalidixicacid-d_5	238	188
Danofloxacin	358	255	Oxolinicacid	262	216
	358	82		262	160
Danofloxacin-d_3	361	255	Oxolinicacid-d_5	267	217
Enrofloxacin	360	286	Flumequine	262	202
Enrofloxacin	360	316		262	126
Enrofloxacin-d_8	365	321	Flumequine-$^{13}C_3$	265	205
Sarafloxacin	386	368	Marbofloxacin	363	320
	386	342		363	72
Sarafloxacin-d_8	394	350			

Table 3. SRM transitions macrolides.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
Tildipyrosin	368	638	Tiamulin	494	192
	368	98		494	119
Tildipyrosin-d_{10}	372.85	108	Natamycin	666	503
Tulathromycin	404	158		666	648
	404	577	Erythromycin	734	98
Lincomycin	407	126		734	158
	407	359	Erythromycin-^{13}C-d_3	738	162
Lincomycine-d_3	410	129	Tylosin	916	174
Spiramycin	422	174		916	772
	422	101	Valnemulin	565	263
Spiramycin-d_3	424	174		565	164
Neospiramycin	699	174	Josamycin	828	174
	699	142		828	109
Pirlimycin	411	112	Tylvalosin	1042	814
	411	363		1042	174
Gamithromycin	777	619	Tilmicosin	435	695
	777	158		435	174
Gamitromycin-d_4	781	623			

Table 4. SRM transitions aminoglycosides and chloramphenicol.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
Chloramphenicol	321	152	Dihydrostreptomycin	584	246
	321	194		584	263
Chloramphenicol-d_5	326	157	Methyldihydrostreptomycin	598	246
Streptomycin	582	246		598	263

Table 5. SRM transitions tetracyclines.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
Tetracycline	445	410	Doxycycline	445	428
	445	154		445	154
Oxytetracycline	461	426	Chlortetracycline	479	154
	461	154		479	444
Demeclocycline	465	448			

Method validation

The method was fully validated according to 2002/657/EC. The following parameters were determined: linearity, trueness, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ), selectivity, robustness and stability. The validation was carried out in a laboratory at RIKILT and in a laboratory at NVWA laboratory with two different UHPLC MS/MS systems by three different analysts with 21 different honey samples. Validation levels are given in Table 6.

Table 6. Target values and MRPL.

Compound group	Aim value	MRPL
Chloramphenicol	-	0.3 µg kg^{-1}
Macrolides	20 µg kg^{-1}	-
Quinolones	20 µg kg^{-1}	-
Sulfonamides	50 µg kg^{-1}	Dapson 5 µg kg^{-1}
Tetracyclines	20 µg kg^{-1}	-
Aminoglycosides	40 µg kg^{-1}	-

Results

For all compounds the method is suitable for screening purpose. For quantification matrix-matched standards can be used for 24 of the compounds. For the other 26 compounds multi level standard addition has to be applied for quantification. A summary of the validation results is given in Table 7.

Table 7. Validation results.

Compound group	Qualitative	Number of compounds	Quantifiable number of compounds using MMS*	Quantifiable number of compounds using MLSA**
Chloramphenicol	Yes	1	0	1
Macrolides	Yes	15	5	10
Quinolones	Yes	10	8	2
Sulfonamides	Yes	18	11	7
Tetracyclines	Yes	4	0	4
Aminoglycosides	Yes	2	0	2
Total	Yes	50	24	26

** MMS = Matrix Matched Standard; ** MLSA = Multi Level Standard Addition.*

Conclusion

A method that discriminates 50 microbials in honey has successfully been developed and fully validated according to 2002/657/EC. The method is applicable for the screening of chloramphenicol, aminoglycosides, tetracyclines, macrolides, quinolones and sulphonamides including dapson and trimethoprim using UHPLC-MS/MS. For the quantification of some compounds a multi-level standard addition is necessary, for the other compounds a mixed matrix standard complies.

Acknowledgements

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DETERMINATION OF THE METABOLITES OF NITROFURAN ANTIBIOTICS IN ANIMAL TISSUES AND ASSOCIATED PRODUCTS BY LIQUID CHROMATOGRAPHY-TANDEM QUADRUPOLE MASS SPECTROMETRY

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Abstract
Nitrofurans are a group of broad spectrum antibiotics employed as feed additives for prophylactic and therapeutic treatment of bacterial and protozoan infections. Due to health concerns, nitrofurans were banned from use in food animal production in many countries including the European Union (EU). Countries with products intended for the EU are bound by the same regulations as locally produced food therefore food imported into the EU should be free of nitrofurans. Over the past twelve years there have been frequent findings of residues in poultry and aquaculture products imported to EU countries leading to product recalls, border rejections and de-listed suppliers. These violations have resulted in an increase in pre-harvest (PHT) and pre-export (PET) testing and analysis of imports at border control. The European Commission prescribed analytical performance limits and criteria to be met to report a sample as non-compliant and a Minimum Required Performance Limit (MRPL) of 1 µg kg^{-1} for furazolidone, furaltadone, nitrofurazone and nitrofurantoin, measured as their respective tissue-bound metabolites. Laboratories must demonstrate that their calculated analytical performance limits (Detection Capability CCα and Decision Limit CCβ) are at or below the MRPL. Although enforcement action is only taken where a residue exceeds the MRPL, non-complaint samples below the MRPL must still be monitored. Meeting these requirements requires the continued development of highly sensitive and specific analytical methodology based upon liquid chromatography-tandem quadrupole mass spectrometry (LC- MS/MS). Examples of performance are given for the analysis of nitrofurans in prawn, fish, honey, egg, poultry muscle and *bovine* kidney.

Introduction
Nitrofurans (NFs) are a group of broad spectrum antibiotics. Due to health concerns, nitrofurans are now prohibited for use in food-producing animals in most jurisdictions. They are still authorized for human medicine and for the treatment of non-food animals. They are widely manufactured, sold and hence available for misuse (Vass *et al*. 2008). There have been frequent findings of residues in honey, poultry and aquaculture products imported to EU countries, which has led to product recalls, border rejections and de-listed suppliers. Violations have resulted in implementation of emergency measures requiring mandatory pre-export testing (PET), widespread voluntary pre-harvest tests (PHT) and an increase both in analysis of imports at border control within the EU and in the frequency of European Commission Food and Veterinary Office (FVO) visits (Points *et al.* 2015).

The EU Minimum Required Performance Limit (MRPL) in poultry meat and aquaculture is 1 µg kg^{-1} for each of the four nitrofurans, measured as their respective tissue-bound metabolites (Commission Decision 2003). MRPLs are 'the minimum content of an analyte in a sample, which at least has to be detected and confirmed'. They are also the reference point for action ('Action levels') when evaluating food consignments. Laboratories must demonstrate that their calculated Detection Capability (CCβ) and Decision Limit (CCα) values (Commission Decision 2002) are at or below the MRPL. Suppliers and importers can set even lower limits for PET based upon trading decisions to provide better warranties to their customers and gain commercial advantage.

Previous studies have demonstrated that parent nitrofurans deplete rapidly in animals and that they are extensively metabolized to tissue-bound metabolites (Cooper *et al.* 2005). Methods have been described for various animal tissues *e.g.* kidney for official control and muscle, honey, shrimp, eggs and milk for consumer risk. Parent nitrofurans are only sought in medicated feeds used for animal production and aquaculture. Commonly sought parent nitrofurans and associated metabolites include: furazolidone as 3-amino-2-oxazolidinone (AOZ), nitrofurazone as semicarbazide (SCA), furaltadone as 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) and nitrofurantoin as 1-aminohydantoin (AHD).

Materials and Methods
The method described here is based upon that originally developed as part of the "FoodBRAND" project (Cooper *et al.* 2005) but as applied for routine surveillance at Fera. Although newer technology has enabled improvements in performance, the principles behind procedure have changed little.

Extraction and clean-up

An aliquot (2.0 g) of sample was transferred into a plastic conical centrifuge tube and 0.2 M hydrochloric acid (10 mL) added. The mixture was homogenised using an Ultra-Turrax. Derivatising agent, a solution of 2-nitrobenzaldehyde (10mg mL^{-1}; 240 µL) was added to the sample. An aliquot of internal standard working solution containing stable isotope analogues was then added to all samples (at 1 µg kg^{-1}) apart from double blank. Matrix-extracted calibrants for screening (Screening Target Concentration [STC] of 0.5 µg kg^{-1}) or confirmation (typically multiple levels over the range 0.25 to 5.0 µg kg^{-1}) were prepared by adding known amounts of a working solution of nitrofuran metabolites. The contents of the tubes were mixed by shaking (*ca.* 15 s) and placed in a water bath at 40°C. The tubes were removed from the water bath after at least 14 h and allowed to cool to room temperature. Extracts were neutralised (*ca.* pH 7.5) by adding 0.2 M dipotassium hydrogen orthophosphate solution (10 mL) and 2 M sodium hydroxide solution (800 µL). The contents of the tubes were mixed again by shaking (*ca.* 15 s) and samples centrifuged (4,500 rpm, ≤ 20°C, 15 min). Supernatant was passed through a syringe filter (PES, 32 mm, 5 µm) prior to solid-phase extraction (SPE). Strata SDB-L (200 mg $3mL^{-1}$) SPE cartridges were conditioned with ethyl acetate (3 mL), methanol (3 mL) and water (2 x 2.5 mL). Sample supernatant was loaded onto the SPE cartridge and passed through the sorbent bed under gravity. The SPE cartridge was washed with water (2 x 2.5 mL). Full vacuum was applied for at least 2 min to dry the SPE cartridges after which the cartridges were eluted with chloroform (2 x 2.5 mL). Chloroform was removed by evaporation and extracts reconstituted in water/methanol (60/40 *v/v*; 1ml) and the contents mixed on a vortex mixer (*ca.* 15 s) before centrifugation (11,500 rpm, 2 min). Sample extract supernatant was passed through a syringe filter (PTFE, 13 mm x 0.45 µm) and collected in a vial for subsequent LC-MS/MS analysis.

Chromatography and mass spectrometry

Chromatography was provided by an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm) fitted in an ACQUITY UPLC H Class with FTN autosampler. For separation a gradient using 0.5 mM ammonium formate in water and methanol, at a flow rate of 0.45mL min^{-1} and temperature of 45°C, was deployed; 20% B initial and hold for 0.2 min, linear gradient to 75% B at 7.0 min, ramped to 100% B at 7.25 min, held until 8.25 min, back to 20% B at 8.26 min and re-equilibrate until 11 min. Injection mode was PLNO and injection volume was 5 µL. A Xevo TQ-S micro mass spectrometer was fitted with electrospray ionisation source, operated in positive ion mode. Various parameters were optimised including capillary voltage (0.5 kV), source temperature (150°C), desolvation temperature (650°C), desolvation gas flow (1,000 L h^{-1}, cone gas flow (150 L h^{-1}) and MRM transitions for the nitrofuran metabolites determined as the nitrophenyl (NP-) derivative (Table 1).

Table 1. MRM parameters for nitrofuran metabolites and stable isotope analogues.

Compound	Retention time	MRM	Cone (V)	CE (eV)
NP-AHD	2.82	249.1>134.0	50	10
		249.1>104.0	50	20
NP-AHD-$^{13}C_3$	2.82	252.1>134.0	50	10
NP-AOZ	2.94	236.1>134.0	50	10
		236.1>104.0	50	20
NP-AOZ-d4	2.91	240.1>134.0	50	10
NP-SCA	3.03	209.1>166.1	30	8
		209.1>192.1	30	10
NP-SCA-$^{13}C^{15}N_2$	3.03	212.1>168.1	30	8
	3.90	335.1>291.1	35	10
NP-AMOZ		335.1>100.0	35	30
NP-AMOZ-d5	3.83	340.1>296.1	35	10

Results and Discussion

The UPLC conditions were taken from an earlier Waters application note (Morphet and Hancock, 2007) but the gradient was adjusted to provide sufficient chromatographic resolution of NP-AHD from isobaric interference on both transitions. The selection of MRM transitions and optimisation of critical parameters was performed by infusion of individual solutions of all the analytes and evaluation of the data by Intellistart™ software to automatically create acquisition and processing methods. The optimum dwell time was set automatically using the autodwell function.

The objective of this work was to evaluate the performance of the Xevo TQ-S micro instrument for nitrofurans analysis rather than development and validation of a new method. Analysis was restricted to batches of different sample types, relevance to routine testing. Performance has been evaluated based upon sensitivity for analytes, the absence of isobaric interference, precision of UPLC-MS/MS measurements, compliance with identification and typical quantification criteria and accuracy and

precision through analysis of proficiency test material. Excellent sensitivity and selectivity was demonstrated by the response for each analyte peaks detected from the analysis of matrix-extracted calibrants prepared at the STC (0.5 µg kg^{-1}) in a range of different sample types; prawn, fish, poultry muscle, *bovine* kidney, egg and honey (see Figure 1). No interfering compounds were detected at the retention times of the analytes in all the tested blank samples. Precision, as measured from the peak areas of replicate (n=8) injections of NP-nitrofuran metabolite standards in solvent (1 ng mL^{-1}), varied from 1.7% to 3.5 % RSD. Overall repeatability, as determined from the peak areas of the stable isotope analogues added to each of the replicate (n=20) extractions of incurred honey (1 µg kg^{-1}), varied from 2.8% to 13 % RSD. Linearity in matrix (honey) was evaluated over the concentration range 0.2-0.5 µg kg^{-1}. The coefficients of determination were satisfactory (r^2 > 0.99) and the residuals <15%. Ten portions of a FAPAS honey proficiency test material contaminated with AMOZ only were prepared and analysed in duplicate. The mean measured concentration of NP-AMOZ (1.41 µg kg^{-1}) compared well with the assigned value (1.50 µg kg^{-1}). The precision for the calculated AMOZ concentration in the replicates of the FAPAS test material was 2.9% RSD. Requirements in 2002/65/7/EC for identification were met. The range of calculated AMOZ ion ratios (0.81 to 0.84) was well within tolerance of ±20% (0.66 to 0.99) and the AMOZ retention time from the replicates of incurred sample showed little variation (0.1% RSD; 3.88-3.89 min), well within acceptance tolerance of ±2.5% (3.79-3.98 min).

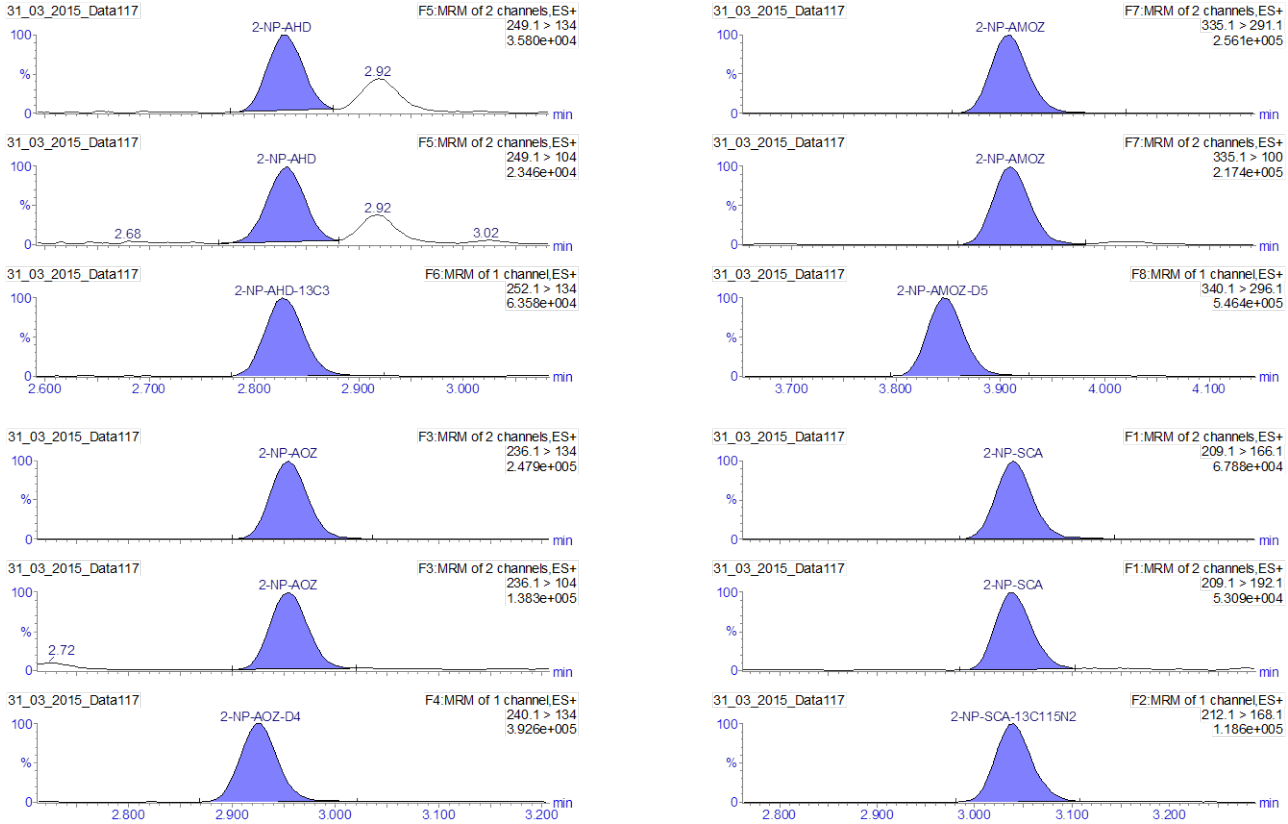

Figure 1. Chromatograms showing nitrofuran metabolites and stable isotope analogues, as NP-derivatives, spiked into prawn matrix at the STC (0.5 µg kg^{-1})

Conclusions

The ACQUITY H-Class with the Xevo TQ-S micro provides sufficient sensitivity for detection, identification and quantification of nitrofuran metabolites in a range of products. The method has the potential for screening and confirmation for official control purposes but also to meet the requirements of pre-export testing, which may demand lower limits of quantification.

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LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF NORTESTOSTERONE GLUCURONIDE IN RAINBOW TROUT MUSCLE

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Abstract

In animal agriculture, steroid hormones are used in food-producing animals to increase growth rate and improve feed efficiency. The FDA approves these drugs only after information and/or studies have shown that the drugs are effective and safe and that food from the treated animals is safe for people to eat. Illegal use of steroid hormones is of concern because it may lead to harmful residues in edible tissues destined for human consumption. Recently, we developed an analytical method to detect and quantitate nortestosterone glucuronide (NT-GLU) in rainbow trout muscle. The method involves extraction of NT-GLU from fish muscle tissue using methanol. The extraction was repeated. Methanol extracts were combined and evaporated to about 1 mL, and the final volume was adjusted to 2 mL using 20/80 acetonitrile/0.1% formic acid. The extracts were centrifuged, filtered through PTFE filters, and analysed on a liquid chromatography-tandem mass spectrometry system equipped with a TurboIonSpray probe in the negative ion mode. Method validation was performed at levels from 1 to 4 ng g^{-1}, with testosterone glucuronide (TEST-GLU) used as an internal standard. The method accuracy is >60% and the coefficient of variation is <20%.

Introduction

The anabolic steroid 19-nortestosterone (NT, Figure 1) is used as a doping agent to enhance performance in sports (Piper *et al.,* 2016). In food-producing animals, NT has been used to improve meat quality and feed efficiency (Poelmans *et al.,* 2005). Due to concerns over the use of NT as a growth promoter, many countries, including the European Union, have prohibited its use in food-producing animals. Illegal use of NT is of regulatory concern because it may lead to harmful residues in edible tissues destined for human consumption. Therefore, analytical methods are needed to monitor NT residues in fish.

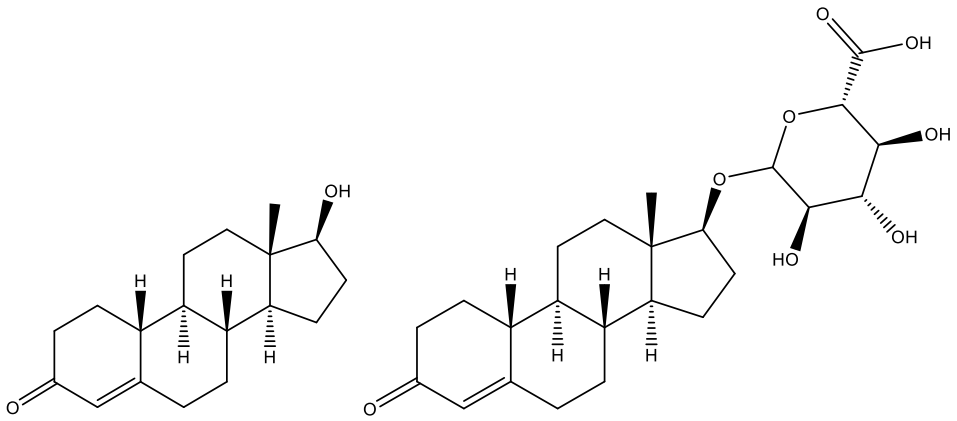

Figure 1. Structures of NT and NT-GLU.

After administration to animals, drugs undergo phase I and phase II metabolism to form more polar and water soluble compounds, which are more readily excreted from the animal's body. In phase I metabolism, drugs are oxidized, reduced, or hydrolyzed; in phase II metabolism, the parent drug and its metabolites are conjugated to amino acids, sulfates, and glucuronic acids to form conjugated metabolites (Parkinson *et al.*, 2012). Glucuronidation and sulfation are two of the most common phase II metabolic pathways. Traditionally, analysis of steroid hormones involves an enzymatic hydrolysis to cleave the aglycones from the glucuronides. The released aglycones are either converted to more volatile derivatives for GC/MS analysis, or are directly analysed by LC-MS (Kuuranne *et al.,* 2000; Gomes *et al.,* 2009). With the advancement of LC-MS/MS, direct analysis of intact steroid hormone glucuronides becomes feasible (Kuuranne *et al.,* 2003). This approach provides a faster and more reliable analysis than the traditional hydrolysis methods.

In the literature, direct analysis methods have been reported for several androgenic anabolic steroid (AAS) glucuronides. Most of these methods were developed for AAS in human urine (Tseng *et al.,* 2006; Strahm *et al.,* 2008); few were developed for aquatic species. Recently, Amarasinghe *et al.* (2012) synthesised a glucuronide of methyltestosterone (MT) and were able to detect its presence in tilapia bile. Subsequently, Nishshanka *et al.* (2015) reported the detection of a glucuronide of a hydroxylated MT in bile. Expanding upon these studies, we describe herein a sensitive LC-MS/MS method capable of determining the intact NT-GLU (Figure 1) in rainbow trout muscle at 2 ng g^{-1}.

Materials and Methods

LC grade water used in preparing solutions was purified in-house with a Milli-Q Plus water system. Methanol (MeOH) and acetonitrile (ACN) were of HPLC grade (Burdick & Jackson, Muskegon, MI, USA). Formic acid (FA) was of reagent grade (Sigma Aldrich, Milwaukee, WI, USA). NT, NT-GLU, and TEST-GLU were obtained from Steraloids (Newport, RI, USA).

Stock Solutions

A primary stock solution (~150 ng μL^{-1}) was prepared by weighing 3 ± 2 mg of NT-GLU into a 20 mL glass volumetric flask. Methanol was added to dissolve the solid. A secondary stock solution of 2 ng μL^{-1} was prepared by dilution of the primary stock solution with MeOH. Working NT-GLU standard solutions at 0.2, 0.08, 0.04, 0.02, 0.01, and 0.005 ng μL^{-1} were prepared by diluting the secondary stock solution (2 ng μL^{-1}) or its diluted solutions with MeOH. Internal standard (TEST-GLU) solutions were prepared in the same manner. These solutions were stored at -10°C or below.

Calibration Standards

A five-point calibration curve of NT-GLU was prepared at the following concentrations: 0.5, 1.0, 2.0, 4.0, and 8.0 ng g^{-1} equivalents (ppb) by adding 100 µL of the appropriate working NT-GLU standard solutions (0.005, 0.01, 0.02, 0.04, and 0.08 ng μL^{-1}) to 50 µL of the TEST-GLU internal standard solution (0.04 ng μL^{-1}) and 850 µL of ACN/0.1% FA (20/80, v/v). Accordingly, each calibration standard contained a fixed level (2 ng g^{-1}) of TEST-GLU. When these standards were prepared, the ng g^{-1} is with reference to a 2 g sample and based on a final volume of 2,000 µL for the sample extracts.

Fish Dosing

Fish used were maintained in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care International (AAALAC), and the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC). Rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial supplier and maintained in recirculating aquaculture systems until use. Fish were housed in 1,800 L fiberglass tanks at a target temperature of 12°C and fed a pelleted commercial trout feed. Individual trout were weighed and then moved into a 550 L experimental tank prior to dosing. Fish were dosed with a combination of NT, MT, and megesterol at either 15 mg kg^{-1} body weight of each compound or with MT and megesterol at 5 mg kg^{-1} body weight and NT at 20 mg kg^{-1} body weight. The three compounds were weighed into a single gelatin capsule which was then administered to fish *via* oral gavage using a feeding tube. After a two-day withdrawal period, the fish were removed from experimental tanks and euthanized *via* cranial concussion followed by cervical dislocation and pithing of the brain. Fillets with adhering skin were then harvested and frozen at -80°C until homogenization with dry ice in a blender. After homogenization, samples were temporarily stored in a -10°C freezer for 2 to 4 days to sublime the CO_2. Thereafter, they were stored at -80°C until analysis.

Extraction Procedure

Homogenized muscle tissue (2.0 ± 0.2 g) was weighed into a 50 mL polyethylene centrifuge tube. Fortification of samples was performed at this step. To each sample was added 6 mL of MeOH and 3–4 glass beads followed by a brief vortex-mixing to disperse the tissue. Samples were then placed on a multi-tube vortexer (VWR, Bridgeport, NJ) for 5 min followed by centrifugation at 3,800 rpm (~3,000 g) for 5 min at 4 °C. After centrifugation, the supernatant (MeOH) was decanted into a 15 mL polyethylene centrifuge tube. The MeOH extraction step was repeated once. The MeOH fractions were combined and evaporated to approximately 1 to 1.5 mL in a TurboVap LV evaporator (Zymark Corp., Hopkinton, MA) set at 40°C. Each sample was then adjusted to a final volume of 2 mL by adding mobile phase (20/80 ACN/0.1% FA) to the 2 mL mark on the centrifuge tube. The sample was centrifuged at 3,800 rpm, 4°C for 2 min and filtered through a Gelman PTFE filter (25 mm, 0.45 µm) into an autosampler vial.

Liquid Chromatographic Conditions

A Shimadzu Nexera LC system (Columbia, MD) consisting of three pumps, a refrigerated autosampler, and a column oven was used. The LC column was a Luna C18(2), 3 µm, 100 x 2.0 mm with a guard column of the same packing (Phenomenex, Torrance, CA). The mobile phase components were: 0.1% formic acid (A) and 100% acetonitrile (B). Extracts were analysed using a 12-min gradient at a constant flow of 0.400 mL min^{-1} — 95A/5B v/v for 0.5 min, a 3-min ramp to 60A/40B v/v, a 1.5-min ramp to 20A/80B v/v for 2 min, and a 0.1 min ramp to 95A/5B v/v for 4.9 min. The injection volume was 10 µL with the autosampler temperature set at 10°C and the column compartment at 40°C. The LC column was equilibrated with the first step

of the gradient for at least one h prior to sample injection. During this time, the mass spectrometer temperature, collision gas, and instrument electronics were also equilibrated. Several standards were injected to equilibrate the gradient and to test the instrument response. A typical injection sequence was as follows: a solvent blank, the standards, a solvent blank, negative and fortified controls, incurred residue samples, a solvent blank, and the standards again to bracket the samples.

Mass Spectrometry Conditions

An AB Sciex (Foster City, CA) 6500 QTRAP triple quadrupole mass spectrometer equipped with a TurboIonSpray probe in the negative ion mode was used. The $[NT\text{-}GLU - H]^-$ ion at *m/z* 449 and $[TEST\text{-}GLU - H]^-$ at *m/z* 463 were selected as the precursor ions for collision-induced dissociation (CID). The MS parameters used during method validation are shown in Table 1, and the MS/MS transitions are shown in Table 2. The dwell time for each monitored transition was 150 ms. Quantitation was performed using peak areas of the quantitative ion and Analyst version 1.6.2.

Table 1. Mass spectrometry operating parameters

	MS system parameters
MS/MS instrument	AB Sciex 6500 QTRAP Triple Quad
Source	Negative TurboIonSpray
Source temperature (TEM) [°C]	550
Curtain gas (CUR) [psi]	30
Ion source gas 1 (GS1) [psi]	50
Ion source gas 2 (GS2) [psi]	60
Collision gas (CAD) [psi]	-3.0
Ion spray voltage (IS) [V]	-4,500

Table 2. MRM MS/MS transition parameters

ID	Q1 Mass (*m/z*)	Q3 Mass (*m/z*)	DP (V)	CE (V)	CXP (V)
NT-GLU	449	85	-40	-42	-37
		113*	-40	-32	-9
		271	-40	-64	-17
		273	-40	-40	-17
TEST-GLU	463	85	-40	-60	-7
		113*	-40	-36	-13
		285	-40	-52	-17
		287	-40	-48	-15

**Quantitation ion*

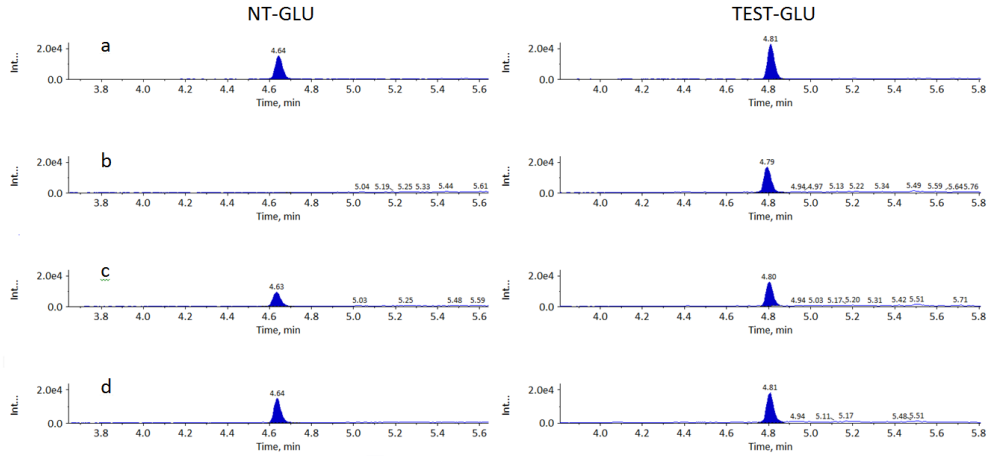

Figure 2. Ion chromatograms of NT-GLU and TEST-GLU extracted from rainbow trout muscle (a) 2 ng g^{-1} standard, (b) control, (c) control fortified at 2 ng g^{-1}, and (d) incurred.

Results and Discussion

The method was validated by fortifying control rainbow trout muscle tissues with NT-GLU at levels from 1 to 4 ng g^{-1}. Typical chromatograms of control, fortified control, and incurred fish for rainbow trout are shown in Figure 2.

A fixed level of TEST-GLU (2 ng g^{-1}) was added to each sample as an internal standard. The results were calculated using both external and internal standard calibration methods. Results calculated with the external standard calibration method are presented in Table 3. The average recoveries of NT-GLU and TEST-GLU were 72.3 and 72.1% from rainbow trout muscle, respectively.

For comparison, results were also calculated using the internal standard calibration method. This quantitation method corrected for procedural recovery loss and compensated for mass spectrometer ionization differences due to matrix effects. As Table 3 shows, the method accuracy for rainbow trout muscle is between 97 and 104% with CVs of less than 10%. The limit of detection (LOD) is estimated as the mean of control samples assay results plus 3 times the standard deviation of the mean, while the limit of quantitation (LOQ) is estimated as the mean of control samples assay results plus 10 times the standard deviation of the mean. The LOD and LOQ were calculated and estimated to be 0.08 and 0.14 ng g^{-1} for NT-GLU.

Table 3. Method validation of NT-GLU for rainbow trout muscle—fortified samples.

	External standard		Internal standard	
Fort. level (ng g^{-1})	Recovery (%)	CV (%)	Accuracy (%)	CV (%)
1.0	75.9	7.9	104	7.4
2.0	70.5	9.1	102	7.5
4.0	70.4	9.5	96.9	6.0

In accordance with the FDA Center for Veterinary Medicine's method development guideline, the method was tested on muscle tissues derived from treated animals. Two levels of incurred residues at the proximity of the target concentration were prepared for rainbow trout muscle and assayed in accordance with the method. Their results are shown in Table 4. NT-GLU was clearly present in the chromatograms of treated fish, demonstrating the effectiveness of the method in recovering NT-GLU from biologically incurred tissues.

Table 4. Method validation of NT-GLU for rainbow trout muscle—incurred residues.

	External standard		Internal standard	
Sample	Level found (ng g^{-1})	CV (%)	Level found (ng g^{-1})	CV (%)
Incurred level 1	0.68	9.3	1.01	4.0
Incurred level 2	1.69	4.6	2.21	6.8

Conclusions

We developed a method suitable for determination of NT-GLU in rainbow trout muscle. The method is simple, involving only extraction, evaporation, dilution, and filtering. Twelve samples and five standards can easily be processed as a batch, and the entire procedure can easily be completed in one day. Using the method, we detected and confirmed the presence of NT-GLU in the muscle of rainbow trout dosed with NT.

Disclaimer

The views expressed in this manuscript are those of the authors and do not necessarily reflect the official policy of the Department of Health and Human Services, the US Food and Drug Administration, or the US Government. The experimental protocol was approved by the IACUC at the Office of Research, Center for Veterinary Medicine, US Food and Drug Administration, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (2011) and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the US Food and Drug Administration.

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A SIMPLIFIED MULTI-CLASS METHOD FOR SIMULTANEOUS DETERMINATION OF GROWTH-PROMOTERS BY LC-MS/MS

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Abstract
Quantitative analysis of multi-class veterinary drugs and growth promoters in animal products are laborious and time-consuming. Extraction of analytes and their metabolites at trace levels in complex food matrices require multiple clean-up steps including solid-phase extraction. In this study, a simple, sensitive and selective multi-class method developed for simultaneous determination of growth-promoters belonging to different classes including stilbenes, steroids and zearanol derivatives. The solvent extraction was followed by dispersive solid-phase extraction. Matrix effects were compensated by matrix-matching. Identification and quantification of the residues were performed by liquid chromatography and tandem mass-spectrometry using electrospray ionization in the positive/negative ion mode. The proposed method was validated according to the Commission Decision 2002/657/EC by the analysis of spiked samples. All of the analytes studied were reliably quantified at their relevant maximum residue limits in *bovine* tissues.

Introduction
Veterinary drugs are widely used in livestock to treat disease, maintain animal health, promote growth, and improve meat quality and yield. The use of these drugs may leave residues in the food. Foods containing the residues may cause adverse health effects in humans. Therefore, government regulatory authorities control the use of veterinary drugs by approving or registering safe uses and monitoring food for unsafe or prohibited residues. In 1981, with Directive 81/602/EEC, the EU prohibited the use of substances having a hormonal action for growth promotion in farm animals. The directives, which came into force afterwards, 96/22/EC and 2003/74/EC, confirmed the prohibition of these substances.

Multi-class multi-residue methods for veterinary drugs are scarce due to a number of analytical challenges including the lack of volatility and chemical stability of most drugs. Wide polarity ranges among the various drugs and some drugs have to be determined at rather low concentration. Because of these difficulties most of the veterinary drug residue methods published focuses on one class of compounds such as β-agonists, quinolones and stilbenes (Lohne 2013). Regarding simultaneous determination of different classes of growth promoters, there are only a limited number of methods published (Yang 2009).

Most of these methods including multi step solid-phase extractions are developed for determination of single class substances and their metabolites. There is a great demand for simple, rapid multi-class multi residue methods. In this study, a multi-class multi-residue LC-MS/MS method was developed for the determination of growth-promoters. The developed method was validated according to the requirements of 2002/657/EC.

Materials and Methods

Reagents and chemicals
HPLC grade acetonitrile (ACN), methanol, glacial acetic acid (Emprove, 100%) and sodium acetate were purchased from Merck (Darmstadt, Germany). *Helix pomatia* β-glucuronidase was from Sigma Aldrich (St. Louis, Missouri, USA). The water used to prepare the solutions was purified in a Milli-Q Plus system (EMD Millipore, Billerica, MA, USA). Magnesium sulphate, sodium chloride, SupelcleanTM primary secondary amine (PSA), anabolic steroids, stilbenes, zeranol and their metabolites, and β-agonists were provided by Sigma Aldrich.

Sample preparation
Tissue samples, upon arrival at our laboratory, were kept at freezer temperature (-25 ± 4°C) until analysis. For preparation, an aliquot of approximately 5 g homogenized tissue sample was weighed in a 50-mL polypropylene centrifuge tube. Tissue sample was mixed with 10 mL 10 mM sodium acetate buffer containing 100 µL β-glucuronidase from *Helix pomatia* and incubated at 37°C for 15 h. The mixture was then cooled to room temperature and 10 mL acetonitrile was added. After vortexing for 3 min, the mixture was centrifuged at 5,000 rpm for 10 min. The upper phase was transferred into a 15-mL tube and was dried under a gentle stream of nitrogen. The residue was reconstituted with 200 µL mobile phase A/ Mobile Phase B (80/20). Then 100 mg PSA was added and the mixture was centrifuged at 5,000 rpm for 10 min. The extract was filtered through a 0.45-µm filter prior to LC-MS/MS analysis.

Chromatographic analysis

The chromatographic analyses were performed using an HPLC system consisting of a binary pump (Shimadzu UFLC LC-20AD model), Shimadzu automatic injector (Auto Sampler SIL-20A HT model) and a column oven (CTO-20AC). Analytical column was an X Terra C18 (150 mm x 2.1 mm id) filled with 5 µm particles (Waters, Milford, MA). Mobile phases were A) 0.1% aqueous formic acid, and B) methanol. The column temperature was maintained at 40°C and the flow rate 0.3 mL min^{-1}. The gradient profile was scheduled as follows: initial proportion (98%A and 2%B) for 0.3 min linear increase to 80% (B) until 7 min, hold 80% (B) for 3 min. The injection volume was 50 µL. The chromatographic system was coupled to electrospray ionization (ESI) source followed by an Applied Biosystems MDS SCIEX 4500 Q TRAP mass-spectrometer. The MS/MS detector conditions were as follows: curtain gas 20 mL min^{-1}, exit potential 10 V, ion source gas 1 and ion source gas 2 were set at 50 mL min^{-1}, ion spray voltage 5,500 V, and turbo spray temperature was set at 550°C. MS data were acquired in the positive ion ESI mode using two alternating MS/MS scan events. Two transitions were monitored for each compound. The selected molecular ion and optimized collision voltages of product ions used for quantification and confirmation were summarized in Table 1. Applied Biosystems Sciex Analyst software version 1.6 was employed for data acquisition and processing.

Results and Discussion

The selected analytes belonging to three different groups were extracted from *bovine* tissues using acetonitrile following enzymatic treatment with β-glucuronidase (*Helix pomatia*) over 15 h. The results of the validation studies (intraday, interday, recovery, CCα and CCβ) obtained were within the acceptable limits.

The validated method was successfully applied to the analysis of more than 200 real samples. Of these 22 were detected positive with 17β-testesteron.

Table 1. MRM transitions, interday, intraday and average recoveries for selected growth-promoters analysed at 0.5 µg kg^{-1}.

analyte	precursor ion (*m/z*)	fragment ions (*m/z*)	intraday precision (RSD%, n=6)	Interday precision (RSD%, n=18)	Average recovery (%, n=18)	CCα (ppb)	CCβ (ppb)
17α-ethinyl estradiol	279.3	133, 158.6	12	16	85	1.1	1.2
17α-estradiol	255	159, 133	10	15	98	1.1	1.0
17β-estradiol	255	159,133	10	15	98	1.1	1.1
diethylstilbestrol	269,5	135,107	16	15	88	1.1	1.2
hexestrol	(-) 269.1	133.5,119	10	17	99	1.0	1.1
progesterone	316	109,97	16	11	101	1.1	1.2
17β-testesterone	290	97,109	9	9	106	1.1	1.2
trenbelone	321,5	303	12	10	110	1.1	1.2
α-zearalanol	323,6	305,287	12	15	86	1.1	1.2
α-zearalenol	(-) 319.3	159.13	11	16	91	1.1	1.0
β-zearalanol	323,6	305,287	10	13	95	1.0	1.0
β-zearalenol	(-) 319.3	159.13	11	14	105	1.1	1.0
zearalenone	321	303,189	16	18	86	1.07	1.00

Conclusions

A simple and reliable multi-class multi-residue LC-MS/MS method was developed for determination of compounds belonging to three different classes including steroids, stilbenes and zearenol and metabolites. The developed method was validated according to 2002/657/EC for simultaneous determination of 13 selected growth promoters in *bovine* tissue.

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DRUGS OF ABUSE AND OTHER INFLUENCES ON STEROID PROFILE USING NEW VALIDATED METHODS

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Abstract

We compared urinary levels of androgen steroids and their ratios such as T/E, A/Etio, A/T, DHT/E and 5α/5β-diols, making part of the steroid profile. The ratios are calculated according the TD and WADA's International standard for laboratories. The comparison is between three groups - control, people using drugs and people with overweight. The testosterone/ epitestosterone ratio was significantly decreased in the narcotics group, the urinary testosterone concentrations were decreased. The group of overweight people shows much higher levels of steroids compared to control group. The abnormal steroid metabolism was investigated. T/E ratios is a parameter used to indicate the suppression of male gonadal function after usage of narcotics. After optimization of sample preparation with new SPE columns, we validated a confirmative mass spectrometric method with GC/C/IRMS. This technique provides the capability to measure the carbon isotopic ratio in order to determine the origin of these steroids. The $^{13}C/^{12}C$ ratio is different whether the steroid is endogenously produced or has its origin from a pharmaceutical preparation. The nature and change in urinary metabolites ratios in humans is of great interest in doping control because it is affected by various drugs, therapeutic medicines and nutritional supplements.

Introduction

Determination of steroid profile parameters is a major interest for doping control specialists for the detection and confirmation of endogenous anabolic steroids. The urinary steroid profile is composed of concentrations and ratios of various endogenous hormones such as testosterone, aldosterone, etiocholanolone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5α-androstane-3α,11β-diol-17-one, DHEA and DHT. Biosynthesis of endogenous steroid is shown in Figure 1.

Sex hormones are often studied as biological basis for gender differences due to their role in the central nervous system regulation, implicating the endocrine system in the pathophysiology of substance use disorders and addictive behaviour. Addictive behaviour is an opioid dependence. The purpose of this study is to examine endocrinal damages at a chronic treatment with opioid agonist (methadone therapy).

The increase in body weight and fat tissue is associated with several abnormalities of steroid balance. Androgens have an important impact on glucose and lipid metabolism and fat homeostasis. It is likely that an androgen imbalance in obesity may play a role in the pathophysiology of the metabolic syndrome and increase the risk for cardiovascular diseases.

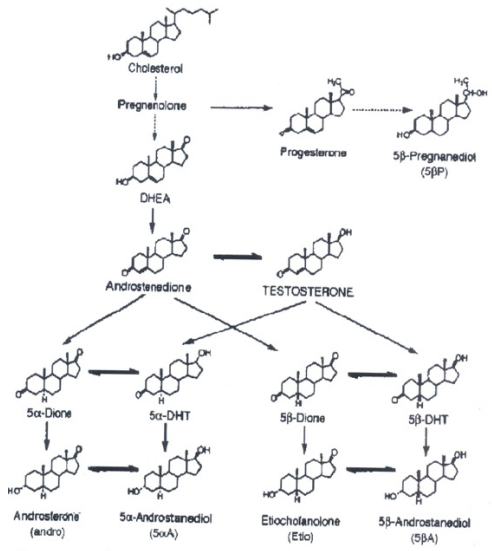

Figure 1. Biosynthesis and conversion of endogenous steroids.

Materials and Methods

All reference standards were obtained from AGNMI (Australia). The GC-IRMS STANDARD $C_{15}/C_{20}/C_{25}$ was obtained from Chiron (Norway). The C_{18} cartridge (200 mg 3 mL^{-1}; 500 mg 10mL^{-1}) were from Thermo Scientific (Bremen, Germany), Silicycle (Canada). All organic solvents were of HPLC grade.

All samples were measured on an Agilent 7890A gas chromatograph and G3170A mass spectrometer. The GC system was equipped with a DB-1MS UI, 20m x 0.180mm x 0.18 µm film thickness column. The injection volume was 2 µL; the injections were performed split 1:5 at 280°C. A constant flow of 1.0mL min^{-1} of helium carrier gas was used. The initial oven temperature was 190°C, followed by a ramp at 2°C min^{-1} to 218°C held for 3 min, followed by a ramp at 2°C min^{-1} to 230°C and 20°C min^{-1} to 300°C and held for 6 min. After hydrolysis with β-glucuronidase (*E. coli*) and liquid-liquid extraction with TBME at pH 9-10 trimethylsilyl derivatives of the steroids were analysed by GC/MS. Table 1 shows the screening method validation data.

Table 1. Screening method: validation data – correlation coefficient, extract recovery, range.

Compound	Correlation coefficient (r^2)	Extraction recovery [%]	Range [ng mL^{-1}]
Androsterone	0.996	91	240-4,445
Etiocholanolone	0.999	92	250-4,664
d_4-Androsterone	1.000	91	50-810
Epitestosterone	0.996	97	2.4-289
Testosterone	0.999	92	2.5-312
5α-Androsterone-3α,17β-diol	0.999	84	5-368
Androstenedione	0.995	91	5-375
5β-Androstane-3α,17β-diol	1.000	81	5-384
Dehydroepiandrosterone	0.997	97	2.4-374
5α-Dihydrotestosterone	0.998	97	2.4-310
5β-Androstane-3α,11β-diol-17-one	0.999	93	100-1,900
5α-Androstane-3α,11β-diol-17-one	0.999	85	100-1,907

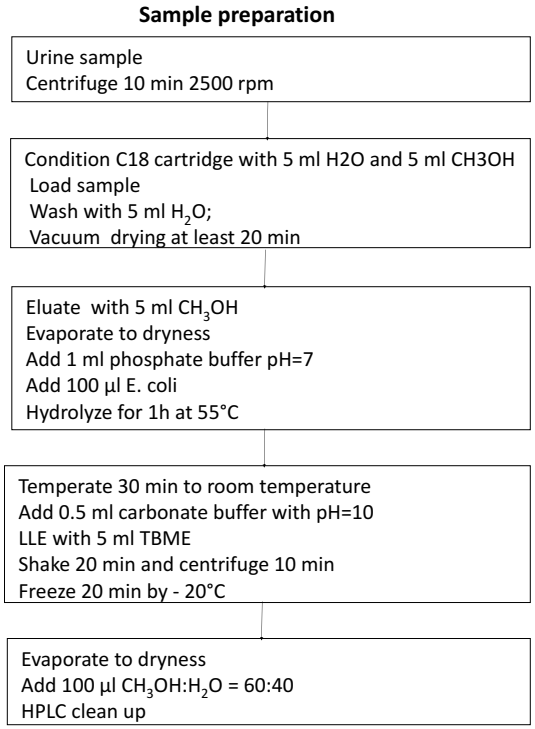

Figure 2. Sample preparation for confirmatory analysis.

The method developed for confirmatory analysis consists of: solid phase extraction (SPE), liquid-liquid extraction (LLE) and preparative – HPLC purification. A 10-25-mL urine sample, depending on the previously measured steroid profile, was prepared by a standard operating procedure for steroids (see Figure 2).

The clean-up was performed on a Dionex Softron GmbH and analytical column BDS Hypersil C18 (250x 4.6 mm, 5 µm) equipped with guard column Hypersil gold (3 µm; 10 x 4 mm). The injection volume was 100 µL, the flow rate 1 mL min^{-1} and the DAD wavelength was set at 196 nm.

For HPLC clean-up, a linear gradient was used increasing from 30:70 acetonitrile/water to 100 % acetonitrile in 25 min; after 3 min at 100% acetonitrile, the column was re-equilibrated for 6 min. Four fractions were collected, two of them (F1 and F4) were dried, reconstituted in acetone and injected in GC/C/IRMS. The other fractions were dried and acetylated by addition of 50 µL acetic anhydride and 50 µL pyridine at 80°C for 2 h. After evaporation of the solvents, the residue was reconstituted in methanol: water 60:40 and submitted to a second HPLC clean-up.

For the second HPLC clean-up, a linear gradient was used increasing from 70:30 acetonitrile/water to 100 % acetonitrile in 33 min; after 3 min at 100% acetonitrile, the column was re-equilibrated for 6 min.

All samples were measured on an Agilent 7890A gas chromatograph coupled through GC Isolink and CONFLO IV to a DELTA PLUS isotope ratio mass spectrometer (Thermo Scientific). The GC system was equipped with a DB-35MS UI, 20 m x 0.180 mm x 0.18 µm film thickness column. Injections of 2 µL were performed split-less at 300°C. A constant flow of 1.2 mL min^{-1} of helium carrier gas was used. The initial oven temperature of 120°C was held for 1.5 min, increased at 40°C min^{-1} to 240°C, followed by a ramp at 2°C min^{-1} to 256°C held for 1 min., followed by a ramp at 2°C min^{-1} to 280°C and 20°C min^{-1} to 300°C and held for 4 min. The oxidation reactor was operated at 940°C and oxidized for 60 min after each sequence of 60-70 injections. The water removal was done by a Nafion membrane. The $\delta^{13}C$ value of the CO_2 reference gas was calibrated towards the GC-IRMS STANDARD $C_{15}/C_{20}/C_{25}$.

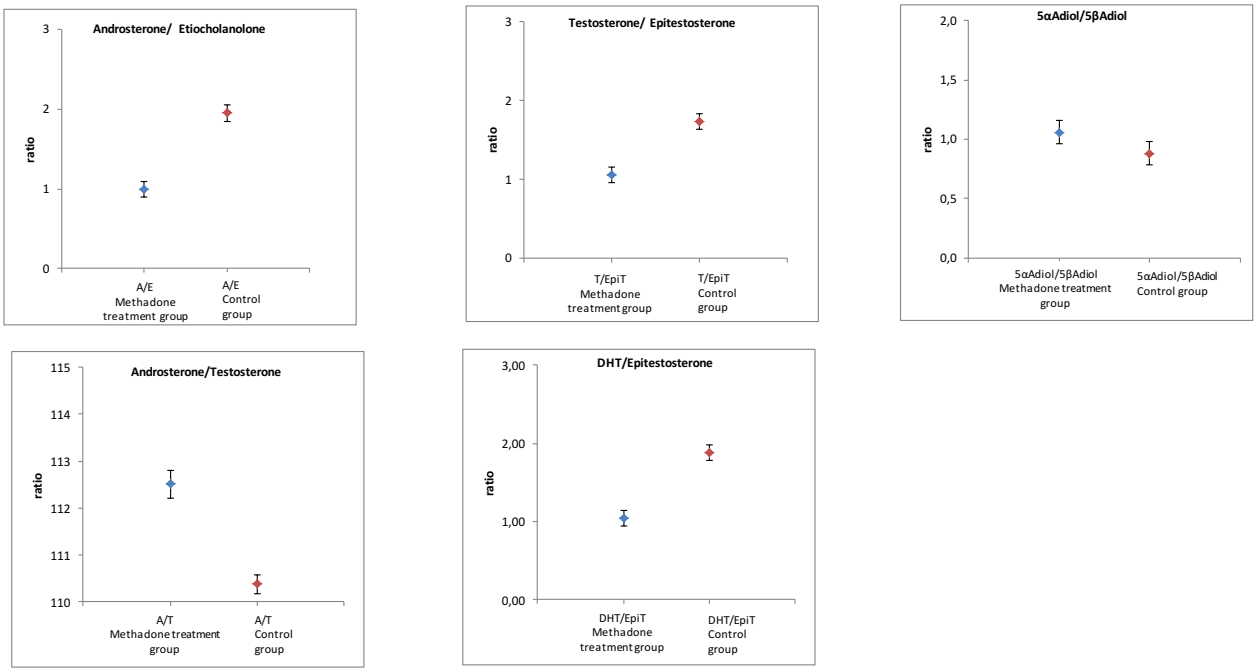

Figure 3. Comparison between control group and methadone treatment group.

Results and Discussion

Using the two validated methods, we analysed three groups: 1) control group, 2) overweight group and 3) methadone group. The ratio between different steroids was compared. Results are presented in Figures 3 and 4.

The period of methadone treatment is from 6 months to 60 months. The concentration of methadone is between 0.94 ng mL^{-1} and 15.35 ng mL^{-1} and depends on the course of treatment. Methadone treatment group had suppressed levels of steroids (A = 2.80 ng $mL^{-1;}$, E = 2.0 ng mL^{-1}; T = 0.03 ng mL^{-1}; 5α-diol = 0.07 ng mL^{-1}; 5β-diol = 0.11 ng mL^{-1}) compared to controls (A = 3.60 ng mL^{-1}; E = 2.03 ng mL^{-1}, T = 0.05 ng mL^{-1}; 5α-diol = 0.08 ng mL^{-1}; 5β-diol = 0.13 ng mL^{-1}).

The levels of steroids in overweight group were A = 6.34 ng mL^{-1}, E = 3.81 ng mL^{-1}, T = 0.07 ng mL^{-1}, 5α-diol = 0.25 ng mL^{-1}, 5β-diol = 0.24 ng mL compared to controls A = 3.60 ng mL, E = 2.03 ng mL, T = 0.05 ng mL^{-1}, 5α-diol = 0.08 ng mL^{-1}, 5β-diol = 0.13 ng mL^{-1}.

The control group had universal steroid profile genotypes namely ins/ins or del/ins. For the other two groups, overweight and methadone show bias, an increase in the ratio of 5α-diol/5β-diol is observed.

Comparing the control group and the methadone group revealed that at the long term methadone users had lower levels of testosterone. Samples were tested by isotope mass spectrometer for determining the origin of steroids and showed that they have an endogenous character.

The overweight group showed higher hormone levels. However, the resulting steroid ratios did not show such large deviations from the control group. Samples were placed on the isotopic mass spectrometer and showed that steroids were of an endogenous nature.

In both groups it was observed that when comparing the ratios of 5α-diol/5β-diol, the ratio of control group was lower than those of the other groups. It can be concluded that this ratio is not affected by the drug's concentration or by abnormalities in the metabolic pathway.

Due to the observed lower levels of testosterone, the ratio of androsterone/testosterone in the methadone group was higher than in the overweight group.

The $\delta^{13}C$ values of the androgenic endogenous steroids androsterone, etiocholanolone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5α-androstane-3α,11β-diol-17-one were measured (Table 2).

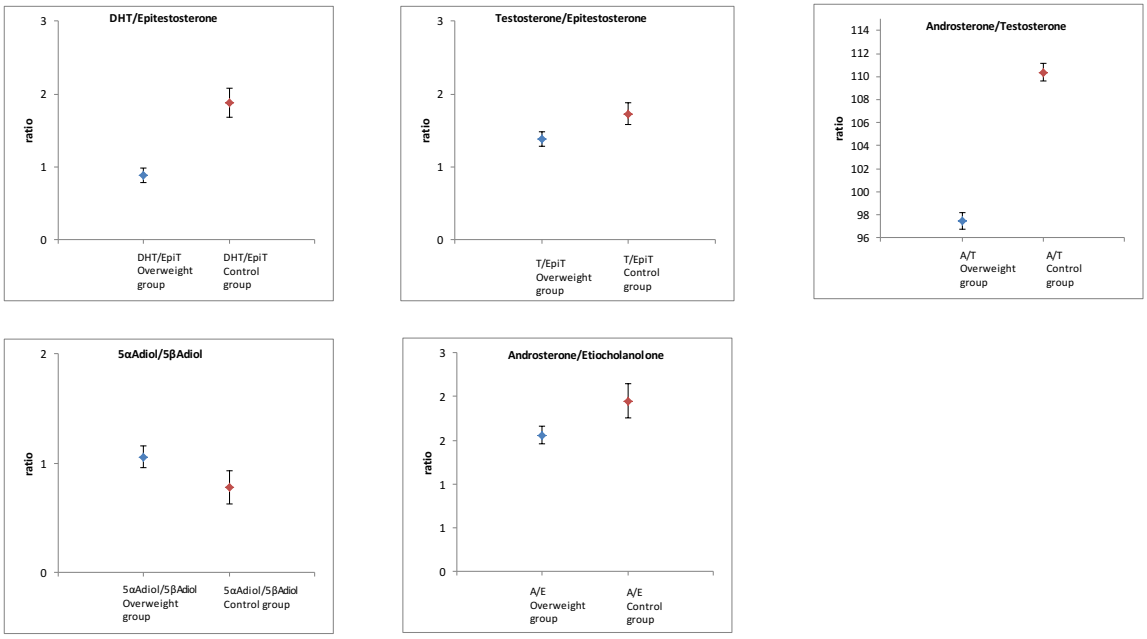

Figure 4. Comparison between control group and overweight group.

Table 2. Confirmation method: $\delta^{13}C$ values

Compound	Reference standards [‰]	Overweight group [‰]	Methadone group [‰]	Control group, [‰]
Androsterone	-32,1	-21,83	-19,78	-18,36
Etiocholanolone	-24,5	-22,85	-22,31	-20,10
5α-Androsterone-3α,17β-diol	-34,7	-24,37	-22,79	-20,47
5β-Androstane-3α,17β-diol	-30,4	-23,72	-22,70	-18,61
5α-Androstane-3α,11β-diol-17-one	-23.6	-22,22	-20,12	-19,57

Conclusions

It can be concluded that chronic use of methadone leads to a direct toxic effect on the sex hormone testosterone. Testing of samples in isotopic mass spectrometry showed that its decrease in concentration is not due to additional intake of prohibited substances. The results confirmed the suppressive effect of methadone on testosterone in individuals subjected to methadone treatment. The average testosterone level in men receiving methadone treatment was significantly lower than controls.

When comparing the two groups, the control and overweight once, the conclusion can be made that slow metabolism leads to increased amounts of endogenous steroids. Testing with the isotope mass spectrometer has confirmed that the higher levels are due to abnormalities in the metabolic pathway.

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(078)

FAST QUANTITATIVE MULTI METHOD FOR ANALYSIS OF PROHIBITED SUBSTANCES USING LC-MS/MS

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Abstract
A new multi-component method based on LC/MSMS is developed and presented. The fast, easy and cheap method is to screen and to confirm various categories of prohibited substances with different analytical characteristics. It is based on a combination of liquid chromatography with sensitive triple-quadrupole or OrbiTrap MS detectors. Using direct injection of urine samples, more than 30 compounds excreted unchanged could be determined quantitatively without a pre-concentration step at concentration levels below the required limits. These compounds included diuretics, stimulants and narcotics. For analysis of glucuronide-conjugated substances, such as the morphine group, samples were prepared using a new type of SPE cartridges from SiliCycle. The method was fully validated according to ISO guidelines [2] and can increase the efficiency of laboratory work.

Introduction
Stimulants include psychomotor, sympathomimetic amines and central nervous system stimulants. For anti-doping purposes, fast analysis can be important during major sports events, where 24-h reporting times are mandatory. From a toxicological point of view, fast analysis facilitates prompt diagnosis which is required in some emergency cases.

Diuretics are drugs that increase the rate of urine flow and sodium excretion to adjust the volume and composition of body fluids. There are several major categories of this drug class and the compounds vary greatly in structure, physicochemical properties, effects on urinary composition and renal hemodynamics, and site and mechanism of action. Because of their abuse by athletes, diuretics have been included on The World Ant-Doping Agency's list of prohibited substances [1]. The use of diuretics is banned both in competition and out of competition.

Liquid-chromatography tandem-mass spectrometry (LC–MS/MS) has become a powerful tool for the quantitative analysis of drugs that does not require derivatisation. DS-LC-MS for the analysis of urine samples has become a trend in the past 10 years in both analytical toxicology and doping-control analysis. In particular, the economic benefits (easy sample preparation and omission of time-consuming extractions) are the driving forces behind this trend.

We developed a sensitive and specific method for the confirmation of more than 25 stimulants and 10 diuretics in urine by using LC/MSMS. The method is fast, easy and cheap.

Materials and Methods

Reagents and chemicals
Stimulants and narcotics were purchased from Cerilliant (USA) as solutions in methanol or acetonitrile, and diuretics from TRC (Canada). Standard stock solutions of each compound were prepared at 1 mg mL^{-1} in methanol. HPLC-grade methanol, *i*-propanol, acetic acid, hexane, ethyl acetate were acquired from Merck (Germany).

Instrumentation
The experiments were performed using a Accela LC system (Thermo Fisher Scientific), interfaced with a TSQ Vantage (Thermo Fisher Scientific) with electrospray ionisation (ESI). The mass spectrometer was operated in positive and negative ion mode. LC separation was performed on Zorbax RRHD Extend-C18 2.1 x 100 mm, 1.8 µm by gradient elution at a constant flow rate of 300 µL min^{-1}. The LC eluents were solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). The mobile phase gradient was programmed as follows: 0 min 95% A, 0-1 min 95% A, 1-7.5 min 95-5% A, 7.5-10.5 min 5% A, 10.5-10.51 min 5-95%. Re-equilibration time was 4.5 min. The total chromatographic run time was 15 min.

Sample preparation
A volume of 200 µL urine and 800 µL mobile phase were mixed with 10 µL 10 µg mL^{-1} internal standard solution (d_5-amphetamine) and directly injected for LC/MSMS analysis.

In case of glucuronide-conjugated substances, SPE pre-treatment was used. For this purpose, an aliquot of 2 mL urine was diluted with 1 mL phosphate buffer pH 7. All calibrators, controls and samples were incubated with 20 µL *E. coli* enzymes at 60°C for 1h. Samples were allowed to cool to room temperature and 10 µL internal standard solutions containing 10 µg mL^{-1}

d_3-amphetamine, 10 µg mL^{-1} d_3 6-acethylmorphine and 10 µg mL^{-1} d_3-benzoylecgonine, was added. Several solid-phase extraction (SPE) sorbents were evaluated for the extraction of glucuronide conjugated substances and its metabolites from urine. The SPE sorbents used for this study were C18, Servo, Oasis SCX, DrugClean (SiliaPrep) and CleanDrud by SiliCycle. Since several different SPE materials were used in this study, Clean Drug by SiliCycle procedure was used to maximize extraction efficiency and minimize carry-over.

An optimal purification was carried out for the urine samples using Clean Drug columns (3 mL; 200 mg, SiliCycle). Each column was conditioned, sample was loaded and the column was washed with 3 mL methanol and 1 mL 0.1 M acetic acid. Washed columns were dried under maximum vacuum for 10 min. Elution was carried out with 3 mL hexane/ethyl acetate (50:50, v/v) followed by 3 mL methylene chloride: *i*-propanol: ammonium 78:20:2. After solvent evaporation at 40°C under a gentle nitrogen flow, the residue was dissolved in 1 mL mobile phase. Ten microliters of the final solution were then injected into the LC–MS system.

Table 1. Summary of validation requirements according to WADA and EC Decision 2002/657.

Characteristic	Wada requirement		EU 2002/657/EC			
	Non-threshold Screening (S)	Threshold substance Confirmation (C)	Qualitative S	Qualitative C	Quantification S	Quantification C
Decision limit (CCα)						
Detection capability (CCβ)	+	+	+	+	+	+
Limit of quantification (LOQ)	-	+				
Precision	-	+	-	-	+	+
Accuracy	-	+	-	-	-	+
Linearity	-	+	-	-	+	+
Specificity	+	+	+	+	+	+
Robustness						

Table 2. Optimized parameters for multiple MRM transitions for various prohibited substances [3].

Compound	Polarity	Parent ion	Product ion	CE	RT min	S-lens
Amphetamine	ESI+	136	119	5	3.16	39
			91	16		
Methylamphetamine	ESI+	150	91	19	3.25	48
			119	8		
Ethylamphetamine	ESI+	164	65	42	3.44	52
			119	11		
MDA	ESI+	180	133	17	3.20	43
			163	7		
MDMA	ESI+	194	163	12	3.26	55
			135	20		
Codeine	ESI+	300	165	41	2.90	93
			215	25		
Oxycodone	ESI+	316	298	19	2.98	75
			241	28		
Morphine	ESI+	286	165	38	1.53	93
			152	58		
Hydromorphone	ESI+	286	185	29	2.26	70
			157	40		
6-Acetylmorphine	ESI+	328	165	37	3.89	98
			211	25		
Benzoilecgonine	ESI+	290	168	18	4.80	82
			105	30		
Oxymorphone	ESI+	302	284	18	1.82	93
			227	28		
Hydromorphone	ESI+	272	152	55	1.33	93

			165	38		
Heroine	ESI+	370	165	48	4.76	109
			268	27		
Fentanyl	ESI+	337	188	24	5.60	89
			105	42		
Cocaine	ESI+	304	182	18	3.77	81
			82	31		
Mepethydine	ESI+	248	220	20	3.95	94
			174	19		
Methadone	ESI+	310	265	14	4.88	74
			105	29		
Ephedrine	ESI+	166	148	11	6.10	50
			115	27		
Pseudoephedrine	ESI+	166	148	11	6.45	50
			115	27		
Cathine	ESI+	134	115	21	4.86	64
			117	27		
Norephedrine	ESI+	134	115	21	5.36	64
			117	27		
Methylephedrine	ESI+	180	91	34	6.50	66
			162	13		
Methcathinone	ESI+	164	131	28	3.40	76
			130	40		
THC	ESI+	315	193	22	7.2	79
			123	32		
THC-COOH	ESI+	345	327	15	7.21	104
			299	18		
JWH-018	ESI+	342	155	24	7.85	110
			127	44		
JWH-250	ESI+	336	121	24	7.40	97
			91	39		
Acetazolamide	ESI+	223	181	14	2.80	60
			164	22		
Piretanide	ESI+	363	236	30	5.48	83
			282	20		
Tolvaptane	ESI+	451	252	17	6.03	97
			119	36		
Metazolamide	ESI+	237	195	14	3.59	62
Benzylhydrochlorothiazide	ESI-	386	294	24	4.74	87
			296	24		
Diclofenamide	ESI-	303	239	19	3.73	73
			267	17		
Polythiazide	ESI-	438	324	22	4.98	95
			398	17		
Furosemide	ESI-	329	205	23	4.90	77
			285	16		
Indapamide	ESI-	364	189	27	5.01	117
			191	27		
Xipamide	ESI-	353	274	27	5.43	113
			127	33		

Results and Discussion

The performance characteristics for the validation of analytical methods according International standard of WADA [1] and Decision 2002/657/ EC are summarised in Table 1 [2].

Screening method

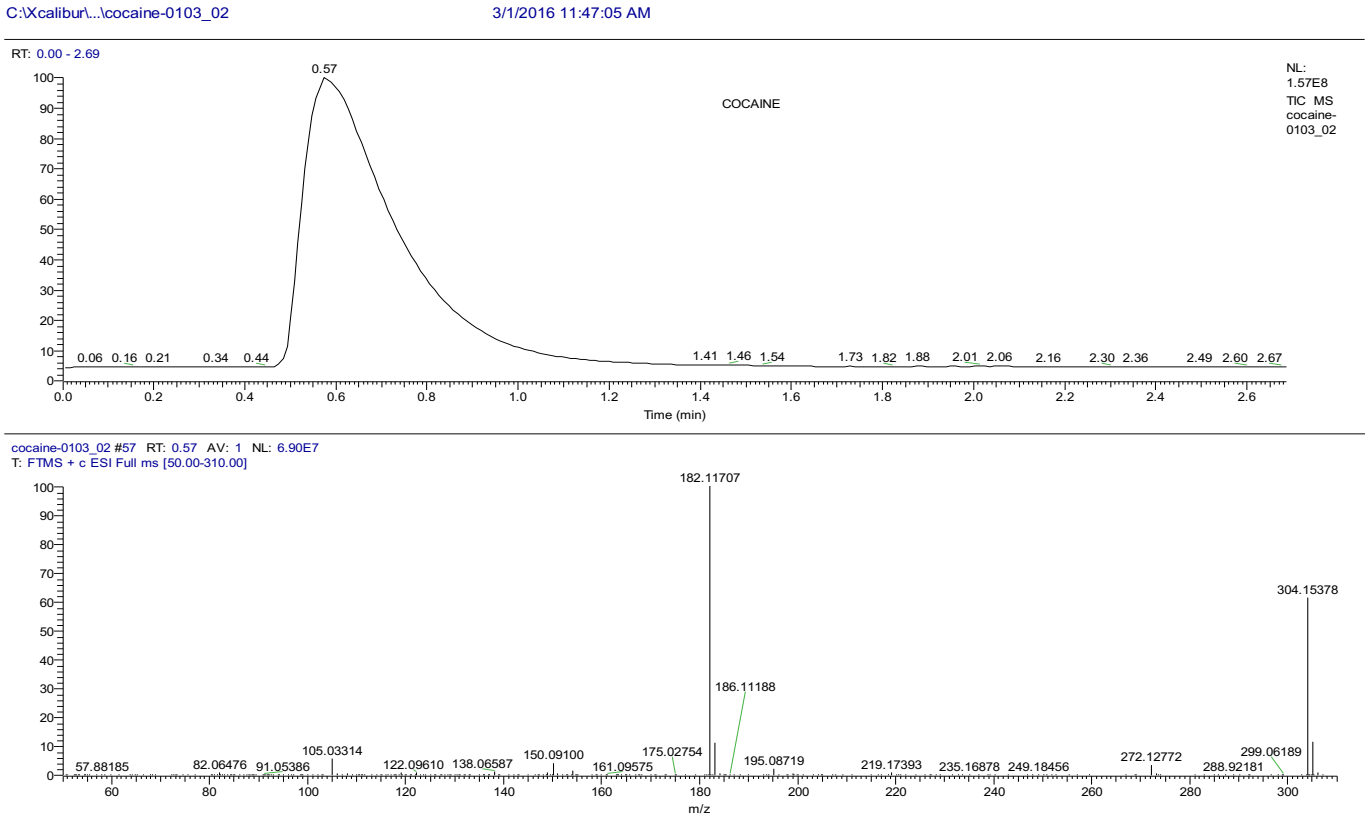
Minimum criteria for chromatographic-mass spectrometric confirmation of the identity of analytes for doping control are:

1. The retention time (RT) of chromatographic peak of the analyte in a sample shall not differ (ΔRT) by more than 1% or ±0.1 min, from that of the same analyte in a spiked sample, reference collection sample, or reference material analysed in the same analytical batch;

2. The relative abundances of any of the diagnostic ions shall not differ by more than the amount specified in WADA technical document from the corresponding relative abundances of the same ions acquired from a spiked positive control urine, reference collection sample, or reference material [4, 5]. Relative ion intensities were calculated on the basis of ion ratios (quantification transition divided by qualifier transition). These values were compared with the mean relative ion intensity of all analytes. Table 2 contains the optimized parameters for multiple MRM transitions for various prohibited substances [3].

Triple-quadrupole mass spectrometry with multiple reaction monitoring (MRM) is the most commonly adapted technique for confirmatory and quantitative drug analysis. We are developing a confirmatory method for the analysis of drugs of abuse in human urine by using a high resolution and high mass accuracy hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap-MS). This method allow the detection of different drugs of abuse, including amphetamines, cocaine, opiate alkaloids, synthetic cannabinoids, hallucinogens and their metabolites [6-8].

In Figure 1 we demonstrated confirmation of some opioid substances.



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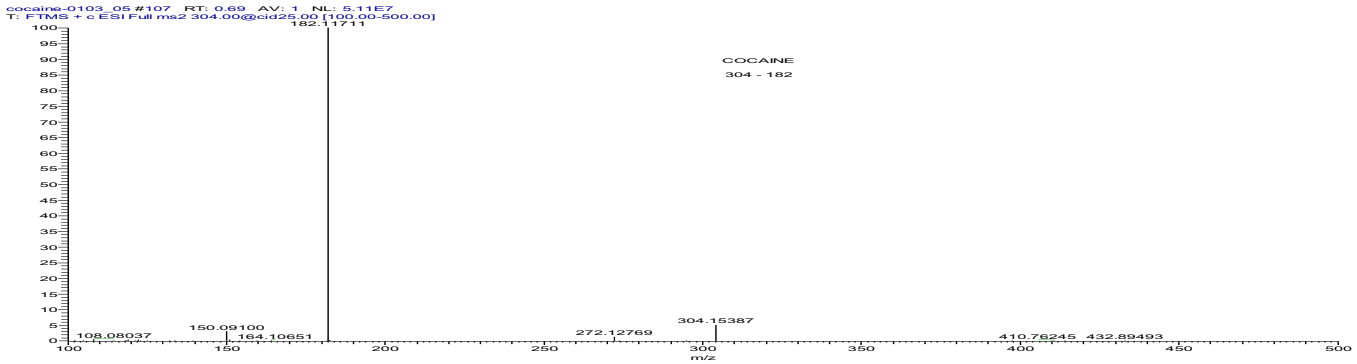

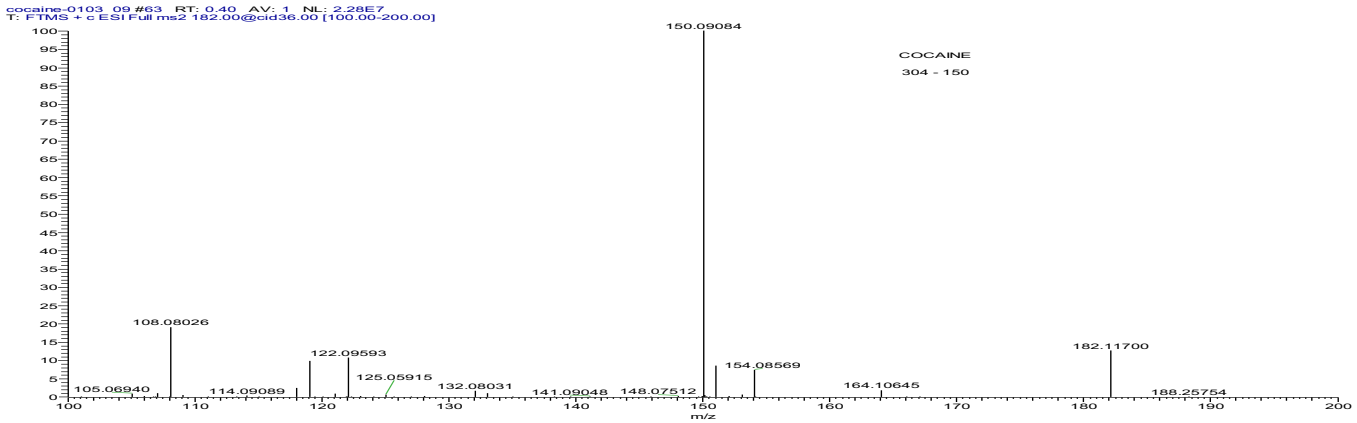

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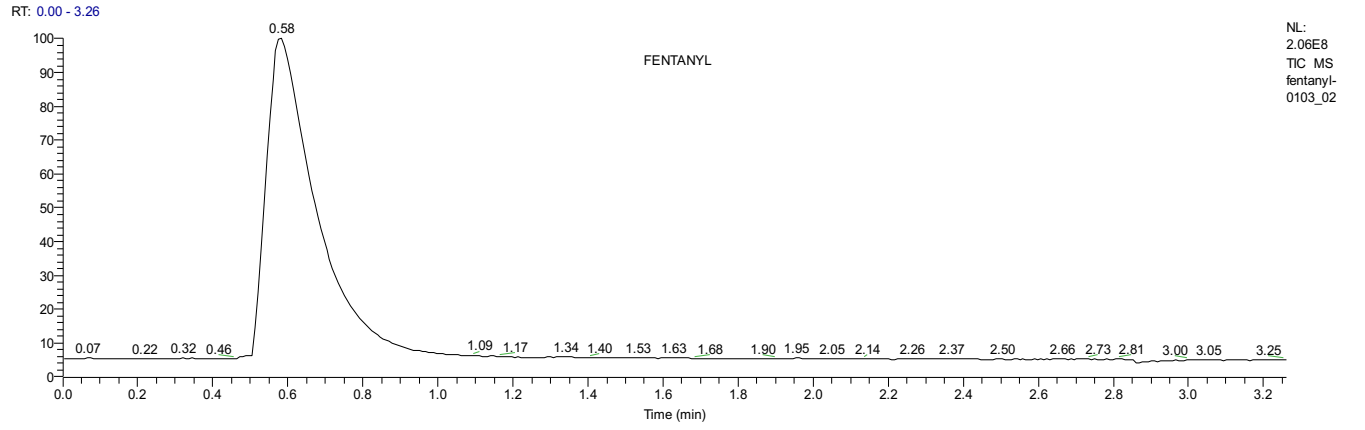

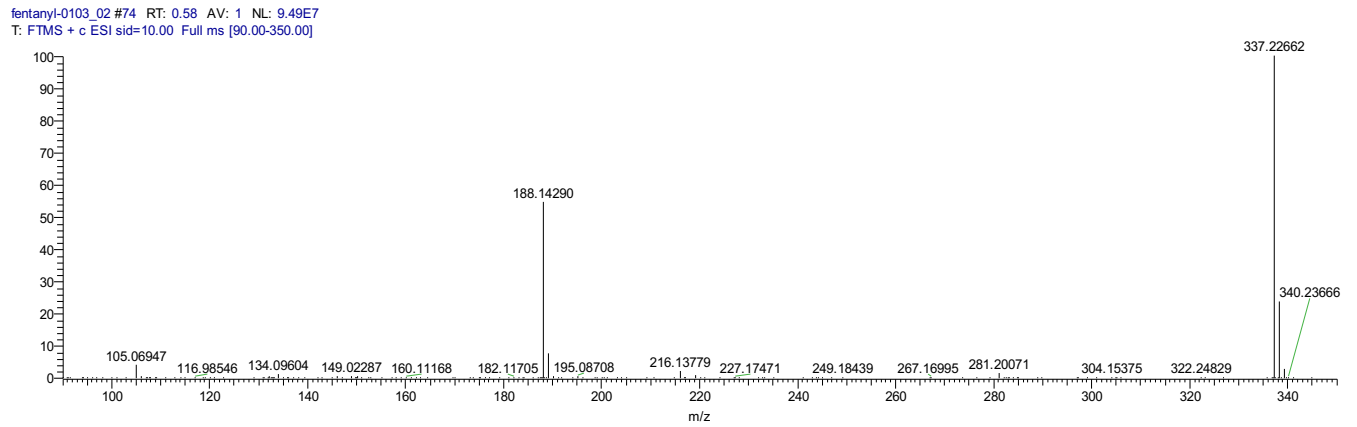

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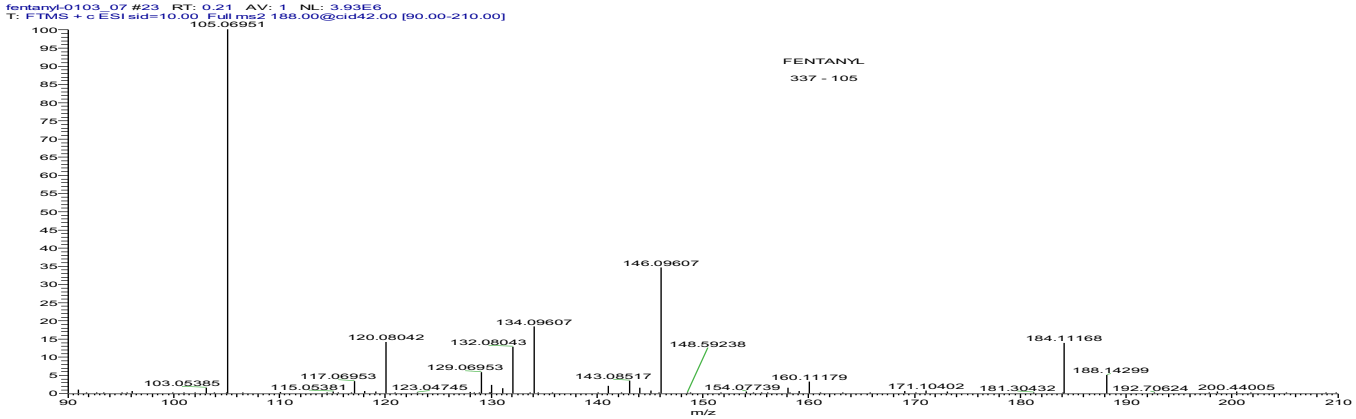

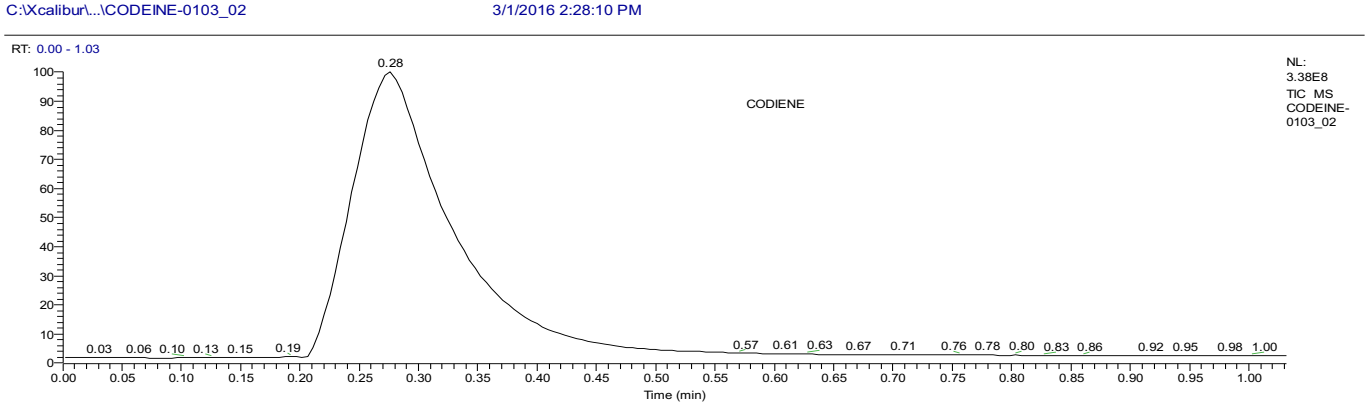

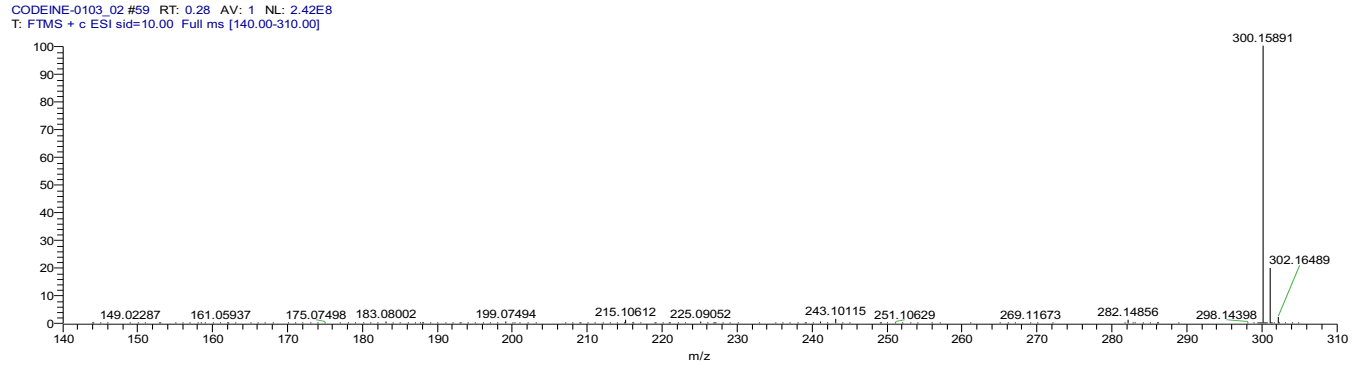

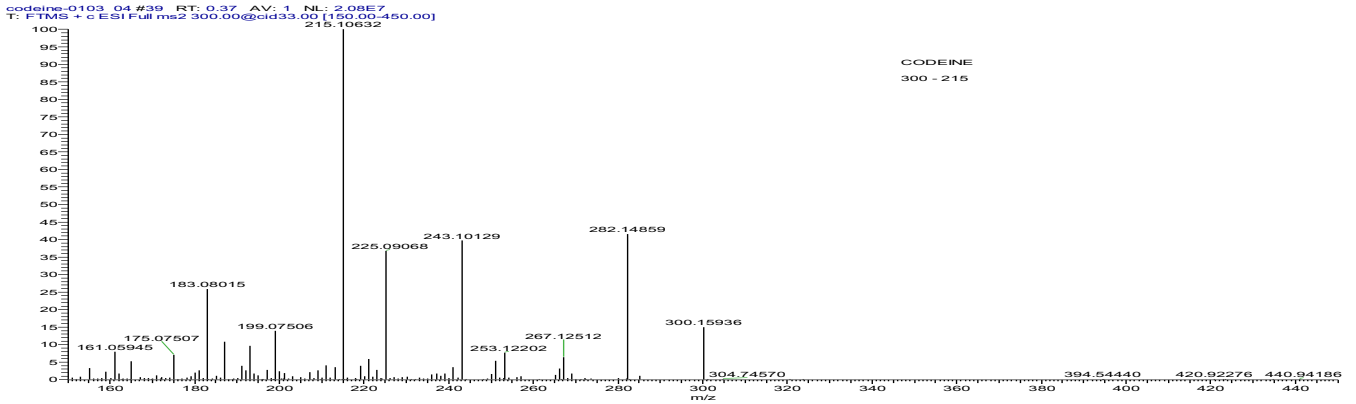

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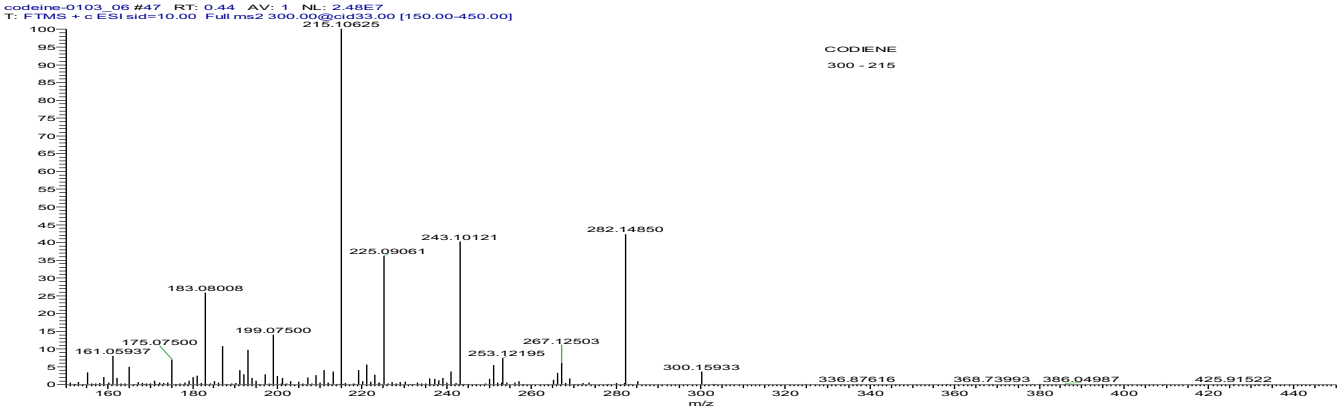

Figure 1. Confirmation of some of the opioid substances. In the panels from top to bottom: xxx

Table 3. Performance parameters of the presented method for the determination of prohibited substances in urine of sportsmen.

Compound	Calibration Concentration (ng mL^{-1})	R^2	Extracting Efficiency/ Output (%)	LOD (ng mL$^{-1)}$	LOQ (ng mL^{-1})	Uncertainty (%)
Amphetamine	10 – 200	0.9985	94	0.150	10.5	4.48
Methylapmphetamine	10 – 200	0.9966	104	0.149	10	4.89
Ethylamphetamine	10 – 200	0.9982	104	1.480	10.5	4.55
MDA	15 – 200	0.9985	102	1.550	15.5	4.37
MDMA	15 – 200	0.9958	102	0.149	14.9	5.73
Codeine	10 – 200	0.9980	99	1.520	10	5.88
Oxycodone	10 – 200	0.9996	94	1.490	10	7.72
Morphine	5 – 100	0.9989	81	0.350	5	5.50
Hydromorphone	5 – 105	0.9989	39	0.362	5.8	9.41
6-Acetylmorphine	5 – 100	0.9982	68	0.350	5	7.97
Benzoilecgonine	5 - 100	0.9958	87	0.350	5	6.74
Oxymorphone	5 - 100	0.9981	58	0.350	5	7.10
Heroine	5 – 100	0.9858	57	0.344	5.5	5.83
Fentanyl	0.22 – 4	0.9965	64	0.034	0.22	5.26
Cocaine	10 – 200	0.9981	99	1.490	10	5.22
Mepethydine	10 – 200	09985	100	1.480	10	5.16
Methadone	15 – 200	0.9991	97	0.151	15	5.42
Ephedrine	20 – 403	0.9962	103	10.3	20.2	6.40
Pseudoephedrine	20 – 400	0.9981	108	9.6	20	7.95
Cathine	20 – 400	0.9969	104	7.56	19.7	8.34
Norephedrine	20 – 400	0.9966	110	8.56	19.8	6.31
Methylephedrine	20 - 300	0.9910	104	14.62	20	7.10
Methcathinone	100 - 400	0.9976	76	35.5	100	6.82
Acetazolamide	50 - 200	0.9984	92	9.97	50	7.58
Piretanide	50 – 202	0.9971	94	1.01	50.4	10.3
Tolvaptane	50 – 200	0.9951	109	0.987	49.3	12.63
Metazolamide	50 - 200	0.9948	106	9.91	50	9.92
Benzylhydrochlorothiazide	50 – 200	0.9984	89	2.97	49.6	10.18
Diclofenamide	51 – 204	0.9965	78	10.2	51	17.26
Polythiazide	50 – 200	0.9960	79	2.96	49.5	12.9
Furosemide	51 - 204	0.9996	73	10.2	51	8.58
Indapamide	50 - 202	0.9960	105	3.03	50.5	11.5
Xipamide	50 – 200	0.9943	75	2.98	49.8	14.02

Quantitative analysis

We developed an LC–MS/MS method for the simultaneous identification and quantification of stimulants, narcotics and diuretics, and we validated the method for linearity, sensitivity, carry-over, extraction efficiency, matrix effects, precision, accuracy, process efficiency, and selectivity. Linear regression with 1/x weighting was used to construct the calibration curves, coefficients of determination (R^2), extraction efficiency, limit of detection, limit of quantification and uncertainty of measurement were determined and summarized in Table 3.

Selectivity was determined by comparing responses of ten spiked urine samples with analytes at the WADA MRPL level with spiked standards at the same concentration prepared in water. No interference was detected at the expected retention times of the analytes. For all compounds, the matrix effect was less than 15%, except for oxycodone, hydromorphone and nor morphine, which gave values of 23%, 24% and 27%, respectively.

Conclusions

The present investigation confirms that direct injection of urine in combination with electrospray LC-MS/MS and a.m. spectrometry can be used for confirmation of various classes of drug residues and prohibited substances in urine. LC/MS/MS multi-compound analysis combined with a simple sample preparation procedure have been developed and validated for the measurement of target substances in urine samples. Based on the results of the proficiency tests, the methods seem to be useful for the rapid and accurate quantitative determination of substances in urine samples.

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UHPLC-MS/MS ANALYSIS OF THYREOSTATS IN *BOVINE* FAECES

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Abstract

A UHPLC-MS/MS method was developed and validated to detect six thyreostatic compounds, tapazole, thiouracil, methylthiouracil, dimethylthiouracil, propylthiouracil and phenylthiouracil, in the *bovine* faeces. Thyreostats were extracted with mixture of methanol and buffer (pH 8) from the matrix and analytes were then derivatised with 3-iodobenzylbromide. The LC separation of derivatives was obtained on a SB-C18 column (50 x 2.1 mm; 1.8 µm, Agilent) with gradient elution using a mobile phase consisting of acetonitrile/0.1% acetic acid within 7.5 min. The analysis was performed on UHPLC Shimadzu NEXERA X2 with triple quadrupole MS 8050 instrument operating in positive electrospray ionization mode. Depending on the target compound, two or three diagnostic signals (multiple reaction monitoring transitions – MRM) were monitored. The procedure was validated according to the Commission Decision 2002/657/EC. The recovery and repeatability satisfy the performance criteria specified for banned compounds. The recovery ranged from 97.5 to 113.5% and repeatability did not exceed 14.1%. The decision limits (CCα) and detection capabilities (CCβ) were below 10 µg kg^{-1}. Highest concentration for CCα observed on PhTU 3.48 µg kg^{-1} and also for CCβ 6.96 µg kg^{-1}.

Introduction

Thyreostats are tioamid antithyroid drugs. Activity of these compounds consists in inhibiting the synthesis of thyroid hormones triiodothyronine (T3) and thyroxine (T4), which favours the processes of animal fattening. Increase in weight of animals is mainly due to the water retention in the tissues and that of the gastrointestinal tract content. The consequence is not only the production of inferior meat, but also the risk of drug residues to human health. According to the International Agency for Research on Cancer some compounds of this group possess carcinogenic and teratogenic properties (IARC 2001). For this reason, the use of thyreostatic drugs for animal fattening purposes is banned in the European Union since 1981 (Directive 81/602/EC 1981). In accordance with the Council Directive 96/23/EC (Directive 81/602/EC 1996) thyreostats belong to the group A2 - compounds with anabolic properties, which must be controlled in slaughter animals.

Latest big progress in the efficiency of analytical methods for thyreostatic drugs determination has lead to the detection of thiouracil (TU) in the urine samples of slaughtered animals (Le Bizec *et al*., 2011; Woźniak *et al*., 2012). Pinel *et al*. (2006) presented a correlation between cruciferous based animal feed and the identification of endogenous TU in urine. EURL guidance paper confirm that thiouracil concentrations below 10 µg L^{-1} might have a natural origin derived from *Brassicaceae* consumption (EURL 2007). Inspired by reports (Le Bizec *et al*. 2011), the EURL suggested to increase this value to 30 µg kg^{-1} (EURL reflection paper 2014). Problem of the endogenous thiouracil formation in pigs was studied by Kiebooms *et al*. (2014), who demonstrated that endogenous formation of TU is *Brassicaceae* induced. It occurs under colonic conditions, most likely through myrosinase-like enzyme activity expressed by different common intestinal bacterial species. In view of these reports, interesting matrix to study the problem of endogenous thiouracil seems to be faeces. With respect to further studies, a fast and sensitive method for the determination of thyreostats in faeces has to be developed. The requirements were an effective way of sample preparation, good chromatographic separation of thyreostats and a short analysis time.

Materials and Methods

Reagent and chemicals

Standards of thyreostats: 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), 1-methyl-2-mercaptoimidazole (TAP), 5,6-dimethyl-2-thiouracil (DMTU) were obtained from Sigma-Aldrich (Steinheim, Germany). Internal standards 1-methyl-2-mercaptoimidazole-d3 (TAP-d3), 6-propyl-2-thiouracil-d5 (PTU-d5) were obtained from Toronto Research Chemicals (Toronto, Canada). All standards were stored at room temperature. Primary standard stock solutions were prepared in methanol at a concentration of 1 mg mL^{-1}. Working solutions were obtained by serial dilution of primary standard solutions to the concentration of 1 µg mL^{-1} in methanol. All working standard solutions were stored at 2-8°C.

Acetic acid (99.5% purity), *ortho*-phosphoric acid (85% purity), diethyl ether, concentrated hydrochloric acid and boric acid were of analytical grade and purchased from POCH (Gliwice, Poland). Sodium hydroxide, methanol (HPLC isocratic grade), acetonitrile (LC-MS reagent) were obtained from Mall Baker (Deventer, The Netherlands). Derivatisation reagent 3-iodobenzyl bromide (3IBBr) was obtained from Sigma-Aldrich. Water was purified (Milli-Q system, Millipore, Bedford, USA).

Britton-Robinson buffer (0.4 M) pH 8.0 was prepared by dissolving boric acid (24.73 g) in warm water (800 mL), then adding *ortho*-phosphoric acid (26.7 mL), acetic acid (23 mL), 50% sodium hydroxide solution and diluting with water to 1,000 mL. A

50% sodium hydroxide solution in water was prepared by dissolving the solid substance (500 g) in water (500 mL). A 3IBBr solution was obtained by dissolving reagent (10 mg) in methanol (4 mL). A 0.1% acetic acid solution was prepared by mixing acetic acid (0.25 mL) with water (249.75 mL).

LC-MS/MS measurement

The chromatographic separation of thyreostatic drugs was achieved on a UHPLC SHIMADZU Nexera2 system with a triple quadrupole MS 8050 instrument operating in positive electrospray ionization mode. The LC separation was obtained on a SB-C18 column (50 x 2.1 mm; 1.8 µm, Agilent) at 45°C. A gradient program using 0.1% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow of 0.6 mL min^{-1} was executed. The gradient increased from 20% B at 0 min to 80% B at 5.5 min, then returned to 20% and equilibrated for 2 min. Injection volume was 15 µL. The totals run time of the analysis was 7.5 min.

Sample preparation

A portion of 0.5 gram faeces was weighed into a centrifuge tube and 10 mL mixtures of methanol and Britton-Robinson buffer (2:8) were added followed by 10 µL of internal standard (1 µg mL^{-1} TAP-d^3, PTU-d^5) to obtain final concentration of 20 µg kg^{-1}. The sample was vortexed, then 5 mL of methanol/buffer phase (equivalent of 0.25 g of faeces) were transferred to a new tube, next 100 µL of methanol 3-iodobenzyl bromide solution was added and the whole sample was mixed. The derivatisation was conducted in the dark at 40°C over 1 h. After derivatisation, the reaction mixture was cooled to room temperature and the pH of the solution was adjusted to 4.5 by adding a few drops of concentrated hydrochloric acid. Then, the mixture was extracted twice with 3 mL diethyl ether. The combined extracts were passed through anhydrous sodium sulphate layer and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted in 500 µL mobile phase consisting of acetonitrile and 0.1% acetic acid aqueous solution (20:80, v/v).

Table 1. LC-MS/MS ions acquisition parameters (MRM) used for identification and confirmation of thyreostats.

Analyte	MRM transition (*m/z*)	Collision energy (eV)
TAP	330,6 > 216,95*	-24
	330,6 > 114,05	-22
	330,6 > 90,10	-40
TU	344,95 > 216,95*	-20
	344,95 > 90,05	-45
MTU	358,70 > 216,95*	-21
	358,70 > 90,10	-45
PTU	386,70 > 217*	-23
	386,70 > 90,10	-50
PhTU	421 > 217*	-24
	421 > 90	-50
DMTU	372,65 > 217*	-24
	372,65 > 90,10	-47
TAP-d3	333,95 > 217,05*	-22
	333,95 > 90,10	-43
PTU-d5	391,95 > 217,10*	-23
	391,95 > 90,10	-48

** Transitions used for quantification.*

Validation

According to Commission Decision 2002/657/EC requirements, the method was validated. The specificity, linearity, precision (repeatability and within-laboratory reproducibility), recovery, decision limit (CCα) and detection capability (CCβ) of the method were evaluated. Three series of analysis, each of which was composed of seven blind matrix samples, seven samples fortified at 5, 10 and 15 µg kg^{-1} was performed. For the factorial effect analysis, software programme "ResVal" (v 3.0; EURL Laboratory, The Netherlands) was used.

Results and Discussion

As the first step, the detection conditions of tandem mass spectrometry were established. The optimal conditions for triple quadrupole analyser (MS 8050) were determined by individually infusing the thyreostats standards and two deuterated analogues as internal standards. For each compounds the parent ion and at least two daughter ions, necessary for confirmation

of analytes according to Commission Decision 2002/657/EC were identified. During the optimization of ion source the following parameters were established: voltage on quadrupole, temperature and flow of the nebulizer gas and collision energy. Also, the composition of mobile phase was compared to obtain the most effective ionization of compounds tested. Special software automatically adjusts the tuning parameters for the best signal intensity. The results of optimization are shown in Table 1.

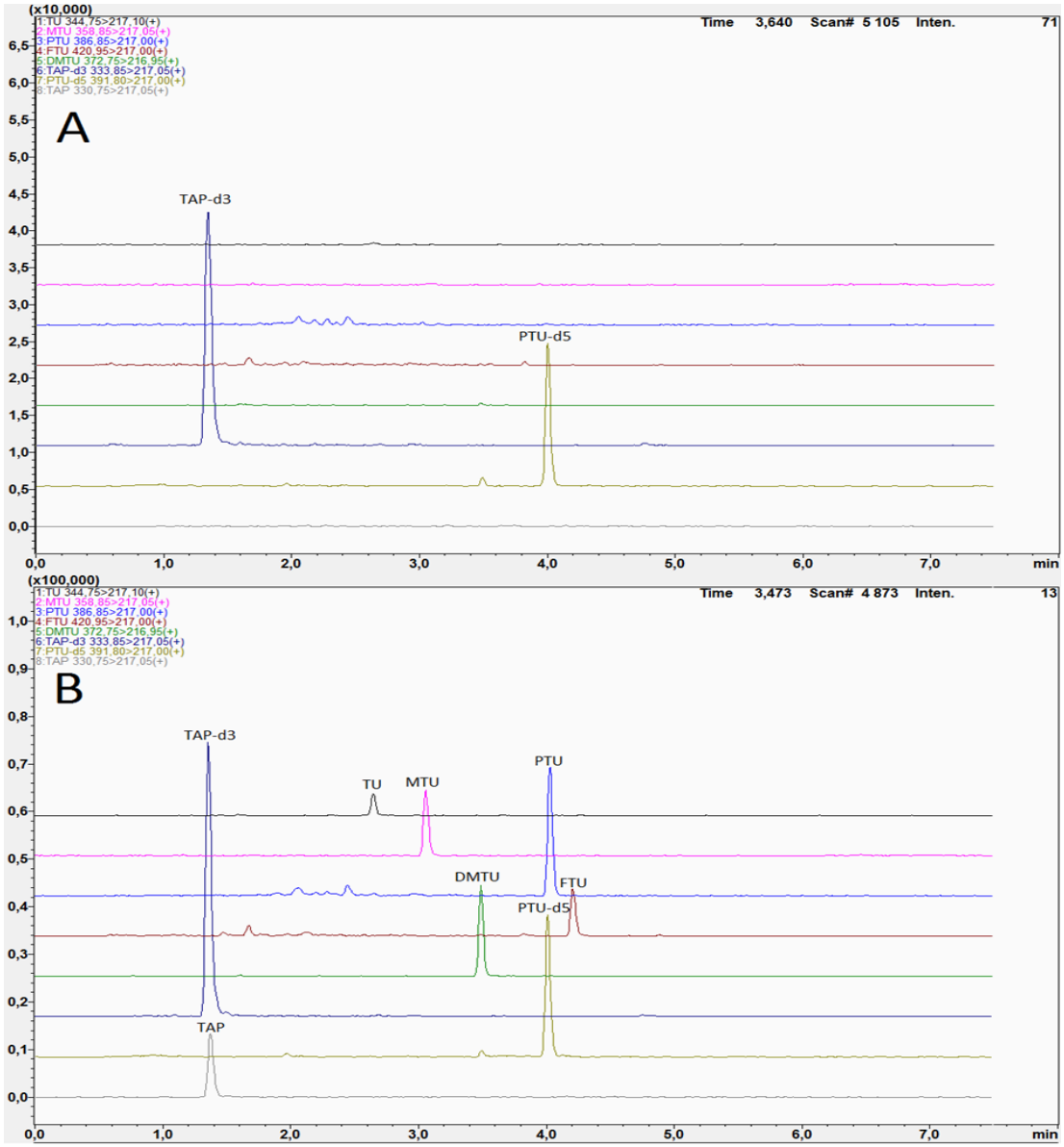

Figure 1. LC-MS/MS ions chromatograms of faeces samples: A – blank sample; B – sample spiked at 5 µg kg^{-1}.

Then, the optimization of chromatographic conditions was performed to obtain good separation of the compounds. Mobile phase gradient and flow, and column temperature were optimized. Obtained chromatographic separation of compounds is shown in Figure 1B. The sample preparation process efficiency of three organic solvents was compared for extraction of thyreostats from faeces. Additionally, the effect of sample size on purity of the extracts was checked. Obtained extracts were clean and no interferences in the blank sample chromatograms were observed.

The preliminary studies of faeces samples have shown high matrix effect for most of the tested compounds. The modification of mobile phase composition, the flow change and the dilution series of the final extract were not effective. Matrix effects were also observed for tapazole, despite the internal standard using. Further studies revealed a significant difference in the suppression of signals from the compounds and their isotope analogues. Since the optimization of the conditions for the detection of radiolabelled standards and standards of thyreostats was conducted at different times, re-tuning performed all

analytes. This procedure eliminated the effect of the matrix. The method performance was investigated with respect to various parameters such as recovery, repeatability, reproducibility, decision limit, detection capability. The results are presented in Table 2.

The results summarized in Table 2 reflect the correct recovery ranging from 97.5% for TAP to 113.5% for MTU with a good CV, less than 25% for most analytes under within-laboratory reproducibility conditions. The specificity of the method was demonstrated by analysis of 21 faeces samples. No interference for any of the transitions was observed around the thyreostats retention times (Figure 1A), therefore, the method was found to be specific. The linearity of the chromatographic response was tested with matrix matched calibration curves for each compound using blank samples spiked from 0 to 30 μg kg^{-1}. The linearity was correct for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) exceeding 0.99 value for all curves. Chromatogram of blank faeces sample and spiked with all thyreostats is presented in Figure 1A.

Table 2. Overview of the results of the validation.

Analyte	Recovery (%)	Repeatability (CV, %)	Reproducibility (CV, %)	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
TAP	97.5	5.9	9.5	3.26	6.52
TU	112.7	4.4	7.1	2.21	4.42
MTU	113.5	10.3	16.5	2.99	5.99
PTU	101.6	11.1	17.8	3.29	6.58
PhTU	112	14.1	22.6	3.48	6.96
DMTU	98.7	13.6	21.8	3.00	6.01

Conclusions

A simple and fast LC-MS/MS method has been developed for thyreostatic drugs in faeces. There are no guidelines for this matrix but the validation results obtained indicate the accordance with requirements of Commission Decision 2002/657/EC. The CCα and CCβ are below the recommended concentration of 10 μg kg^{-1}, defined for urine samples. The MRM transition ratios meet the criteria for confirmation.

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DETERMINATION OF POLYPEPTIDE ANTIBIOTIC RESIDUES IN MUSCLE AND MILK SAMPLES BY LIQUID-CHROMATOGRAPHY TANDEM MASS-SPECTROMETRY

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Abstract

A liquid chromatography tandem mass spectrometric (LC-MS/MS) method for the determination and quantification of polypeptide antibiotics (bacitracin and colistin) in muscle and milk samples was developed. Prior to the instrumental analysis, sample preparation involved extraction with ammonia in acetonitrile followed by evaporation, reconstitution and filtration steps. The chromatographic separation was performed on C18 column with gradient elution. Mass spectral acquisitions were performed using selective multiple reaction monitoring on a triple quadrupole mass spectrometry detector. The method was characterized by testing its precision, accuracy, specificity, decision limit (CCα) and detection capability (CCβ) according to Commission Decision 2002/657/EC. The calibration curves were linear in the range of 10-500 µg kg^{-1}. The recoveries ranged from 95.9% to 102.3%. Repeatability was below 9.9% and within-laboratory reproducibility was lower than 11.6%. Calculated CCα and CCβ values were slightly higher than MRL value. The developed method fulfils the criteria for confirmatory methods and thanks to its labour efficiency, it may be used also for screening purposes.

Introduction

Polypeptides are a group of antibiotics with a variety of actions against many Gram-negative and Gram-positive bacteria. Members of the polypeptide family are bacitracin and colistin. Bacitracin is produced by *Bacillus licheniformis* and *B. subtilis*, and is a mixture of several closely related polypeptides, mainly consisting of bacitracin A, and in a lower extent of bacitracin B1, B2, C and F (EMEA 2001). The chemical structure of the major component, bacitracin A consist of a heptapeptide ring that linked to a pentapeptide chain. Bacitracin has a narrow but potent therapeutic spectrum against Gram-positive cocci and bacilli because of the inhibition of cell-wall biosynthesis by immobilizing lipid pyrophatase during the process of trans-membrane transport of peptidoglycan precursors (Sin and Wong, 2003). Because bacitracin is highly nephrotoxic after parenteral administration, it is generally only used in the topical treatment of superficial infections of the skin and mucosal surfaces (Dowling 2013). In veterinary medicine, bacitracin is used in combination with tetracycline, neomycin and prednisolone for intramammary treatment of mastitis in lactating cows. In rabbits, this antibiotic is indicated for the treatment of enzootic rabbit enterocolitis, and is given orally with drinking water or in feed (EMEA 2002a).

Colistin (also known as polymyxin E) is an important member of the polymyxin group of cationic peptide antibiotics and is produced by cultures of *Bacillus polymyxa* var. *colistinus.* It features a cyclic heptapeptide and tripeptide side chain acylated at the N-terminus by a fatty acid. At least thirty different components were found in commercially available colistin. The major components are colistin A (polymyxin E1) and colistin B (polymyxin E2) which, together, account for more than 85% of total weight of the raw material. The two substances differ in the length of the fatty acid side chain by one methylene group (Ma *et al.,* 2008). Colistin is effective primarily against Gram-negative microorganisms. It causes disorganisation of the bacterial cell membrane with leakage of intracellular materials and inhibits bacterial oxidative metabolism. In veterinary medicine is used for prevention and treatment of diseases caused by sensitive bacteria (e.g. *Salmonella* and *Escherichia coli*) in pigs, cattle, poultry, sheep, goats and rabbits. Even though parenteral and intramammary administration occasionally occurs in veterinary medicine, colistin is mainly used in oral preparations (EMEA 2002b).

The therapeutic use of polypeptide antibiotics in food producing animals must be assessed not only in terms of good clinical efficacy but also considering the risk of the presence of residues in edible tissues. Therefore, to ensure human food safety, the EU has set a tolerance level for these compounds as the maximum residue limit (MRL). The European Commission regulation 37/2010/EU has set MRLs for bacitracin at the level of 100 µg kg^{-1} and for colistin at the level of 50 µg kg^{-1} in milk. In muscle tissues both polypeptides have MRL of 150 µg kg^{-1}.

Over the past years, very few LC-MS/MS methods have been published for analysis of bacitracin and/or colistin in food of animal origin, such as milk (Kaufmann and Widmer 2013; Sin *et al*., 2005; Wan *et al*., 2006; Zang *et al.,* 2015), muscle (Boison *et al*., 2015; Kaufmann and Widmer 2013; Wan *et al*., 2006; Zang *et al.,* 2015) and other animal food samples (Kaufmann and Widmer 2013; Sin *et al*., 2005; Wan *et al*., 2006; Xu *et al*., 2012; Zang *et al.,* 2015). All these methods are based on a low pH extraction to liberate the analytes from the matrix. After acidified extraction, the majority of the previously mentioned methods employed a polymer-based reversed phase solid phase (SPE) extraction step for extract purification.

The aim of this work was to develop a reliable LC-MS/MS method with fast and simple sample pre-treatment, suitable for extraction of bacitracin and colistin residues from muscle and milk samples. The method was validated in accordance with

the Commission Decision 2002/657/EC and demonstrates to be suitable for detection and quantitation of polypeptide antibiotic residues in food of animal origin.

Materials and Methods

Reagents, materials and standards

All solvents used were of analytical grade. Acetonitrile and methanol were obtained from JT Baker (Deventer, The Netherlands). Ammonia solution 25% was purchased from POCH (Gliwice, Poland). Formic acid was obtained from Fluka (Newport News, VA, the United States). Ultra-pure water (resistance > 18 mΩ) was obtained from a Milli-Q system (Millipore, France). Syringe filters 0.22 µm PVDF were from Restek (Bellefonte, PA, the United States) The analytical reference standard of bacitracin A, colistin sulfate salt, polymyxin B sulfate were bought from Sigma-Aldrich (St. Louis, MO, USA).

Stock standard solutions (1,000 µg mL^{-1}) of analytes were prepared separately by weighting of 10 mg reference standard and dissolving in 10 mL methanol (bacitracin A) or 10 mL of mixture of methanol and 0.1% formic acid in water (1:1, v/v) in case of colistin sulfate and internal standard (IS), polymyxin B sulfate. The stock solutions were stored in the dark glass bottles at < -18°C and were stable for at least 3 months. The working standard solutions of bacitracin and colistin, used for sample fortification were prepared by the dilution of these solutions with water and were stored in the dark at 2-10°C for at least one week. Working solution of internal standard (10 µg mL^{-1}) in water was prepared separately and stored in the dark at 2-10°C for at least one week.

Sample preparation

Two grams homogenised muscle or 5 g milk were weighed into 50 mL polypropylene centrifuge tube and fortified with 30 µL of 10 µg mL^{-1} working internal standard solution. Then, 8 mL of mixture of acetonitrile : ammonia solution 25% (9:1, v/v) was added into the tube and the sample was mixed on a vortex-mixer and shaken for about 10 min on rotary stirrer. After centrifugation at 4,500 rpm for 10 min at 4°C, 6 mL supernatant was taken and placed in a 10-mL tube. The extract was evaporated to dryness under a weak stream of nitrogen at 45°C. The dry residue was reconstituted in 0.5 mL of a mixture of 1% formic acid in acetonitrile : 1% formic acid in water (1:1, v/v) and filtered through 0.22 µm PVDF syringe filters into LC vial for LC-MS/MS analysis.

Liquid chromatography–tandem mass spectrometry analysis

An Agilent Series 1200 HPLC system (Agilent Technologies, Waldbrom, Germany) was connected to QTRAP 5500 mass-spectrometer (AB SCIEX, Ontario, Canada). The Analyst 1.6 software controlled the LC-MS/MS system and processed the data. The mass spectrometer was operated in electrospray positive ionisation mode (ESI+) and multiple reaction monitoring (MRM) mode was used to quantify the analytes. The mass spectrometer settings were optimised and following parameters were used: resolution Q1 and Q3: unit; curtain gas: 20, collision gas: medium, ion source gas 1: 50, ion source gas 2: 60, ion spray voltage: 5,500 V; temperature of ion source: 400°C.

The chromatographic separation was performed on a Kinetex 2.6 µm XB-C18 column (2.1 x 100 mm, Phenomenex, Torrance, CA, the United States) coupled with SecurityGuard ULTRA holder and cartridge UHPLC C18 for 2.1 mm ID columns (Phenomenex, Torrance, CA, the United States). The mobile phase consisted of a solvent A: 1% formic acid in acetonitrile and solvent B: 1% formic acid in water. The elution was performed in a gradient mode. The starting conditions for the mobile phase were 95% of eluent B and then decreased to 50% within 5 min and in 5.01 min decreased to 10% for 1 min to completely elute analytes. At 6.01 min eluent B returned to initial 95% and with the following equilibration time of 7 min, the resulting total run was 13 min. The column was operated at 45°C with a flow rate of 0.30 mL min^{-1}; the injection volume was 5 µL.

Method validation

The method was validated according to the recommendations of the Commission Decision 2002/657/EC on the basis of an in-house validation concept. The validation study was performed in terms of linearity, specificity, accuracy, precision (repeatability and within-laboratory reproducibility), decision limit (CCα) and detection capability (CCβ). Matrix-matched calibration curves were prepared at six concentrations by plotting the response of analyte/internal standard peak area ratio versus the analyte concentration. In the precision and accuracy study blank muscle and milk samples were spiked with bacitracin and colistin at 0.5, 1.0 and 1.5 times of the MRL level. In the repeatability study, three series were analysed under the identical conditions (six samples for each fortification level). Standard deviation (SD) and coefficient of variation (CV, %) were calculated for each level. The within-laboratory reproducibility was obtained by analysis of two additional series (on three levels) under reproducibility conditions (two different occasions, another technician) and overall SD and CV were calculated. The overall mean concentrations obtained in the reproducibility study were used to calculate accuracy expressed as percent. The parameters of CCα and CCβ were calculated using within-laboratory reproducibility results, in accordance with the procedure described in the Commission Decision 2002/657/EC.

Results and Discussion

Sample preparation

In order to assess the occurrence of polypeptide antibiotics in food of animal origin, sensitive analytical methods are required which enable to determine these drug residues at quite low concentration levels. One important requirement of the extraction method was that it should be fast and robust enough to allow the rational analysis of a large number of samples.

Mostly, the sample preparation procedure involved deproteinization step (referred to as acidic pre-treatment) which is performed by organic solvents (methanol and acetonitrile) and acids (trifluoroacetic acid, trichloroacetic acid, hydrochloric acid) in different combinations. After that step, generally solid phase extraction is performed on polymeric-based SPE cartridges.

The extraction procedure of our method, as described above, was optimized after evaluating the performance of different mixtures of solvents in order to remove selectively many possible interfering substances from biological matrices like muscle and milk with maintaining high recovery of analytes. Because polypeptide antibiotics are polar, hydrophilic compounds with amino groups in their structure we investigated a different approach to sample preparation by the use of alkaline extraction. We chose acetonitrile as organic solvent because generally is better protein precipitation than methanol (Polson *et al.,* 2003) and ammonia solution as basic reagent because it is usually used to basic acetonitrile solutions and evaporate fairly quickly.

In this study, the efficiency of isolation of analytes after the use of different combination of extraction solvents consisting of acetonitrile with different concentration of ammonia solution in acetonitrile were compared. After the comparison study, mixture of acetonitrile : ammonia solution 25 % (9:1, v/v), was chosen as an extraction solvent. Experimentally studying impact of this extraction solvent on the degree of isolation of analytes from matrices provided the optimum levels of analytes recovery.

Liquid chromatography–tandem mass spectrometry analysis

The fragmentation reactions used for monitoring of bacitracin, colistin A and B and IS were selected on the basis of their significance in product ion spectra during the optimisation by injecting working standard (100 ng mL^{-1}) into a mass spectrometer. Polypeptide antibiotics can form multiple charged ions in ionization process. The most common were triply charged ions and doubly charged ions. During optimisation it was noted that triply charged ions had better ion response than doubly charged ions. Therefore, the triply charged ions were used as the precursor for establishing MRM analysis. Two MRM transitions of bacitracin, colistin A and B and one transition for polymyxine B1 (IS) were monitored in order to comply with the criteria needed for a confirmatory method and for unequivocal identification. The parent ion, products ions, declustering potential, entrance potential, collision energy, cell exit potential for each compound are listed in Table 1 whereas Figure 1 shows chromatograms of analytes and internal standard in spiked muscle sample. Identification of analytes was carried out by retention times and relative ion ratio of selected MRM transition.

During preliminary work we test different C18 chromatographic column and mobile phase for separation of analytes. Finally, chromatographic analysis of polypeptides was performed on Kinetex 2.6 u XB-C18 column after optimisation of mobile phase composition and gradient elution program. The optimal separation of analytes was obtained with mobile phase, consisting of 1% formic acid in acetonitrile and 1% formic acid in water. Using these conditions, we obtained sharp, symmetrical chromatographic peaks with minimum band broadening. The compounds of interest were eluted in order of increasing retention times as follows: colistin B = 6.2 min, colistin A = 6.4 min, IS = 6.5 min and bacitracin = 7.2 min, and the complete running time was 13 min. Ion chromatograms obtained from muscle sample spiked with polypeptides are presented in Figure 1.

Method validation

The whole procedure was validated in-house according to the Commission Decision 2002/657/EC. Because isotope labelled polypeptide analytical standards were not available, we used polymyxin B1 as an internal standard as it has a structure and chemical properties similar to colistin. Colistin is a mixture of compounds, with two major components (colistin A and B). Although colistin A and B are the major components in commercially available colistin, the proportion of colistin A and B differs between batches and manufactures. Therefore, the percentages of colistin A and B in the reference sample of colistin were calculated as the ratio of peak areas for colistin A and B and found to be 33 and 67%, for colistin A and B, respectively. Because the ratio of colistin A to B was estimated at 1:2, we quantified both colistins in our assay.

The specificity of the method was checked by analysing 20 blank muscle and milk samples and no peak was detected in these samples at the retention time corresponding to each analyte. Matrix-matched calibration curves with 1/x weighting factor were used for quantification with acceptable linearity (correlation coefficient, $r > 0.995$) over the ranges 10-500 µg kg^{-1}. Accuracy, repeatability, within-laboratory reproducibility, decision limit (CCα) and detection capability (CCβ) of polypeptide antibiotic were calculated and presented in Table 2 for muscle and in Table 3 for milk. Validation results show that accuracy was high in the range of 95.9 – 102.3%. Results from assessment of repeatability and from the within-laboratory reproducibility study (CV = 5.6 – 9.9% and 6.1 – 11.6%, respectively) show that the precision of the determination of bacitracin and colistin was acceptable. Calculated CCα and CCβ values were slightly higher than MRL value.

Table 1. MS/MS parameters used for monitored compounds.

Analyte	MRM transition (*m/z*)	Declustering potential (V)	Entrance potential (V)	Colision energy (V)	Cell exit potential (V)
Bacitracin A	475.0 → 199.0[a]	100	5	35	26
	475.0 → 227.0	100	5	28	10
Colistin A	390.7 → 384.6[a]	85	10	16	15
	390.7 → 101.1	85	10	23	15
Colistin B	386.0 → 380.0[a]	85	10	16	15
	386.0 → 101.1	85	10	23	15
Polymyxin B1 (IS)	402.0 → 396.3	95	10	16	15

[a] *ion transition used for quantification*

Table 2. Validation results of the method for the determination of polypeptides in muscle.

Parameter	Bacitracin			Colistin A			Colistin B		
Spiking level (µg kg^{-1})	75	150	225	75	100	150	75	150	225
Accuracy (%)	99.6	102.2	99.1	99.3	101.4	99.1	98.4	102.3	98.5
Repeatability (CV, %)	7.8	7.3	5.6	8.4	7.4	5.7	8.8	7.6	5.7
Within-lab Reproducibility (CV, %)	8.8	7.9	6.2	9.1	8.3	6.1	9.3	8.7	6.3
CCα (µg kg^{-1})	174			175			175		
CCβ (µg kg^{-1})	196			196			198		

Table 3. Validation results of the method for the determination of polypeptides in milk.

Parameter	Bacitracin			Colistin A			Colistin B		
Spiking level (µg kg^{-1})	50	100	150	25	50	75	25	50	75
Accuracy (%)	95.9	97.4	97.7	96.8	98.2	99.3	96.7	99.0	99.2
Repeatability (CV, %)	9.2	7.5	5.7	9.9	9.0	8.1	9.6	8.9	7.9
Within-lab Reproducibility (CV, %)	10.8	9.1	6.1	11.6	10.2	9.5	10.9	9.9	9.2
CCα (µg kg^{-1})	116			58			58		
CCβ (µg kg^{-1})	130			68			69		

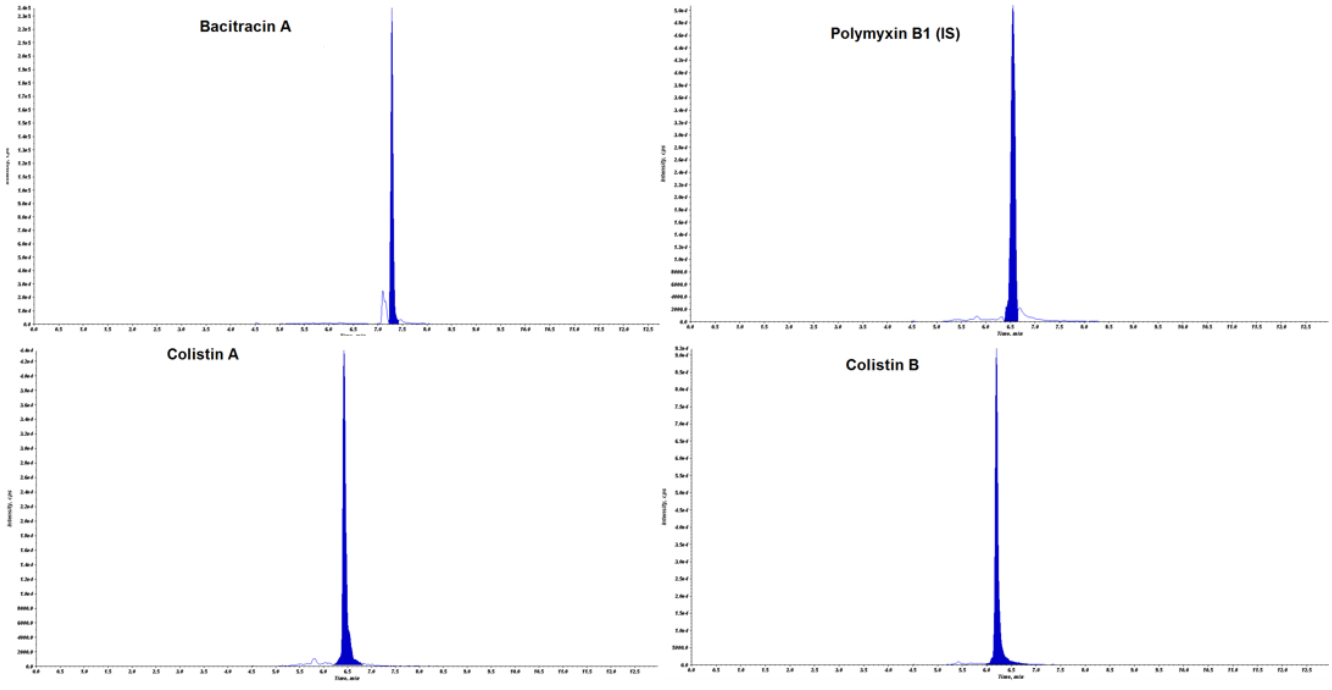

Figure 1. LC-MS/MS chromatograms in MRM mode of muscle sample spiked with polypeptide antibiotics at the level of 150 µg kg^{-1}.

Conclusions

An analytical method for the determination of polypeptides antibiotics in muscle and milk samples has been successfully developed. A simple sample preparation and 13-min single chromatographic run allow to perform multiple analyses within one working day. The method validation parameters demonstrate its reliability with good response linearity, high recovery and precision and good selectivity for polypeptide antibiotics determination in food matrices. The developed method fulfils the criteria for confirmatory methods and thanks to its labour efficiency, it may be used also for screening purposes.

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TRANSFER OF NITROIMIDAZOLES FROM CONTAMINATED BEESWAX TO HONEY

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Abstract

Nitroimidazoles are not authorised for the treatment of honey bees in the European Union. However, they can be found in honey largely because they are illegally used in apiculture for the treatment of nosemosis. The aim of the study was to examine the possible transfer of nitroimidazoles (metronidazole, ronidazole, dimetridazole and ipronidazole) from contaminated beeswax to honey. The wax foundation fortified with a mixture of 4 nitroimidazoles at the concentration level of 10 mg kg^{-1} was placed in beehive to let the honeybees (*Apis mellifera* L.) draw out the contaminated wax foundation to a honeycomb. At 1 month from the start, the frames filled with capped honey were removed from the hive for a first sampling of honey. Next, the honeycomb was further incubated for 5 months in the laboratory at 35°C and sampled monthly. In the sampled honey, the concentrations of nitroimidazoles and their main metabolites (hydroxymetronidazole, 2-hydroxymethyl-1-methyl-5-nitroimidazole, hydroxyipronidazole) were determined by LC-MS/MS and compared to those determined in the nitroimidazole-containing wax foundation. Each of the tested nitroimidazoles could migrate from beeswax to honey kept in the contaminated comb. The highest concentration for metronidazole (368 µg kg^{-1}) and ronidazole (233 µg kg^{-1}) was observed at the fourth month whereas for dimetridazole (8 µg kg^{-1}) and ipronidazole (8 µg kg^{-1}) the highest content was found in the second month from the start of the experiment. When we took into account that a frame completely filled with honey on both sides of the comb contained 110 g of beeswax and 2,488 g of honey (assuming that this ratio was constant), then the maximum amount of initial metronidazole, ronidazole, dimetridazole and ipronidazole that migrated from contaminated wax foundation to honey was 89.38%, 54.62%, 2.65% and 2.02%, respectively.

Introduction

Bacterial and protozoal diseases of honeybees (*Apis mellifera* L.) may severely decrease the honeybee population, honey and other bee products production causing significant damage to the beekeeping industry. One of the most destructive and widespread diseases affecting adult honey bees, and thus are major economic problems due to the large losses in apiaries, is disease caused by nosema (Michalczyk *et al*., 2014). This infection has usually been treated in bee colonies by using nitroimidazoles (Bogdanov, 2006; Mitrowska *et al*., 2014). However, the use of nitroimidazoles and other antimicrobial substances in commercial beekeeping is prohibited by law in the European Union (EU) because there are no maximum residue limits (MRLs) for these drugs in honey (European Commission, 2010). In addition, metronidazole (MNZ), the most popular nitroimidazole, has been classified by the International Agency for Research on Cancer (IARC) into Group 2B as possibly carcinogenic to humans (IARC, 1987). As a consequence, metronidazole and other nitroimidazoles such as dimetridazole (DMZ) and ronidazole (RNZ) were banned for use in food-producing animals and inserted into Annex IV of Regulation (EEC) No. 2377/90 repealed by Commission Regulation (EU) No. 37/2010 where these drugs are listed in Table 2 (European Commission, 2010).

In addition, as a result of Council Directive 96/23/EC, these nitroimidazoles are classed as A6 compounds and their monitoring is required within the EU as well as by the countries exporting to the EU (European Commission, 1996). Other nitroimidazoles including ipronidazole (IPZ) have never been issued a veterinary licence for use in food-producing animals and therefore are also prohibited. The nitroimidazoles are known to be rapidly metabolised in animals. For MNZ, the major metabolite formed by hydroxylation is hydroxymetronidazole (MNZOH). In the same way, DMZ and RNZ produce 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI) and IPZ is metabolised into hydroxyipronidazole (IPZOH). The metabolites may have similar mutagenic potential as the parent compounds and thus the analysis of these compounds is recommended by the European Union Reference Laboratory. The "recommended concentration" for analytical methods used for the determination of parent nitroimidazoles and their metabolites in food of animal origin is 3 µg kg^{-1} (CRL, 2007).

Despite the introduction of such regulations in the EU, a small percent of the results obtained from honey samples examined in the period of 2013 - 2015 as a part of the National Residue Monitoring Plan in Poland were found to be non-compliant *i.e*. they were found to contain residues of nitroimidazoles (EFSA, 2015). In the majority of the cases, the non-compliant results corresponded to the presence of MNZ. According to Yanovych, 20% out of 250 samples of Ukrainian honey analysed contained MNZ at the concentration level above 0.5 µg $kg^{-1}$and in individual cases above 40 µg kg^{-1} (Yanovych, 2013). Commercial formulations containing MNZ such as Nozemacid or Nosemat produced in Russia are widely used in Ukraine although MNZ, like in the EU countries, is prohibited to use in beekeeping. These formulations are found to be easily available on the black market in Poland as well. Therefore, they might be illegally used by Polish beekeepers as well. Moreover, there have been four notifications in the Rapid Alert System for Food and Feed (RASFF) about residues of MNZ in honey imported from Ukraine, China, India and Guatemala.

In honey, there is no time-dependent depletion of residues based on pharmacokinetic behaviour as it is in mammalian or avian tissues. Residues once present in honey largely remain there (EMEA, 2006). As a consequence, illegal use of antibacterials could result in an accumulation of their residues in honey and other honeybee products such as beeswax.

Beeswax is one of the most valuable honeybee products in addition to honey. It finds important applications in food, cosmetics and pharmaceutical industries, which requires good quality beeswax. Beeswax is listed in the Pharmacopeia of different countries (Council of Europe, 2013; URPLWMiPB, 2013) and it is an authorised food preservative in the EU under the name of E901. It is a natural product and no additives should be present. However, any toxic substances dissolved or incorporated in beeswax can be released much later when the beeswax is used in the production of cosmetics or pharmaceuticals, consumed as food or given to bees in the form of wax foundations sheets.

The results of the study carried out by (Reybroeck *et al*., 2010) indicate that after the use of sulfamethazine in the hive, residues of this drug remain in the wax of the comb and they can contaminate honey during the next honey season. Regarding nitroimidazoles, to our knowledge, no such studies have been performed. Therefore, the aim of the study was to investigate whether nitroimidazole-contaminated beeswax could lead to contamination of the honey.

Materials and Methods

Chemicals

All chemicals and solvents used were of analytical grade and suitable for liquid chromatography. The reference standards of MNZ, DMZ and RNZ were from Sigma-Aldrich (Seelze, Germany), whereas IPZ, MNZOH), HMMNI, IPZOH, metronidazole-$^{13}C_2$, $^{15}N_2$ (MNZ-$^{13}C_2$, $^{15}N_2$), dimetridazole-D_3 (DMZ-D_3), ronidazole-D_3 (RNZ-D_3), ipronidazole-D_3 (IPZ-D_3), hydroxymetronidazole-D_2 (MNZOH-D_2), hydroxymethyl-1-methyl-5-nitroimidazole -D_3 (HMMNI-D_3), and hydroxyipronidazole-D_3 (IPZOH-D_3) were sourced from Witega Laboratorien Berlin-Adlershof (Berlin, Germany). All isotopically labelled nitroimidazoles were of chemical and isotopic purity > 98% and used as internal standards (IS).

Beeswax foundation and treatments

We started with the preparation of nitroimidazole-containing wax foundation by melting blank beeswax at 80°C and adding a mixture of 4 nitroimidazoles dissolved in methanol at the concentration level of 10 mg kg^{-1}. Following mixing, hot liquid contaminated beeswax was poured into a wax foundation mould and the beeswax was allowed to cool. After moulding, a small amount of beeswax foundation was taken for nitroimidazole analysis by LC-MS/MS. Next, a nitroimidazole-containing wax foundation was placed in a rectangular wooden frame (360 x 260 mm) and at the beginning of the summer blossoming season (mid-June) the frame was placed in a "wielkopolski" hive to let the honeybees (*Apis mellifera* L.) draw out the contaminated wax foundation to honeycomb. After 1 week from the start, the frame was transferred to the super box of the hive and after next 3 weeks the frame filled with capped honey was removed from the beehive for the first sampling of honey and was further incubated for 5 months in the laboratory at 35°C. In monthly sampled honey the concentrations of nitroimidazoles were determined by LC-MS/MS and compared to those determined in the contaminated beeswax foundations. Honey from other honeycombs present in the hive participating in the experiment as well as from other hives placed in the same apiary was also sampled and analysed on the presence of residues of nitroimidazoles.

Standard solutions

Individual stock solutions of analytical standards at 1,000 µg mL^{-1} were prepared in acetonitrile (stable for at least 12 months at -20°C). These solutions were combined and further diluted with 0.1% acetic acid to prepare working standard solutions of nitroimidazoles and their metabolites (MNZ, DMZ, RNZ, IPZ, MNZOH, HMMNI and IPZOH) as well as internal standards (MNZ-$^{13}C_2$,$^{15}N_2$, DMZ-D_3, RNZ-D_3, IPZ-D_3, MNZOH-D_2, HMMNI-D_3, and IPZOH-D_3) and stored in amber glass at 4°C for 6 months when not in use.

Analytical methods

An ISs-fortified honey sample (2 g) was extracted with 2% acetic acid and cleaned-up on a strong cation-exchange solid-phase extraction (SPE) cartridge. The obtained extract was evaporated to dryness, reconstituted in 400 µL 0.1% acetic acid and 10 µL aliquot was injected into the LC-MS/MS system.

Beeswax samples containing nitroimidazoles < 100 µg kg^{-1} were analysed using liquid–liquid extraction (LLE) followed by SPE while beeswax samples containing nitroimidazoles > 100 µg kg^{-1} were analysed using only LLE without SPE step. After adding ISs and a mixture of *n*-hexane and isopropanol (8:2, v/v), a 1-g beeswax sample was subjected to ultrasonication at 45°C for 10 min. After the beeswax melted 2% acetic acid was added and the sample was subjected to ultrasonication for another 10 min. Next, the extract was centrifuged for 10 min at 2,200 *g* at -4°C and the water phase with the analysed compounds (100 µL) were directly diluted with 0.1% acetic acid (900 µL), passed through a syringe filter and a 10-µL aliquot was injected into the LC-MS/MS system. In case of beeswax sample containing ≤ 100 µg kg^{-1} the water phase was further preconditioned

on a strong cation-exchange SPE cartridge. The obtained extract was evaporated to dryness, reconstituted in 500 µL 0.1% acetic acid and a 10-µL aliquot was injected into the LC-MS/MS system.

The LC-MS/MS system comprised a Shimadzu Triple Quadrupole LCMS-8050 system (Shimadzu, Kyoto, Japan) equipped with a system controller (CBM-20A), a column oven (CTO-20AC), an autosampler (SIL-30AC), two pumps (LC-30AD) and an MS detector with ultrafast polarity switching (LCMS-8050). The instrument was controlled by LabSolutions LCMS version 5.60 SP2 software. Electrospray ionisation (ESI) in positive-ionisation mode was used with the following parameter settings: nebulizing and drying gas flow: 3 L min^{-1}, heating gas flow: 17 L min^{-1}, interface temperature: 230°C, DL temperature: 210°C and heat block temperature: 210°C. For the quantification the multiple reaction monitoring (MRM) mode was used and for each nitroimidazole two transitions were monitored, one for quantitative determination and the other for identification, whereas for ISs only one transition was monitored. Ions monitored were *m/z* 172, 128, 82 for MNZ, *m/z* 142, 96, 81 for DMZ, *m/z* 201, 140, 55 for RNZ, *m/z* 170, 109, 124 for IPZ, *m/z* 188, 126, 123 for MNZOH, *m/z* 158, 140, 55 for HMMNI, *m/z* 186, 168, 122 for IPZOH and *m/z* 176, 132 for MNZ-$^{13}C_2{}^{15}N_2$, *m/z* 145, 99 for DMZ-D_3, *m/z* 204, 143 for RNZ-D_3, *m/z* 173, 112 for IPZ-D_3, *m/z* 190, 128 for MNZOH-D_2, *m/z* 161, 143 for HMMNI-D_3, *m/z* 189, 171 for IPZOH-D_3.

The chromatographic separation of nitroimidazoles was performed on a Luna pentafluorophenyl (PFP) analytical column (3 µm, 150 × 2 mm, Phenomenex, Torrance, CA, USA). The mobile phase A containing 0.01% acetic acid in acetonitrile and the mobile phase B containing 0.01% acetic acid in water were used to perform the gradient elution. The flow rate of the mobile phase was controlled at 300 µL min^{-1}. The column temperature was maintained at 35°C whereas the injection volume was 10 µL for all the samples injected.

During the analysis of honey and beeswax samples, quality control samples comprising blank and fortified samples were run for each sample series. Analyte concentrations in samples were calculated by comparing the ratio of an analyte base peak response to its corresponding isotopically labelled internal standard response with the same ratio in matrix-matched calibration curve standards. The limits of quantification for all analysed nitroimidazoles in honey and beeswax were 0.5 µg kg^{-1} and 2.0 µg kg^{-1}, respectively.

Results and Discussion

The results of the analyses of nitroimidazoles in the wax foundation made of beeswax fortified with MNZ, RNZ, DMZ and IPZ at the level of 10,000 µg kg^{-1} showed that some of the initial amount of the added drugs was lost due to heat degradation taking place in heated liquid wax at 80°C (Table 1). Ronidazole turned out to be the most stable analyte with loss of 4% whereas dimetridazole was the least stable sulfonamide with degradation of 28%. Besides parent compounds, HMMNI was detected in the wax foundation at a very low concentration level (22 µg kg^{-1}) which is 0.22% of the initial fortification concentration of the parent drugs. The presence of this metabolite in the wax foundation might be explained by heat degradation of DMZ and/or RNZ.

Table 1. Concentrations of nitroimidazoles in the wax foundation made of beeswax fortified with MNZ, RNZ, DMZ and IPZ at the level of 10,000 µg kg^{-1}.

	Concentration found in wax foundation (µg kg^{-1})	Recovery (%)
MNZ	9,324	93
RNZ	9,646	96
DMZ	7,175	72
IPZ	8,926	89
HMMNI	22	n.a.
MNZOH	n.d.	n.a.
IPZOH	n.d.	n.a.

n.d., not detectable; n.a., not applicable.

In case of the analyses of nitroimidazoles in honey, the results showed that each of the tested analytes could migrate from contaminated beeswax to honey (Figures 1 and 2). Nitroimidazole residues seem to be quite persistent in wax throughout the monitored period. The highest concentration for MNZ (368 µg kg^{-1}) and RNZ (233 µg kg^{-1}) was observed at the fourth month whereas for DMZ (8 µg kg^{-1}) and IPZ (8 µg kg^{-1}) the highest content was found in the second month from the start of the experiment. In addition, low concentrations of HMMNI (0.63 – 1.75 µg kg^{-1}) were found in honey samples collected at each time point except for the first month. MNZOH and IPZOH residues were not detected in any of the analysed honey samples. The control honey samples collected from the hive participating in the experiment and from other hives placed in the same apiary were free of nitroimidazoles which showed that nitroimidazoles found in honey samples completely originated from the fortified beeswax.

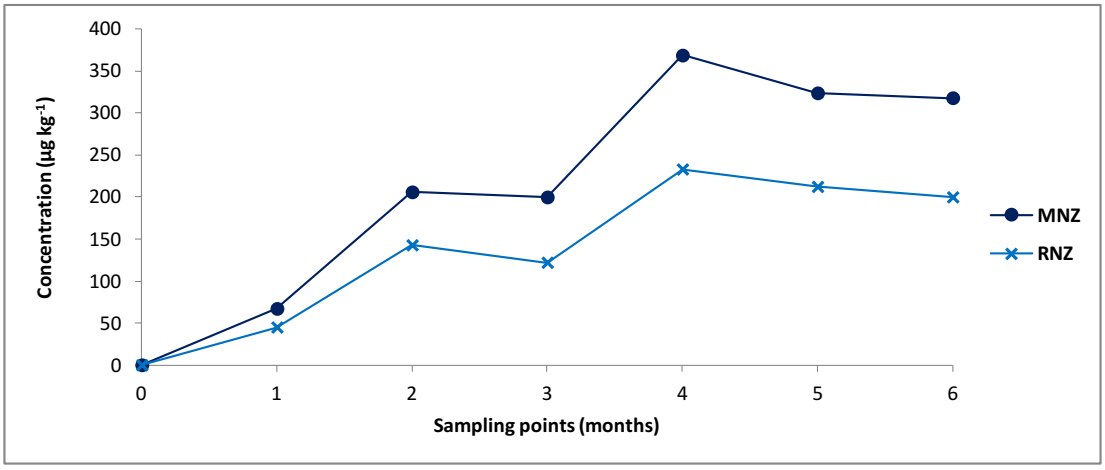

Figure 1. Concentrations of MNZ and RNZ in honey sampled from a honeycomb drawn out on a wax foundation contaminated with MNZ, RNZ at the level of 9,324 µg kg^{-1} and 9,646 µg kg^{-1}, respectively.

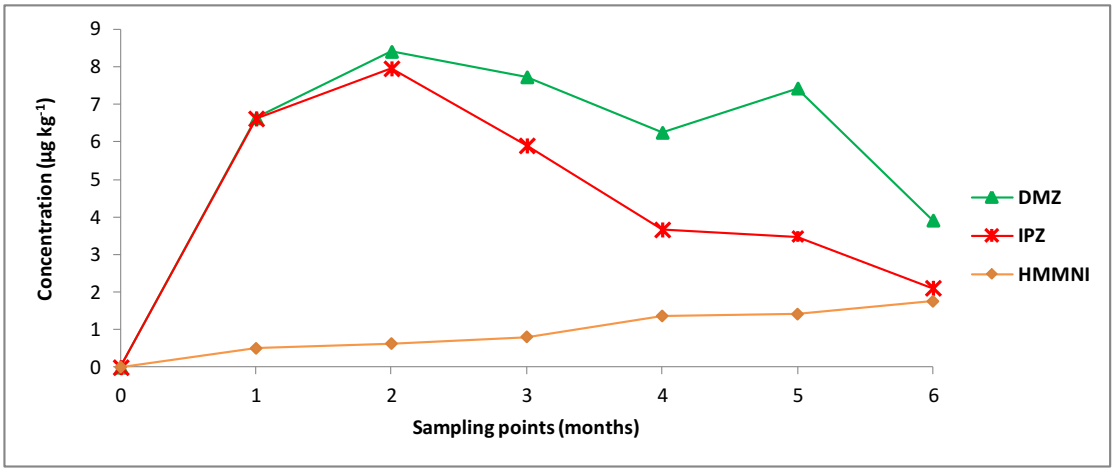

Figure 2. Concentrations of DMZ, IPZ and HMMNI in honey sampled from a honeycomb drawn out on a wax foundation contaminated with DMZ, IPZ and HMMNI at the level of 7,175 µg kg^{-1}, 8,926 µg kg^{-1} and 22 µg kg^{-1}, respectively.

Nitroimidazoles percentage transfer from beeswax to honey was based on the analyte concentration differences and the wax and honey masses in relation to the nitroimidazole concentrations determined in wax foundation. When we took into account that a frame completely filled with honey on both sides of the comb contained 110 g of beeswax and 2,448 g of honey and that this ratio was constant, then the maximum amount of MNZ, RNZ, DMZ, IPZ and HMMNI that migrated from contaminated wax foundation to the honey was 89.38%, 54.62%, 2.65%, 2.02% and 90.06%, respectively (Table 2). These results are comparable with literature data. Reybroeck *et al*. (2010) showed that the maximum transfer of sulfamethazine from contaminated beeswax to honey was 56.9 % of the same initial fortification concentration (10,000 µg kg^{-1}). However, the transfer of sulfamethazine was lower than MNZ, HMMNI and higher than RNZ, DMZ and IPZ.

Redistribution among the different compartments of the hive such as beeswax and honey is determined by the physicochemical properties of the compound and the main characteristics of the hive matrices. As beeswax is a hydrophobic material and honey a hydrophilic sugar solution, a partition process of nitroimidazoles between beeswax and honey can be comparable to that between *n*-octanol and water. A correlation between a logarithm of *n*-octanol/water partition coefficient ($logP_{oct/water}$) and the transfer of nitroimidazoles from beeswax to honey is possible but insignificant because of the lack of available experimental data and the variability of theoretical predicted $logP_{oct/water}$ values.

Table 2. Transfer (%) of nitroimidazoles that migrated from beeswax contaminated with MNZ, RNZ, DMZ, IPZ and HMMNI (at the level of 9,324 µg kg^{-1}, 9,646 µg kg^{-1}, 7,175 µg kg^{-1}, 8,926 µg kg^{-1} and 22 µg kg^{-1}, respectively) to the honey stored in the contaminated comb [a].

Analyte	Transfer (%) of nitroimidazoles at:					
	1 month	2 months	3 months	4 months	5 months	6 months
MNZ	16.27	49.92	48.44	89.38	78.49	77.08
RNZ	2.09	2.65	2.44	1.97	2.34	1.23
DMZ	10.54	33.47	28.60	54.62	49.75	46.85
IPZ	1.68	2.02	1.50	0.93	0.88	0.54
HMMNI	<25.70	32.28	41.46	70.03	72.55	90.06

[a] Based on the assumption that a frame completely filled with honey on both sides of the comb contains 110 g of beeswax and 2,488 g of honey and this ratio is constant.

Conclusions

From the obtained results of this study we have concluded that each of the tested nitroimidazoles could migrate from beeswax to honey kept in the contaminated comb. However, metronidazole has the most and ipronidazole has the least migration potential from contaminated beeswax to honey. Thus, the control of nitroimidazoles in beeswax should be carried out to ensure high quality and safety of beeswax itself, beeswax foundation and honey.

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TISSUE DISTRIBUTION AND RESIDUE DEPLETION OF METRONIDAZOLE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Abstract
Tissue distribution and residue depletion of metronidazole (MNZ) was studied in rainbow trout (*Oncorhynchus mykiss*) following oral administration of MNZ in feed at the average dose of 25 mg kg^{-1} body weight day^{-1} for 7 days. The concentrations of MNZ and its main metabolite, hydroxymetronidazole (MNZOH), in fish tissues were determined by LC-MS/MS. The drug was well distributed in tissues with maximum concentrations on day 1 post-administration. At this time, the mean MNZ concentrations in muscle, skin, kidney, liver and gill were 14,999, 20,269, 15,070, 10,102 and 16,467 µg kg^{-1}, respectively. MNZ was converted into MNZOH with the ratio of MNZOH:MNZ up to 7% in all fish tissues. It shows that MNZ itself is the main residue in rainbow trout. MNZ was detected at the level close to the decision limit (0.20 µg kg^{-1}) in muscle, skin and muscle with adhering skin up to 42 days while in kidney, liver and gill it was up to 28 days post-administration. MNZOH was eliminated more rapidly from fish tissues and it was present in muscle up to 21 days. The elimination half-lives of MNZ and MNZOH in rainbow trout tissues were 1.8-2.5 and 1.2-2.1 days, respectively.

Introduction
Metronidazole (MNZ) belongs to the group of 5-nitroimidazoles and has been used for the treatment of anaerobic bacterial and parasitic infections in veterinary and human medicine. It has been traditionally used for both prevention and treatment of histomoniasis and coccidiosis in poultry, genital trichomoniasis in cattle or haemorrhagic enteritis in swine (EMEA, 1997). In aquaculture, this compound appears to be highly effective as an oral medication for the treatment of hexamitosis, spironucleosis and ichthyobodosis (Noga, 2010). MNZ has been classified as suspected mutagen and carcinogen (International Agency for Research on Cancer, 1987). Because its residues, at whatever limit, in foodstuffs of animal origin might constitute a hazard to the health of the consumer, no MRL has been established for MNZ. As a consequence, it was banned for use in food-producing animals in 1998 (European Commission, 1998) and inserted into Annex IV of Regulation (EEC) No. 2377/90 repealed by Commission Regulation (EU) No. 37/2010 where this drug is listed in Table 2 (European Commission, 2010). In addition, as a result of Council Directive 96/23/EC, MNZ is classed as an A6 compound and its monitoring is required within the EU as well as by the countries exporting to the EU (European Commission, 1996).

In poultry, beef, swine and fish, MNZ is rapidly metabolised by oxidation of the side-chain in the C2 position of the imidazole ring to hydroxymetronidazole (MNZOH), which may have comparable toxicity to the parent form. Therefore, both the parent and the metabolite should be included for residue control purposes. In spite of the fact that the whole group of 5-nitroimidazoles is prohibited or not licensed for use in food-producing animals within the EU, cases of food products containing their residues on the EU market are quite common as evidenced by the EU reports of national monitoring plans and RASFF notifications. The non-compliant results for the most frequently reported 5-nitroimidazole compound - MNZ - have mainly been found in pigs and poultry, but there have been a few cases that have reported the presence of MNZ in rainbow trout (Wagil *et al*., 2015). MNZ seems to be more often considered a useful chemotherapeutic in aquaculture because it can be very effective at controlling particular infections, is cheap in comparison with other nitroimidazoles and is readily available for treating non-food fish (Noga, 2010). This could explain the occurrence of prohibited veterinary drug residues in the human food chain.

Limited data on MNZ residue depletion in fish are available. Sorensen and Hansen (2000) have reported the presence of MNZ and MNZOH residues in muscle and skin tissues of rainbow trout given feed containing MNZ shortly after the administration period but not 3 weeks later. However, no reports on the residue distribution of MNZ in other fish tissues have been published. Thus, the aim of the study was to evaluate the distribution and residue depletion of MNZ in rainbow trout muscle, skin, kidney, liver and gill after multiple oral administration. Moreover, the influence of sampling and storage conditions on the level of MNZ and its metabolite in muscle and skin were investigated.

Materials and Methods

Chemicals

All chemicals and solvents used were of analytical grade and suitable for liquid chromatography. Acetonitrile, acetone, methanol, formic acid, acetic acid and ammonium hydroxide (25%) were provided by JT Baker (Deventer, The Netherlands). Anhydrous sodium sulphate was purchased from Sigma-Aldrich (Steinheim, Germany). Pure water was obtained from a Milli-Q plus system from Millipore (Bedford, MA, USA). The reference standard of MNZ was from Riedel-de Haën (Seelze, Germany), whereas MNZOH, MNZ-13C2, 15N2 and MNZOH-D2 were sourced from Witega Laboratorien Berlin-Adlershof (Berlin, Germany). All isotopically labelled nitroimidazoles were of chemical and isotopic purity > 98% and used as internal standards (IS). Strata SCX (strong cation exchange) SPE cartridges (500 mg, 3 mL) were from Phenomenex (Torrance, CA, USA).

Animals and treatments

The rainbow trout exposure study was performed at the facility of the Department of Fish Diseases, National Veterinary Research Institute in Pulawy. The study protocol was approved by the II Local Ethics Committee on Animal Experimentation of the University of Life Sciences in Lublin, Poland (Decision No. 34/2014). One hundred and eighty rainbow trout (*Oncorhynchus mykiss*) with an average body weight of 150 ± 20 g were housed in glass tanks in a flow-through system with dechlorinated and aerated water before and during experiments. Water temperature was maintained at 11 ± 2°C. The fish were acclimatised for 14 days during which they were fed commercial antibiotic-free diet (BioMar). After 1 day of starvation, fish were orally treated with the average dose of 25 mg MNZ kg^{-1} body weight (bw) day^{-1} for 7 days, as recommended by Gratzek and Matthews (1992). To obtain a nominal dose, the concentration of MNZ in feed (0.25%) was calculated on the basis of an assumed. feed intake rate (1% of bw day^{-1}). Samples of muscle with and without adhering skin in natural proportions, skin alone, kidney, liver and gill were collected from 15 fish, which had been euthanised with a lethal dose of ethyl 3-aminobenzoate methane sulfonate (MS-222, Aldrich) at scheduled time points (1, 2, 3, 5, 8, 11, 14, 21, 28, 36 and 42 days posttreatment). The samples were placed immediately into ice and transferred to -25°C within 15 min. In addition, the samples of muscle without skin and skin alone were first stored at RT for 2 h, then at 4°C for 18 h and transferred to -25°C. Moreover, 15 rainbow trout were euthanised and killed before the start of drug administration and used as the control group.

Standard solutions

Individual stock solutions of analytical standards at 1,000 µg mL^{-1} were prepared in acetonitrile (stable for at least 12 months at -20°C). These solutions were combined and further diluted with 0.1% formic acid in water to prepare working standard solutions of nitroimidazoles (MNZ and MNZOH) and internal standards (MNZ-$^{13}C_2$, $^{15}N_2$ and MNZOH-D_2) and stored in amber glass at 4°C for 3 months when not in use.

Sample preparation

Sample preparations for LC-MS/MS were performed as previously described by Mitrowska *et al*. (2010) with some modifications. Fish sample (5 g) was weighed into a 100-mL polypropylene centrifugal tube and fortified with IS at the level corresponding to 2 µg kg^{-1} (samples containing ≤ 20 µg kg^{-1}) or 200 µg kg^{-1} (samples containing > 20 µg kg^{-1}). Following this, 50 mL acetonitrile were added into the tube and the sample was stirred for 1 min. Next, 5 g of anhydrous sodium sulphate were added and the tube was closed and shaken for 1 min followed by centrifugation at 2,200 *g* for 10 min at 4°C. Extracts of samples containing ≤ 20 µg kg^{-1} were passed through anhydrous sodium sulphate (10 g in Whatman No. 4 filter paper) directly into an SCX SPE cartridge preconditioned with the mixture of acetonitrile and acetic acid (95:5, v/v). To this 2.5 mL of acetic acid were added and the content was mixed and allowed to run through the SPE cartridge. The cartridge was washed sequentially with 2.5 mL of acetone, 5 mL of methanol and 5 mL of acetonitrile. After the last wash step, a strong vacuum was applied to dry the cartridge. To elute the analytes, 5 mL of the mixture of acetonitrile and ammonium hydroxide (95:5, v/v) were used. The eluate was evaporated until dry under a stream of nitrogen at 40°C and the residue was reconstituted in 0.2 mL 0.1% formic acid in water. In case of samples containing > 20 µg kg^{-1}, the SPE step was omitted and 2.5 mL of the acetonitrile extract obtained after centrifugation were evaporated until dry under a stream of nitrogen at 40°C and the residue was reconstituted in 1 mL of 0.1% formic acid in water.

The applicability of the sample preparation (previously validated for poultry tissues) to fish tissues (skin, kidney, liver and gill) was verified in each matrix by analysing blank fish samples fortified with MNZ and MNZOH at 2, 20, 200 and 2,000 µg kg^{-1} and calculating recovery and repeatability (European Commission, 2002).

LC-MS/MS analysis and quantification

The LC-MS/MS system comprised an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a degasser, a binary pump, a column heater, a switching valve and a QTRAP 5500 linear ion-trap quadrupole mass spectrometer with a Turbo V source (Applied Biosystems, Sciex Instruments, Toronto, ON, Canada). The instrument was controlled by Analyst software in version 1.6.2 (AB Sciex, Canada). Electrospray ionisation (ESI) in positive-ionisation mode was used with a spray voltage of 3,500 V and a source temperature of 400°C. Nitrogen was used as the nebuliser gas

(40 psig), turbo gas (40 psig) and curtain gas (30 psig). Quantification was done using MRM mode to monitor precursor to product ion transitions for all standards using unit resolution. Ions monitored were *m/z* 172, 128, 82 for MNZ, *m/z* 188, 126, 123 for MNZOH, *m/z* 176, 132 for MNZ-$^{13}C_2$ and *m/z* 190, 125 for MNZOH-D_2. The chromatographic separation was performed on a Kinetex XB octadecyl analytical column (2.6 µm, 150 × 2.1 mm) with an octadecyl guard cartridge (Phenomenex, Torrance, CA, USA). The mobile phase A (15%) containing 0.1% formic acid in acetonitrile and the mobile phase B (85%) containing 0.1% formic acid in water were used to perform the isocratic elution for 3 min. The flow rate of the mobile phase was controlled at 300 µL min^{-1}. The column temperature was maintained at 35°C whereas the injection volume was 20 µL for all the samples injected.

During the analysis of incurred samples of each matrix, quality control samples comprising blank and fortified samples were run for each sample series. Analyte concentrations in samples were calculated by comparing the ratio of an analyte base peak response to its corresponding isotopically labelled internal standard response with the same ratio in matrix-matched calibration curve standards in the range 0.2-20 µg kg^{-1} or 20-20,000 µg kg^{-1}. Highly concentrated samples were diluted in 0.1% formic acid in water as required and reanalysed.

Statistical analysis

Differences between MNZ and MNZOH concentrations obtained from the trout samples kept at different storage conditions were tested for significance using a paired t-test. Comparisons were considered statistically significant at the $p < 0.05$ level.

Results and Discussion

Quantification and method validation

The method used in this study in both low and high concentration ranges was validated according to EU Commission Decision 2002/657/EC criteria (European Commission, 2002). The results of the validation confirmed the versatility of the extraction and clean-up method, previously applied to poultry muscle, plasma, eggs (Mitrowska *et al*., 2010), and milk (Mitrowska *et al*., 2014) and proved its applicability to fish tissues. Additionally, the recovery and repeatability results demonstrated good suitability of the method to samples with high nitroimidazole concentration. The specificity of the method was evaluated by the analysis of 10 control samples of rainbow trout muscle, skin, kidney, liver and gill. No interfering peaks from endogenous compounds were found in the retention time of the target analytes. Matrix-matched calibration curves showed a good linearity for both MNZ and MNZOH in low (0.2-20 µg kg^{-1}) and high (20-20,000 µg kg^{-1}) concentration ranges with correlation coefficient above 0.998. The decision limit (CCα) for MNZ and MNZOH in each matrix was 0.2 µg kg^{-1}. This value was confirmed experimentally by injecting the extracts obtained from different fish samples fortified at the critical concentration. The percentage recoveries of MNZ and MNZOH in all tested matrices at two low (2 and 20 µg kg^{-1}) and two high (200 and 2,000 µg kg^{-1}) concentrations were in the range from 96.7% to 107.8% with CV < 6.6% under repeatability conditions. These values indicated that the proposed method applied in both low and high concentration ranges was accurate and precise and fit for the purpose of conducting depletion studies.

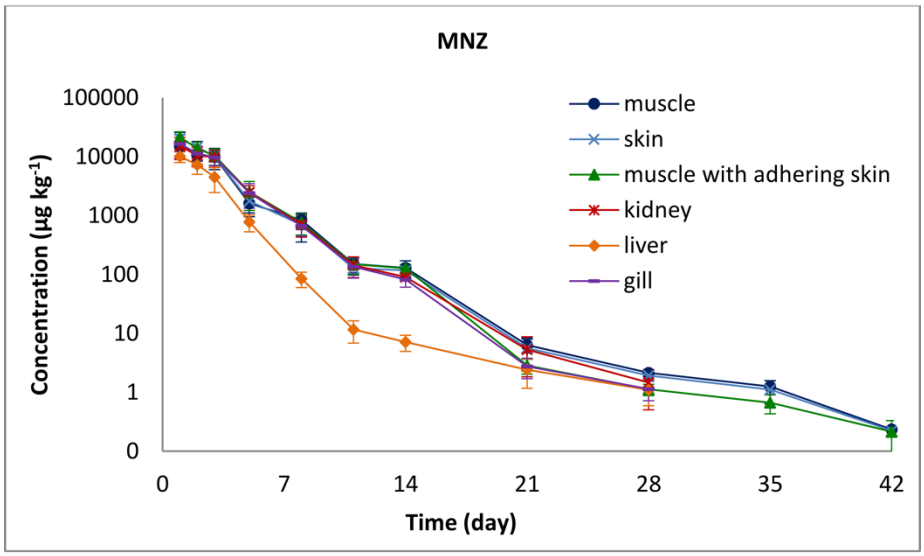
Distribution and depletion of metronidazole residue in different rainbow trout tissues

Figure 1 illustrates the depletion of MNZ and its main metabolite, MNZOH, from rainbow trout muscle, skin, muscle with adhering skin, kidney, liver and gill following oral administration of MNZ in feed at a dose of 25 mg kg^{-1}bw day^{-1} for 7 consecutive days. The drug was well distributed in tissues with relatively high concentrations found in muscle, skin and muscle with adhering skin. MNZ was rapidly converted into MNZOH. The ratio of MNZOH:MNZ was up to 7% in all fish tissue samples throughout the withdrawal period. Maximum MNZ and MNZOH concentrations in all tested tissues were found 1 day after the final dose of MNZ and decreased over time. The highest MNZ concentrations were present in muscle with adhering skin (21,533 µg kg^{-1}) while MNZOH accumulated at the highest concentrations in muscle alone (553 µg kg^{-1}). The lowest concentrations of both analytes were found in liver (10,101 µg kg^{-1}for MNZ and 202 µg kg^{-1} for MNZOH). MNZ residues were detectable in muscle, skin and muscle with adhering skin at the level close to the decision limit (0.20 µg kg^{-1}) up to 42 days. The parent drug was eliminated more rapidly from kidney, liver and gill where it was present up to 28 days. In case of the metabolite, its residues were observed after 21 days only in muscle alone. MNZOH was still found in skin alone, muscle with adhering skin, kidney and gill up to 14 days, while in liver up to 11 days post-administration. The elimination half-lives ($t_{1/2\beta}$) of MNZ and MNZOH were also determined in individual tissues assuming a single compartment model and first-order kinetics (Table 1). The calculated MNZ $t_{1/2\beta}$ (1.83-2.53 days) were similar to those of MNZOH (1.24-2.12 days), however MNZOH concentrations were lower than MNZ in all tissues of rainbow trout which received MNZ medicated feed.

When muscle was analysed with and without skin, higher MNZOH concentrations were determined in muscle alone than muscle with adhering skin at each sampling point (Table 2). Moreover, no MNZOH residues at all were detected in muscle with adhering skin on day 21 post-treatment, while MNZOH was still present in muscle alone at the mean concentration of 0.46 µg kg^{-1}. As can be seen in Table 2, MNZ is not distributed equally in muscle and skin throughout the withdrawal period. Within 5 days following the end of the treatment (days 1, 2, 3 and 5), the mean concentrations of the parent compound were

higher in muscle with adhering skin than in muscle alone, whereas at later sampling points (days 8, 11, 14, 21, 28, 35 and 42) the opposite situation occurred and higher MNZ levels were found in muscle alone. For fish, the edible products are defined as muscle and skin in natural proportions (European Commission, 2010). Therefore, they are the target tissue for determining withdrawal periods in fish. From the results obtained in this study it could be seen that when only muscle was analysed, higher MNZ and MNZOH concentrations occurred, and for a longer period of time, than in muscle with adhering skin. Thus muscle without skin could be more appropriate for the efficient residue control of MNZ in rainbow trout.

(A)



(B)

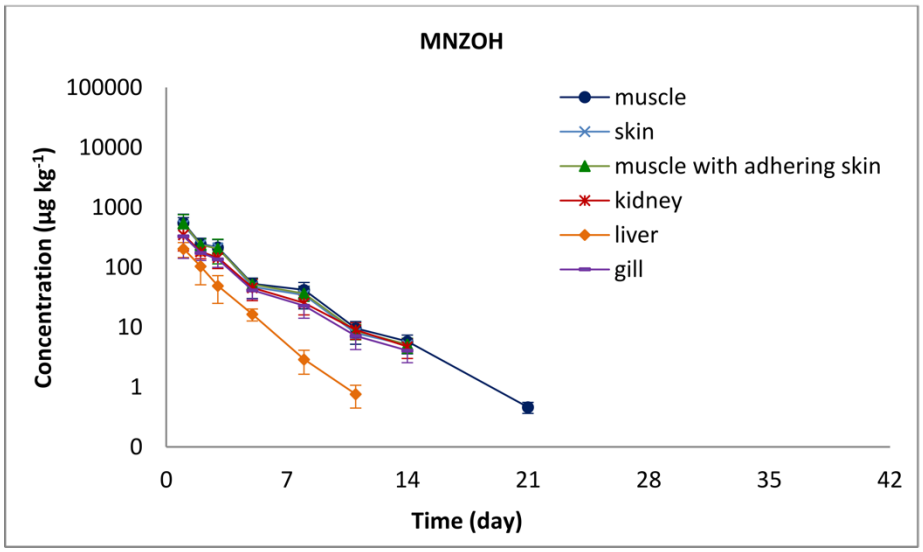


Figure 1. Depletion of (A) MNZ and (B) MNZOH in tissues of rainbow trout orally treated with a dose of 25 mg kg^{-1} bw day^{-1} for 7 days. Data are the mean of 15 fish per time point ± standard error of the mean (SEM).

Table 1. The elimination half-lives of MNZ and MNZOH in tissues of rainbow trout after oral administration of metronidazole in feed at a dose of 25 mg k^{-1} bw day^{-1} for 7 days.

Tissue	Elimination half-life[a] (day)	
	MNZ	MNZOH
Muscle	2.53	2.05
Skin	2.47	1.70
Muscle with adhering skin	2.37	1.98
Kidney	1.93	2.12
Liver	1.91	1.24
Gill	1.83	2.06

[a] *Elimination half-lives were calculated by fitting individual data from all data points after MNZ administration to $C_t = C_0 \times e^{-\beta t}$ where C_t is the mean concentration of the analyte at time t, C_0 is the mean concentration at the beginning of the decrease, β is the elimination rate in days^{-1}. Elimination half-lives ($t_{1/2\beta}$) in different tissues were calculated from the k_e values by the equation $t_{1/2\beta} = ln2/\beta$.*

Table 2. MNZ and MNZOH concentrations in rainbow trout muscle with and without adhering skin, and their ratio after oral administration of metronidazole in feed at a dose of 25 mg kg^{-1} bw day^{-1} for 7 days.

Time (day)	MNZ			MNZOH		
	Muscle (µg kg^{-1})	Muscle with skin (µg kg^{-1})	Ratio[a] (%)	Muscle (µg kg^{-1})	Muscle with skin (µg kg^{-1})	Ratio[a] (%)
1	14,999 ± 2,869	21,533 ± 4,906	1.44	553 ± 216	539 ± 218	0.98
2	10,722 ± 2,295	13,818 ± 3,751	1.29	245 ± 55	237 ± 61	0.97
3	9,918 ± 3,895	19,315 ± 3,177	1.04	214 ± 76	204 ± 90	0.95
5	1,603 ± 647	2,510 ± 1,288	1.57	53 ± 13	52 ± 12	0.97
8	841 ± 261	764 ± 296	0.91	42 ± 14	36 ± 9.3	0.87
11	150 ± 43	149 ± 48	0.99	9.5 ± 2.9	8.6 ± 2.4	0.90
14	129 ± 42	128 ± 21	0.99	5.7 ± 1.6	5.1 ± 1.4	0.89
21	6.3 ± 1.6	2.8 ± 0.8	0.45	0.46 ± 0.10	n.d.	
28	2.13 ± 0.29	1.2 ± 0.2	0.53	n.d.	n.d.	
35	1.25 ± 0.32	0.7 ± 0.2	0.54	n.d.	n.d.	
42	0.23 ± 0.07	0.21 ± 0.12	0.91	n.d.	n.d.	

[a] *Ratio of the compound concentration in muscle with adhering skin to the compound concentration in muscle alone; n.d., not detectable.*

There have been only two reports on depletion of MNZ residues in farmed aquaculture species following treatment (Sorensen *et al*., 2000; Gadaj *et al*., 2015). Sorensen and Hansen (2000) conducted his research on rainbow trout with a weight of approximately 100 g (smaller than those in the present study) kept at temperature conditions similar to those in this experiment (11°C). However, dosage of the drug was 10 times lower (2.5 mg kg^{-1}bw day^{-1}) and the administration period was shorter (5 days) in comparison with the treatment used in this study (25 mg kg^{-1} bw day^{-1} for 7 days). Those studies were limited only to two matrices, namely muscle and skin, in which the highest MNZ concentrations were found on the first day after the administration period (2,690 and 2,360 kg^{-1}, respectively). MNZOH was detected in muscle and skin samples at the same sampling point at the level of 44 and 31 µg kg^{-1}, respectively. The concentration levels in muscle and skin tissues were not significantly different and the ratio of MNZOH:MNZ was less than 2%. No residues could be detected 3 weeks after the administration of the drug. It should be pointed out that the LOD of the analytical method used for both compounds in muscle was 2.8 µg kg^{-1}, whereas LOD for MNZ and MNZOH in skin was 3 and 5 µg kg^{-1}, respectively. It is more than 10 times higher than the limit of the method used in this study.

Considering that residue concentrations and their depletion profiles are inevitably linked to the administered dose of the drug, it is not surprising that the results from the current study showed longer persistence of MNZ in rainbow trout following treatment since higher dosage and longer administration was applied to fish. Thus, we demonstrated that MNZ residues were still detectable at the level very close to the LOD of the method in both muscle and skin 6 weeks post-administration. Also, maximum MNZ and MNZOH concentrations found 1 day after the final dose of MNZ were approximately 10 times higher than those previously reported by Sorensen and Hansen (2000). In comparison with other aquaculture species, namely shrimp, the maximum concentration of MNZ measured within the individual shrimp tissue were found immediately after the immersion (equivalent to the dosage 1 mg kg^{-1} bw day^{-1}) and were in the range 361-4,189 µg kg^{-1}, whereas MNZOH residues were only detectable at that time point at the concentration level ranged from 0.28 to 6.6 µg kg^{-1} MNZ at a concentration between 0.12 and 1.00 µg kg^{-1} was still detected at 8 days post-administration.

The results reported here show that for animals treated with MNZ the parent drug itself is the most persistent and thus the most relevant marker residue and is in line with those previously reported in rainbow trout (Sorensen *et al.*, 2000), shrimp (Gadaj *et al*., 2015), and turkey (Polzer *et al*., 2004).

Moreover, the findings of this residue depletion study indicate that MNZ and MNZOH residues persist for prolonged periods of time after MNZ administration. Therefore, it should be underlined that the use of this nitroimidazole in rainbow trout is clearly inappropriate as significant drug residues will occur.

Influence of sampling and storage conditions

As indicated in Table 3, less careful sampling and storing conditions as those simulated in this study (muscle without skin and skin alone after sampling were kept at RT for 2 h following storing at 4°C for 18 h prior to deep-freezing at -25°C) could significantly influence the concentrations of both MNZ and MNZOH in target tissues. The decrease in the concentrations of target analytes in muscle and skin not directly frozen varied throughout the withdrawal period and was significant ($p < 0.05$) in some sampling points evaluated in this study. Moreover, due to the degradation process, no residues of the parent compound at all were detected in muscle not directly stored at -25°C at the last sampling point (day 42) when the mean concentration of MNZ at the level of 0.23 µg $kg^{-1}$was still present in muscle directly frozen. As was the case with turkey muscle (Polzer *et al*., 2004), MNZ and its metabolite also degrade in fish muscle and skin during storage in non-freezing conditions and care must be taken to ensure direct cooling immediately after sampling.

Table 3. MNZ and MNZOH concentrations in rainbow trout muscle and skin after oral administration of metronidazole in feed at a dose of 25 mg kg^{-1} bw day^{-1} for 7 days kept at different storage conditions (mean, n =15).

Time (day)	Muscle				Skin			
	Directly frozen at -25°C		Stored at room temperature for 2 h following storing at 4°C for 18 h prior deep-freezing at -25°C		Directly frozen at -25°C		Stored at room temperature for 2 h following storing at 4°C for 18 h prior deep-freezing at -25°C	
	MNZ	MNZOH	MNZ	MNZOH	MNZ	MNZOH	MNZ	MNZOH
1	14,999	553	14,756	535	20,269	508*	20,115	378*
2	10,722	245	10,482	237	14,518	238	12,337	207
3	9,918	214	8,033	209	10,144	212*	9,418	138*
5	1,604	53	1,520	50	1,760	47	1,710	42
8	842	42	690	36	701	34*	665	15*
11	150	9.5	148	8.0	127	7.9	120	6.4
14	129	5.7	123	5.3	117	5.0	113	4.8
21	6.3*	0.46*	3.8*	0.29*	5.6*	ND	3.4*	ND
28	2.1	ND	1.9	ND	1.9	ND	1.6	ND
35	1.2*	ND	0.34*	ND	1.1	ND	1.0	ND
42	0.23	ND	ND	ND	0.22	ND	0.22	ND

[a] *Statistically significant difference (paired t-test, $p < 0.05$); n.d., not detectable.*

Conclusions

The results of the current study demonstrate that MNZ is well distributed in rainbow trout muscle, skin, kidney, liver and gill and is rapidly converted into MNZOH after oral administration of MNZ in feed. The depletion studies show that the parent drug occurs at higher concentrations and is more persistent than MNZOH in all trout tissues, thus it is the most relevant marker residue. MNZ was detected at the level close to the decision limit (0.20 µg kg^{-1}) in muscle, skin and muscle with adhering skin up to 42 days, while MNZOH was present in muscle exclusively up to 21 days post-administration. For the reason that MNZ and MNZOH residues persist for prolonged periods of time after MNZ administration, the use of this nitroimidazole in rainbow trout is clearly inappropriate, as significant concentrations of drug residues will occur. When muscle without skin was analysed, higher MNZ and MNZOH concentrations were detected, and for a longer period of time, than in muscle with adhering skin. Thus, muscle alone could be more appropriate for the effective residue control of MNZ in rainbow trout. For the same reason, it is essential to ensure direct cooling immediately after sampling since MNZ and its metabolite degrade in fish muscle and skin stored in non-freezing conditions.

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CONFIRMATORY ANALYSIS OF ANTIBACTERIAL RESIDUES IN FOOD OF ANIMAL ORIGIN IN POLAND

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Abstract

The widespread and off-label use of antimicrobials in veterinary medicine may results in a risk of their residues in food of animal origin. The residues may pose a risk factor for public health. To consumer health protection a monitoring programmes for the control of the presence of these substances in food chain is defined and implemented in EU countries. In the national control programme of antibiotics and chemotherapeutics in Poland, the confirmatory analyses are performed by a multi-residue LC-MS/MS method. The method has been validated according to European Decision 2002/657/EC. The analytical determination was carried out in different biological material like muscle, milk and eggs. The confirmatory control of antibacterials include the ten groups of antibiotics and chemotherapeutics (sulphonamides, fluoroquinolones, tetracyclines, aminoglycosides, penicillins, cephalosporins, macrolides, lincosamides, diaminopyrimidines, pleuromutilins). During the period of 2013 and 2014, the residues of antibacterial substances were detected in 0.24% and 0.25% of tested samples, respectively. The most often determined groups of antibacterials were tetracyclines and aminoglycosides.

Introduction

In contemporary intensive animal production, many classes of antibiotics and antibacterial compounds are widely administered to food-producing animals for a variety of purposes. However, overuse of veterinary medicinal products, the misuse and failure in the following the label direction, as well as illegal use of some substances on farms can lead to their residues in edible tissues and animal origin products for consumption. Despite the positive effects of veterinary medicaments, inadequate use of antibiotics poses a potential health risk for consumers. A number of possible adverse effects of veterinary drug residues have been suggested, including: allergic/toxic reactions to residues, chronic toxic effects occurring with prolonged exposure to low levels of antibiotics or disruption of normal human flora in the intestine. Additionally, the widespread use of agricultural antibiotics can provoke the development of antibiotic-resistant bacteria in treated animals, what might then cause difficulty to treat human infections.

To public health protection, the European Union established the obligatory control of antibacterial residues in food chain with verified and validated methods. Additionally, the EU legislative framework defines maximum permitted limits of certain substances in food. Regulation (EU) No 37/2010 establishes maximum residues limits (MRLs) for veterinary medicinal products in food-producing animals and animal products (Commission Regulation (EU) No 37/2010).

For the screening detection of antibacterials in food in Poland, the microbial growth inhibition tests are used as the primary screening approach. Microbial methods allow determination of the presence on antibacterials in the samples. The results of microbiological screening system are always further examined by more specific and sensitive, chemical confirmatory methods used to identify individual antibiotics. The positive result in the screening test make the sample suspect and this result must be confirmed to identify the substance and establish whether its concentration is above the MRL or not. The confirmation must lead to negative result (compliant sample) or positive results (non-compliant sample).

In the analysis of antibacterials many liquid chromatography coupled with UV and/or fluorometric (FLD) detection has been applied for qualitative and quantitative analysis of single antibiotic groups (Bailac *et al*. 2006; Gajda *et al*. 2009). But over the last years LC-MS methods have been developed for analysis and screen a large number of analytes from a range of different classes of veterinary drugs (CiaoChan *et al*. 2010; Granelli *et al*. 2007; Gaugin-Juhel *et al*. 2009; Martos *et al*. 2010). This paper reports an investigation and results of the confirmatory control and contamination with antibacterial substances of *bovine*, *porcine*, poultry, milk and eggs in Poland. In the confirmatory analyses, a reliable and simple multi-class LC-MS/MS method for the analysis of 56 antibiotics from ten different classes (sulphonamides, fluoroquinolones, tetracyclines, aminoglycosides, penicillins, cephalosporins, macrolides, lincosamides, diaminopyrimidines, pleuromutilins) was used.

Materials and Methods

Sample collection

Sampling was carried out according to the requirements of Council Directive 96/23/EC. The calculation of number of samples to be analysed every year include the data about animal production, as well as the number of non-compliant results, detected in the preceding year. The samples were collected by district veterinary inspectors within the framework of official control, according to National Residues Control Plan. Samples were sent to the National Reference Laboratory (NRL) for drug

residues or to one of the six regional laboratories approved to performed analyses. During a period of two years (2013-2014) a total of 14,471 of *bovine*, *porcine*, poultry muscle and kidney samples, 3,759 milk samples and 572 eggs samples were analysed for antibacterials.

Reagents, materials and standards

All reagents used were of analytical grade. Acetonitrile, and methanol were obtained from J.T. Baker (the Netherlands). Ethylenediaminetetraacetic acid (EDTA) and oxalic acid were from POCH (Poland), trichloroacetic acid (TCA) was from Sigma – Aldrich (USA). Heptafluorobutyric acid (HFBA) was from Fluka, (USA). Water was deionised (>18MΩ cm^{-1}) by the Millipore system. Amoxicillin (AMOX), ampicillin (AMPI), penicillin G (PEN G), penicillin V (PEN V), oxacillin (OXA), cloxacillin (CLOX), nafcillin (NAF), dicloxacillin (DICLOX), cephapirin (CFPI), ceftiofur (CFT), cefoperazone (CFPE), cephalexin (CFLE), cefquinome (CFQ), cefazolin (CFZ), cefalonium (CFLO), sulfaguanidine (SGU), sulfadiazine (SDZ), sulfathiazole (STZ), sulfamerazine (SME), sulfamethazine (SMT), sulfamethoxazole (SMA), sulfamethoxypyridazine (SMP), sulfamonomethoxine (SMM), sulfadoxine (SDX), sulfaquinoxaline (SQX), sulfadimethoxine (SDMX), tylosin (TYL), erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), josamycin (JOS), tulathromycin (TUL), danofloxacin (DAN), difloxacin (DIF), enrofloxacin (ENR), ciprofloxacin (CIP), flumequine (FLU), sarafloxacin (SAR), marbofloxacin (MAR), norfloxacin (NOR), oxolinic acid (OXO), nalidixic acid (NAL), chlortetracycline (CTC), tetracycline (TC), doxycycline (DC), oxytetracycline (OTC), streptomycin (STRP), dihydrostrepromycin (DISTRP), gentamycin (GEN), paromomycin (PAR), spectinomycin (SPEC), kanamycin (KAN), neomycin (NEO), lincomycin (LIN), tiamulin (TIA), trimethoprim (TRM) and sulfaphenazole (IS) were from Sigma - Aldrich. Oasis HLB (60 mg, 3 ml) cartridges were obtained from Phenomenex, (USA), syringe filters 0.22 µm PVDF were from Restek (USA).

Analytical methods

Extraction – muscle and milk. For the measurement of antibacterials in muscle and milk, a liquid chromatography – tandem mass spectrometry (LC-MS/MS) method was used, previously described by Bladek *et al*. (2011). For the isolation of penicillins, sulphonamides, fluoroquinolones, penicillins, cephalosporins and macrolides, an extraction with acetonitrile was used. Two grams of muscle or milk sample were weighed in a polypropylene centrifuge tube and 8 mL of acetonitrile was added. Samples were mixed and centrifuged for 10 min at 4,200×g and 6 mL of the supernatant were transferred to glass tubes and placed in a nitrogen evaporator at 45°C to dryness. The dry residues were dissolved in 600 µL of 0.025% HFBA and filtered through 0.22 mm PVDF filters before analysis. The isolation of tetracyclines, aminoglycosides, lincosamides were performed with 5% trichloroacetic acid. To the two grams of muscle or milk, a 6 mL of solution of 5% TCA in water was added and the samples were mixed and centrifuged for 10 min at 4,200×g. The TCA extracts (1 mL) were taken and filtered through 0.22 mm PVDF filters.

Extraction – eggs. The analysis of antibacterials in eggs was previously presented by Bladek *et al*. (2012). Briefly, 1 g of previously homogenized egg was placed in a polypropylene centrifuge tube and 1 mL of 0.02 M of oxalic acid (pH 4), 0.5 mL of 0.1 M Na_2EDTA and 8 mL of acetonitrile were added. The samples were mixed and centrifuged for 10 min at 4,200xg and separated solutions were cleaned by passing through an OASIS HLB cartridge (60 mg). The cleaned extracts were evaporated to dryness in nitrogen evaporator at 45°C. The dry residues were reconstituted in 1 mL of of 0.025% HFBA and filtered through 0.22 mm PVDF filters.

Liquid chromatography - mass spectrometry analysis. The LC–MS/MS analysis was performed on the Agilent 1200 HPLC system (Agilent Technologies, Germany) connected to the AB Sciex API 4000™ triple quadrupole mass spectrometer (AB Sciex, Canada). The MS instrument was operated in the positive ESI mode. The following parameters were used in the tune mode: resolution Q1 and Q3 – unit; temperature – 500°C, curtain gas (N_2) – 20; nebuliser gas (N_2) – 40; collision gas (N_2) – 3; auxiliary gas – 50; ion spray voltage – 5,500 V. The Analyst® 1.5 (AB Sciex, Canada) software controlled the LC-MS/MS system and processed the data. MS data acquisition was performed in the multiple reaction monitoring (MRM) mode. The chromatographic separation was performed on the Luna octadecyl C18 column 150 × 2.0 mm, 3 µm (Phenomenex, USA) coupled with an octadecyl guard column (2 × 4 mm) (Phenomenex, USA), which was maintained at 30°C. The flow rate of mobile phase was 250 µL min^{-1}, the injection volume – 30 µL. The optimal composition of mobile phase A and B consisted of acetonitrile (A) and 0.025% HFBA (B). The elution was performed in a gradient mode. The mobile phase flow started at 95% of B, 60% B at 2 min, and 10% B at 9 min held for 2 min. The analytical column returned to the initial state and was recalibrated within 10 min before the next analysis, total run time was 24 min.

Validation

The method used in presented study was fully validated in accordance to Decision 2002/657/EC (Commission Decision 2002/657/EC). Detailed validation data are presented by Bladek *et al*. (2011 and 2012). Linearity, precision (repeatability and intra-laboratory reproducibility), recovery, decision limit (CCα), detection capability (CCβ), and the limit of quantification (LOQ) were evaluated. During validation process a good linear response was observed. The correlation coefficients for all analytes were over r > 0.99. Precision was determined by the repeated analysis (n=6) of samples spiked with analytes at con-

centrations corresponding to 0.5, 1.0 and 1.5 x MRL/VL (validation level), from run-to run during 1 day and 3 days, respectively. An overall coefficient of variations (CVs) for all analytes were in the range of 5% – 13.6% for repeatability and 9.2% - 18.2% for within-laboratory reproducibility. The validation results showed good accuracy with the average recoveries ranged from 88 % to 105%, depending on the analytes and matrix. The procedure was satisfactory sensitive with LOQ = 2 µg kg^{-1} for penicillins, LOQ at the range of 10 – 50 µg kg^{-1} for cephalosporins, LOQ = 5 µg kg^{-1} for sulphonamides, fluoroquinolones, macrolides, tetracyclines, lincomycin, trimethoprim and LOQ = 1 µg kg^{-1} for tiamulin. The CCα and CCβ were determined according to the MRL values established. For compounds with no MRL values, the decision limits at the level of LOQ were established. The decision limits for all analysed antibacterials are presented in Table 1.

Table 1. Decision limits (CCα) for all compounds depending on the analysed matrix.

Analyte	CCα [µg kg^{-1}] *bovine* muscle	CCα [µg kg^{-1}] *porcine* muscle	CCα [µg kg^{-1}] poultry muscle	CCα [µg kg^{-1}] milk	CCα [µg kg^{-1}] eggs
AMOX	60	60	60	5	30
AMP	59	59	59	5	30
PEN G	61	61	61	4	28
PEN V	2	28	28	2	26
OXA	322	322	322	35	30
CLOX	333	333	333	36	30
NAF	335	2	2	37	29
DICLOX	331	331	331	34	28
CFPI	57	25	25	71	29
CFT	1062	1062	1062	110	28
CFPE	32	32	32	55	30
CFLE	234	50	50	114	27
CFQ	56	56	10	25	27
CFZ	31	31	31	57	28
CFLO	13	13	13	24	27
SMT	114	114	114	118	27
SGU	103	103	103	105	27
SDZ	105	105	105	108	29
STZ	106	106	106	105	25
SME	109	109	109	118	28
SDMX	113	113	113	117	26
SMA	114	114	114	111	25
SMM	114	114	114	114	28
SDX	106	106	106	109	26
SQX	111	111	111	117	26
SMP	110	110	110	112	29
DC	115	115	115	23	28
OTC	110	110	110	113	238
TC	113	113	113	114	235
CTC	110	110	110	111	232
ERY	234	234	234	47	178
SPI	224	280	224	221	26
TYL	109	109	109	54	231
TIL	55	55	82	56	25
JOS	5	5	5	5	28
TUL	332	885	25	-	-
STRP	589	589	25	224	-
SPEC	325	325	325	228	-
DISTRP	583	583	25	217	-

Table 1. Continued.

PAR	572	572	572	250	-
KAN	115	115	115	160	-
NEO	570	570	570	1594	-
GEN	56	56	25	116	-
FLU	223	223	446	55	26
CIP	105	105	105	106	28
ENR	110	110	110	110	27
DIF	471	471	353	5	28
DAN	212	106	212	36	27
NOR	67	67	67	5	27
SAR	35	35	35	5	27
MAR	168	168	5	82	27
OXO	110	110	110	5	26
NAL	61	61	61	5	25
LIN	114	114	114	167	58
TIA	42	111	111	-	1028
TRM	58	58	58	-	-

Results and Discussion

A multi-residue method for determination of ten different classes of antibacterials was implemented in the National Residue Control Programme of Poland. The analyses were performed in the National Veterinary Research Institute, as well as in Regional Laboratories. The percentage of non-compliant samples for antibacterials in 2013 was 0.24% (9,230 tested samples, targeted samples). A similar result was observed in 2014, when only 0.25% of samples were reported non-compliant (9,572 tested samples). The number of tested samples in different species in 2013-2014 was as follows: 2,938 *bovine*, 7,483 *porcine* and 4,050 poultry samples. The highest percentages of non-compliant results (0.47 and 0.62% in 2013 and 2014, respectively) were reported for *bovine* samples. In case of *porcine* samples, 19 samples with violative residue concentrations were recorded, which is 0.25% of all *porcine* samples. The smallest number of non-compliant samples with antibacterials was for poultry (0.15% in 2013 and 0.09% in 2014). In milk only 5 non-compliant results among 3,759 analysed milk samples were reported (0.13%), while in eggs one sample positive for antibiotic was detected out of the 572 samples analysed (0.17%).

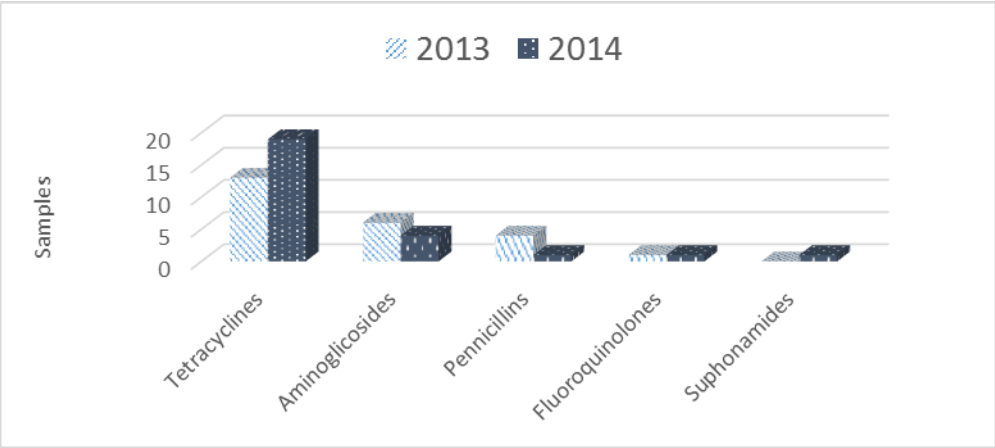

Figure 1. The groups of antibacterials confirmed during 2013-2014.

Within the period of 2013 - 2014, the most common detected group of antibacterials were tetracyclines. In the other samples, the presence of aminoglycosides and penicillins was observed. A few incidents of fluoroquinolones (enrofloxacin) and one result above MRL for sulphonamides (sulfadoxine) were also reported. In the group of tetracyclines, the most often detected compound was doxycycline. In the aminoglycosides group, dihydrostreptomycin and neomycin were found most frequently. Violative residues of amoxicillin and penicillin G, belonging to the group of penicillins, were also detected. The graphical presentation of the non-compliant results, depending on the group of antibacterials confirmed are presented in Figure 1. The highest percentage of non - compliant results in *bovine* concerned oxytetracycline, while in poultry and swine

the highest frequency was observed for doxycycline. In milk samples, mainly amoxicillin and penicillin G were detected. One doxycycline-positive egg sample was reported in 2014, which is a forbidden antibiotic in laying hens.

The European Union every year summarises and presents the monitoring data on the presence of residues of veterinary medicinal products and certain substances in live animals and animal products in the 28 Member States. The EU reported the total number of analyses carried out in 2013 for antimicrobials in targeted samples as 124,262, of which 258 (0.21 %) were non-compliant (EFSA Report, 2013). According to the latest EU Report, the frequency of non-compliant samples for antimicrobials were comparable to previous years (0.18 – 0.29%). For antibacterials in *bovine*s, 12 EU Member States reported a total of 74 non-compliant results. Similar to Poland, among the substances identified in *bovine*, oxytetracycline was most frequently detected. For antibacterials in pigs, a total of 89 non-compliant results were reported. The most frequent substances reported were: sulfonamides, dihydrostreptomycin, doxycycline and enrofloxacin. In poultry, 10 Member States reported a total of 30 non-compliant results. Similar to previous years, the most frequent substance reported was doxycycline. For antibacterials in milk a total of 16 non-compliant results with most commonly detected residues coming from the use of penicillins. In eggs, 11 non-compliant results were reported. Substances found were mainly: enrofloxacin, oxytetracycline and sulfadiazine. The percentage of non-compliant samples for antibacterials in different animal products reported by Member Stated in 2013 are presented in Figure 2.

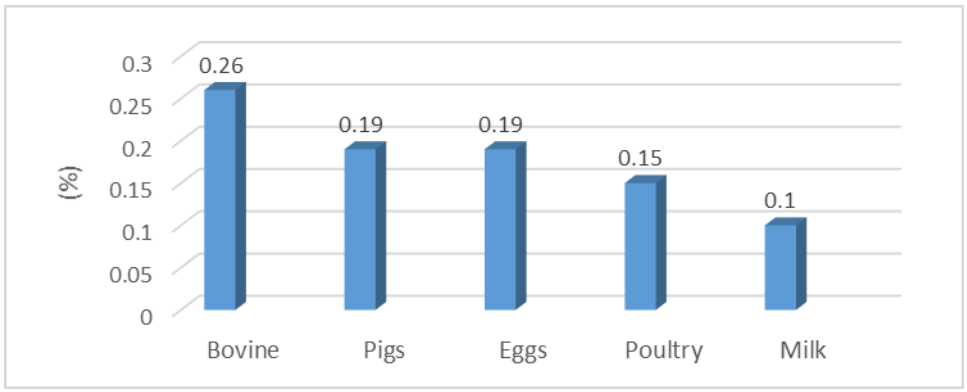

Figure 2. The percentage of non-compliant samples for antibacterials in animal products reported by Member States in 2013 (targeted samples).

The presented LC-MS/MS method, used in the official control, fulfil all criteria for confirmatory analytical methods established in the Council Directive 96/23/EC. The confirmatory methods must provide the information on chemical structure of analysed compounds. Thanks to mass spectrometry technique applied, this requirement is meet. The peak width at half maximum height within 90-110% range of the original width and the tolerance of ±2.5% the relative retention time (RRT) of analyte correspond to that of the calibration solution is controlled, as well as the internal standard in developed LC-MS/MS is used. Additionally, 4-points matrix matched calibration curve including blank sample are always analysed with each batch of tested samples. For each analytes, two multiple reaction monitoring (MRM) transitions are monitored in order to give four identification points. Besides, the satisfactory results of validation proved that the presented multi-residues LC-MS/MS method is efficient, precise, reliable and useful for confirmatory identification of many antibacterials in different animal origin products.

The proficiency testing is one of the most important elements of laboratory quality assurance. Participation in these testing allows comparison of in-house analytical results with results from other laboratories and gives confidence on the reliability of the method. The LC-MS/MS method, used as the confirmatory analytical tool, was checked in many proficiency testing (PT) programmes organised by European Union Reference Laboratory ANSES (Fougères, France), RIKILT Wageningen UR (The Netherlands) as well as commercial organizer FAPAS - Food Analysis Performance Assessment Scheme (Central Science Laboratory, UK). The antibacterials were tested in muscle and milk. In all studies, satisfactory z-score results were obtained. The results prove good accuracy, reproducibility, and selectivity of the developed method, implemented in the Polish National Control Programme.

Conclusions

Monitoring and control of antibacterials in food ensure the safety of supplied food. The use of an official and validated method is an important analytical tool to guarantee a good level of consumer health protection. The two years on monitoring results (2013-2014) indicate the small percentage of non-compliant results both in Poland and in European Union Member States. These results indicate that antibiotics and chemotherapeutics are rationally used in European Union countries. It should be kept in mind, that prudent use of antimicrobials will minimize the development of antimicrobial resistance, maximizes therapeutic effect and influences positively food safety assurance.

Acknowledgements
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COMBINING STANDARD ADDITION WITH BLANK ADDITION

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Abstract

A limitation of the well-known standard addition method is that only data points with abscissa values at and above zero are available so that there is no information on whether linearity holds below the addition level zero. This limitation can be overcome by combining standard addition with „blank addition". An illustrative example is provided.

Introduction

The standard addition method is a well-known approach to quantification in analytical chemistry. It is used in order to circumvent systematic errors that are due to the sample preparation procedure or are caused by matrix effects. The standard addition technique involves adding varying amounts of the analyte to sample portions of fixed mass or fixed volume and submitting those portions to the sample preparation procedure. After the final extract solutions are measured, the observed signals are linearly regressed on the spiked amounts. The original unknown amount is estimated by the opposite of the abscissa intercept of the fitted straight line. A limitation of this method is that only data points with abscissa values equal to and greater than zero are available so that there is no information on whether linearity holds below the spiking level zero. To overcome this limitation, standard addition can be combined with blank addition (Steliopoulos 2015). Blank addition means that defined mixtures of blank matrix and sample material are subjected to sample preparation to give final extract solutions. This contribution presents the use of the combined approach to quantify nandrolone in a male *bovine* urine sample.

Methods

For derivation of formulae and a description of the properties of the combined approach, see Steliopoulos (2015). Suppose that a liquid sample contains a particular compound of interest at unknown concentration. To determine this concentration, n aliquots of the sample are spiked with the analyte at concentration levels $(x_i)_{i=1,\ldots,n}$. Furthermore, sample material is diluted with blank matrix to give mixtures with sample volume fractions of $(k_j)_{j=1,\ldots,m}$ (ratios of sample volume to volume of total mixture). From each solution of the two series an aliquot of the same volume is taken and submitted to chemical analysis. On the basis of the data, the estimate for the ordinate intercept (b_0), the estimate for the slope (b_1), the estimate for the originally present unknown concentration (x^*) and the abscissa values of the blank addition points ($x_j^{\#}$) can be computed as follows:

$$b_0 = \frac{\sum_i x_i^2 (\sum_i y_i + \sum_j y_j k_j) - \sum_i x_i y_i \sum_i x_i}{\sum_i x_i^2 (n + \sum_j k_j^2) - (\sum_i x_i)^2}$$

$$b_1 = \frac{\sum_i x_i y_i \left(n + \sum_j k_j^2\right) - \sum_i x_i (\sum_i y_i + \sum_j y_j k_j)}{\sum_i x_i^2 (n + \sum_j k_j^2) - (\sum_i x_i)^2}$$

$$x^* = \frac{b_0}{b_1}$$

$$x_j^{\#} = (k_j - 1) x^*$$

y_i denotes the ith standard addition signal and y_j denotes the jth blank addition signal.

Example case

A male *bovine* urine sample was analysed for its content of the steroid nandrolone by an LC-MS assay. A series of six aliquots of the urine sample were spiked with the target analyte at levels of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 µg L^{-1}. Additionally, dilutions of the urine sample with nandrolone-free urine were prepared at sample volume fractions of 0.1, 0.3, 0.5, 0.7 and 0.9. Table 1 lists the observed signals, the estimated parameters, the estimate for the originally present unknown concentration, the 0.95 confidence interval for the originally present unknown concentration and the calculated abscissa values of the blank addition data points. Figure 1 displays the data points, the straight line fitted to the combined data set and the 0.95 confidence bands.

Table 1. Quantification of nandrolone in a bovine urine sample (all concentrations in µg L^{-1}).

x_i	y_i	k_j	y_j	b_0	b_1	$x_j^{\#}$	x^*	0.95 confidence interval
0.0	5394708	0.1	464139	4950060	2696536	– 1.65	1.84	[1.62; 2.08]
0.5	6061705	0.3	1497300			– 1.29		
1.0	7872740	0.5	2492913			– 0.92		
1.5	8822356	0.7	3069726			– 0.55		
2.0	10548385	0.9	4348496			– 1.65		
2.5	11587569							

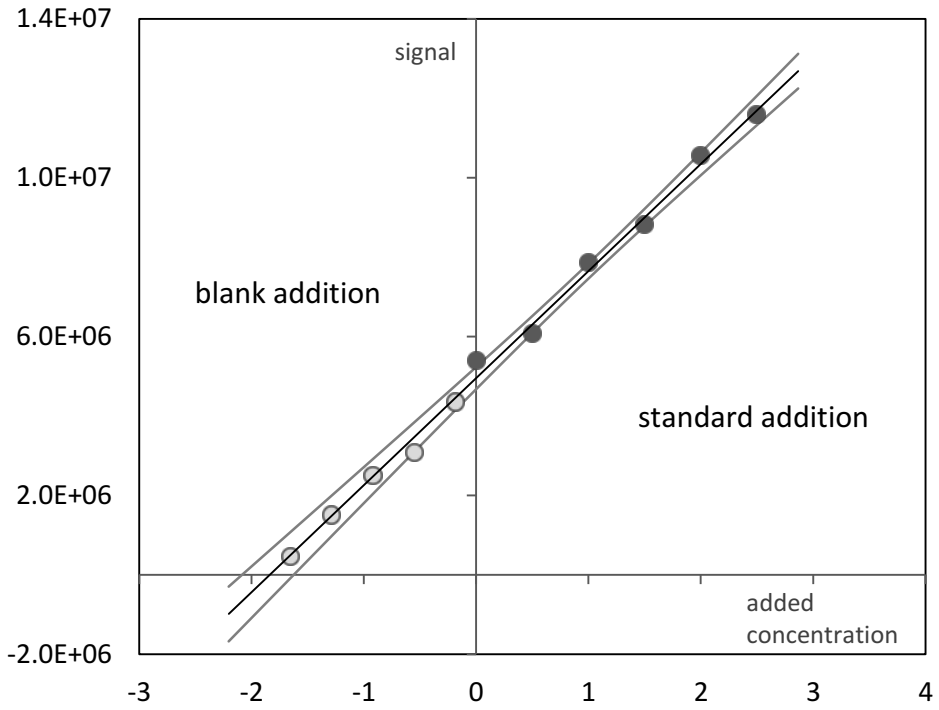

Figure 1. Quantification of nandrolone in a bovine urine sample by standard addition combined with blank addition (plot of the standard and blank addition line, the 0.95 confidence bands and the data points).

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DETERMINATION OF FLUBENZURONS IN SEAFOOD BY LC-MS-QQQ

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Abstract

Teflubenzuron and diflubenzuron are insecticides used against crustacean sea lice in aquaculture. Flubenzurons act by interfering with chitin synthesis and are thus effective against crustaceans during moulting. However, concerns about possible toxic effects to non-target marine species that undergo moulting have been raised. Hence, the use of flubenzurons was terminated in Norwegian aquaculture in 2002. However, due to reduced sensitivity for other anti-sea lice agents, flubenzurons were reintroduced in 2009. Both teflubenzuron and diflubenzuron are used for Salmonid fish species, and have a MRL of 500 and 1,000 ng g^{-1}, respectively (EU 37/2010). We have developed a method to determine both agents in fish by LC-MS-QQQ. Diflubenzuron-d4 is used as internal standard and extraction is performed with acetone. The analytes are detected and quantified by LC-MS-QQQ in negative multiple reaction monitoring (MRM) mode, using electro spray ionization (ESI). Each run lasts 4 min. The limit of quantification is 1.0 ng g^{-1} for both teflubenzuron and diflubenzuron. Preliminary results from the validation show linearity up to 1,000 ng g^{-1} for teflubenzuron and 20 ng g^{-1} for diflubenzuron. Assay imprecision is ≤ 17% and recovery ranges from 87% to 113%. The method has successfully been applied on samples of crustaceans, and for regulatory control of farmed fish.

Introduction

Salmon lice (*Lepeophtheirus salmonis*, Krøyer 1837) are ectoparasites that feed on blood, skin and mucous on salmon and trout (Pike, 1999). A large number of lice on a single fish may lead to reduced growth and welfare, and can cause mortality by osmotic stress and secondary infections by bacterial and viral pathogens. Therefore, sea lice are considered a major problem for the Atlantic salmon (*Salmo salar*) industry in Norway (Costello, 2006; Burka *et al*., 2012).

Fish infected with sea lice are treated by antiparasitic agents that are dissolved in water as bath treatment or administered orally via the feed (Lunestad *et al*., 2015). In Norway, diflubenzuron and teflubenzuron were frequently in use from late 1990`s until 2001. From 2002, emamectin and the pyrethroids, cypermethrin and deltamethrin were preferred, but due to reduced sensitivity for these drugs, the flubenzurons were reintroduced in 2009 (Norwegian Institute of Public Health, Oslo, Norway, www.fhi.no). Drug resistance has become an increasing problem over the last years (Aaen *et al*., 2015). To prevent development of drug resistance, it is recommended to alternate between different drugs and to avoid long-term and recurrent use of the same or similar drug. However, this is challenging with the few alternatives for drugs that are currently available.

Flubenzurons are administered via fish feed, and act by interfering with the synthesis of chitin in the salmon lice. Diflubenzuron and teflubenzuron are therefore effective against all stages of seas lice that undergo moulting (Campbell *et al*., 2006). However, concerns are raised about the potentially highly toxic effects on non-target marine biota that undergo moulting within their life cycle, including species like lobster, crabs and shrimps (Burridge *et al*., 2010; Samuelsen *et al*., 2014). In Atlantic salmon, both bioavailability and metabolism are low. Hence, in the periods of medication and immediately following a treatment most of the drug will be released from the fish as parent compound via faeces (EMEA, 1998; EMEA, 1999). Furthermore, a major part of the drug will end up in the surrounding environment associated with faeces or uneaten pellets (Selvik *et al*., 2002; Samuelsen *et al*., 2015).

Salmon lice have become an increasing problem over the last years, leading to an increasing use of antiparasitic agents (Norwegian Institute of Public Health, Oslo, Norway, www.fhi.no). Hence, official monitoring of flubenzurons in non-target marine biota as well as residues of diflubenzuron and teflubenzuron in farmed fish is required. The purpose of this work was to develop a method able to measure flubenzurons in non-target marine biota in addition to fish.

Materials and Methods

Chemicals and solutions

Diflubenzuron (analytical standard), teflubenzuron (analytical standard), heptane, acetone, acetonitrile and diethyl ether (all HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Diflubenzuron-d4 was purchased from CND Isotopes (Quebec, Canada). Tetrahydrofuran (HPLC grade) was purchased from Merck (Darmstadt, Germany). The water used was purified with a Milli-Q water purification system from Millipore. Stock solutions of teflubenzuron and diflubenzuron were prepared in tetrahydrofuran, at a concentration of 1 mg mL^{-1} and stored at 4°C. Working standards were prepared by dilution of stock solutions with a mix of acetonitrile:water (50:50, v/v).

Sample processing

Homogenised samples were spiked with internal standard (diflubenzuron-d4) and extracted with 5 mL acetone. The samples were homogenised using a whirl mixer, and placed in an ultrasonic bath (40 kHz) for 10 min before the samples were centrifuged for 3 min at 4,000 *g* (Eppendorf Centrifuge 5810 R, EppendorfAG, Hamburg, Germany). The resulting extracts were evaporated to dryness under nitrogen at 40°C, the residue dissolved in heptane (5 mL) and cleaned-up by solid phase extraction using a GX-271 ASPEC system from Gilson (Middleton, USA), as previously described (Samuelsen *et al*., 2004). Briefly, a solid-phase extraction column, Bond Elut Si, was conditioned with heptane prior loading of the sample. After loading, the column was washed with heptane, heptane/diethyl ether (95:5 v/v) and heptane/diethyl ether (90:10 v/v). The analyte and internal standard were eluted with heptane/diethyl ether (60:40 v/v). The eluate was evaporated to dryness using nitrogen at 40°C and dissolved in 250 mL of a solution of acetonitrile:water (75:25 v/v). The samples were filtered through a 0.45 µm syringe filter prior to analysis.

Instrumentation

The samples were analysed using an Agilent 1260 LC-system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent triple quadrupole mass spectrometer (Agilent Technologies). Masshunter software (Agilent Technologies) was used for LC-system control and data acquisition and processing. The analytical column used was an Agilent SB C18 2.1 x 50 mm, 1.8 µm (Agilent Technologies). The injection volume was 2 µL. The mobile phase was a mixture of acetonitrile and water (75:25 v/v) at an isocratic flow rate of 0.4 mL min^{-1} at 25°C. Each run lasted for 4.0 min. The analytes and internal standard were analysed by negative electrospray ionization (ESI) with Agilent jet stream in multiple reaction monitoring (MRM) mode. The following experimental parameters were used: drying gas temperature, 300°C; drying gas flow, 5 L min^{-1}; nebulizer pressure, 45 psi; capillary voltage, 3,500 V; sheath gas heater, 250°C; sheath gas flow, 11 L min^{-1}. Mass transitions and other instrument parameters are detailed in Table 1.

Table 1. Overview of parameters.

Compound	Transition (*m/z*)	Collision energy (V)	Fragmentor (V)	Comment
Diflubenzuron	309.0 -> 289.0	5	100	Quantifier
	309.0 -> 93.0	50	100	Qualifier
Teflubenzuron	379.0 -> 339.0	4	100	Quantifier
	379.0 -> 195.9	18	100	Qualifier
Diflubenzuron-d4	313.0 -> 293.1	5	100	Internal standard

Method validation

We assessed the linear range and limit of quantification (LOQ) by adding the analytes to salmon muscle, shrimps and lobster. Limits of quantification (LOQ) was defined as the lowest concentration that gave peaks with signal-to-noise (S/N) ratios of 3, and LOQ were defined as the lowest concentrations that gave peaks with S/N ratios of 9.

Results and discussion

The flubenzuron assay is based on LC-MS-QQQ and is optimized to detect and quantify diflubenzuron and teflubenzuron in seafood (Figure 1). Each analyte was identified by two transitions, the ratio between the fragment ions, and the retention time. The criteria for the ions and retention time according to 2002/657 were fulfilled. The sensitivity for diflubenzuron was highest with positive ionisation whereas the sensitivity for teflubenzuron increased with negative ionisation. Although switching between negative and positive ionisation is possible, this led to a low reproducibility for teflubenzuron, probably due to the use of diflubenzuron-d4 as internal standard for teflubenzuron. Therefore, both diflubenzuron and teflubenzuron were analysed in negative ionisation (Figure 1). The mobile phase consisted of acetonitrile and Milli-Q water. Other buffers like acetic acid, formic acid, ammonium acetate, and ammonium hydroxide were tested. However, the use of Milli-Q water gave good intensity for both flubenzurons, and increased reproducibility for teflubenzuron compared to the other buffers.

The method development of the flubenzuron assay is still in progress. Hence, the presented results are preliminary. The LODs were determined to 0.3 ng g^{-1}, and the LOQs were determined to 1.0 ng g^{-1} for both flubenzurons. Teflubenzuron is linear up to 1,000 ng g^{-1} (R = 0.993). Diflubenzuron has been tested for linearity up to 20 ng g^{-1} (R = 0.999), but the linearity will be examined up to a concentration of 1,000 ng g^{-1}. Preliminary results for imprecision at the LOQ level give a repeatability of 8% and 11%, and within laboratory reproducibility of 10% and 17% for diflubenzuron and teflubenzuron, respectively. Recovery varies between 87-112% for diflubenzuron and 95-113% for teflubenzuron.

Conclusions

In summary, we are developing a LC-MS-QQQ method for the determination of diflubenzuron and teflubenzuron in seafood. Preliminary results show that the method is suitable for official monitoring of residues of diflubenzuron and teflubenzuron in farmed fish, and for studying the outcome of flubenzurons in non-target marine biota.

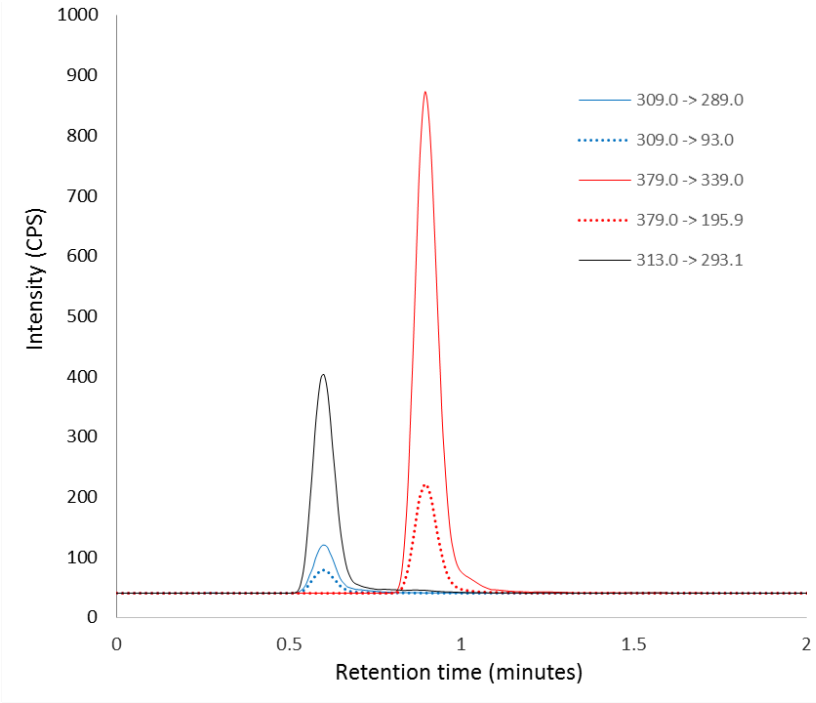

Figure 1. LC-MS-QQQ chromatogram of diflubenzuron and teflubenzuron in Atlantic salmon.

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CONFIRMATORY METHOD FOR THE DETERMINATION OF ACIDIC AND BASIC NSAIDS IN MILK BY UPLC-MS/MS

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Abstract
A confirmatory method was developed and validated for NSAIDs in milk based on QuEChERS, which includes an enzymatic hydrolysis with β-glucuronidase/aryl sulfatase, a clean-up by dispersive SPE and a measurement by UPLC-MS/MS. The method is suited to control several NSAIDs, including authorised substances with established MRLs and non-authorised substances with recommended concentrations. For validation, the alternative approach according to Commission Decision 2002/657/EC was used and the method proved to be rugged against *e.g*. changes with regard to the operator, the extraction procedure and the storage of the final extract. Analyte recoveries from 92.1 % to 112.1 % and within-laboratory reproducibilities from 4.5 % to 16.5 % could be reached with this method.

Introduction
Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs with anti-inflammatory, analgesic and antipyretic effects. They are routinely used in veterinary medicine. As a consequence of animal treatment and of sometimes inadequate withdrawal times, residues of NSAIDs can be found in various tissues or liquids and may, therefore, enter the human food chain. To ensure food safety, established maximum residue limits (MRLs) for authorised substances as well as a potential misuse of prohibited or non-authorised substances must be subject to controls. For this control the European Union (EU) laid down MRLs for numerous substance-matrix combinations, including some NSAIDs in milk, in Commission Regulation (EU) No. 37/2010 (European Commission, 2009). In addition, for some of the non-authorised NSAIDs recommended concentrations were laid down in the CRL guidance paper (7 December 2007) (CRLs, 2007) in order to improve and harmonise the analytical methods for NSAIDs throughout the EU.

To date, most methods available for non-steroidal anti-inflammatory drugs (NSAIDs) in milk consider either basic or acidic NSAIDs because of their different chemical characteristics. Two separate sample preparation procedures are applied very often to cover both NSAID classes. Up to now, only few methods allow the combined analysis of acidic and basic NSAIDs (Kaufmann *et al.*, 2014; Van Pamel & Daeseleire, 2015). However, these methods could be further improved by: *i*) an enzymatic hydrolysis step which is milder than a chemical hydrolysis and *ii*) a clean-up step. A hydrolysis step potentially releases residues from matrix components (Cooper *et al.*, 2001, Jedziniak *et al.*, 2013); the clean-up step is expected to remove major interferences causing *e.g*. matrix effects.

The aim of this study was the development of a confirmatory method for the determination of acidic and basic NSAIDs in milk by UPLC-MS/MS. QuEChERS is a comprehensive extraction method commonly used in pesticide residue analysis covering compounds with very heterogeneous chemical characteristics. For this reason, the application of QuEChERS for the extraction of acidic as well as basic NSAIDs in milk appears promising. In addition, the NSAID analysis should comprise a hydrolysis and a clean-up step.

Materials and methods

Standards and Chemical Reagents
The NSAIDs antipyrine (A), aminoantipyrine (AA), celecoxib (CLX), formylaminoantipyrine (FAA), ketoprofen (KTP), methylaminoantipyrine (MAA), oxyphenbutazone (OPB), phenylbutazone (PBZ) and rofecoxib (RCX) were bought from LGC Promochem (Wesel, Germany). Sigma Aldrich (Taufkirchen, Germany) provided carprofen (CPF), dimethylaminoantipyrine (DMAA), flufenamic acid (FFA), flurbiprofen (FIPF), indoprofen (IDP), meclofenamic acid (MCA), mefenamic acid (MFA), niflumic acid (NFA), 4-hydroxyantipyrine (OH-A), tolfenamic acid (TFA), ibuprofen (IP)-$^{13}C_6$ and salicylic acid (SA)-$^{13}C_6$. 5-hydroxyflunixin (FLUOH), isopropylaminoantipyrine (IPAA), meloxicam (MLX), A-D_3 and DMAA-$^{13}C_2$ were supplied by Essex Tierarznei (Munich, Germany), Hangzhou Dayangchem (Hangzhou City, China), Chemos GmbH (Regenstauf, Germany), Dr. Ehrenstorfer (Augsburg, Germany) and Euriso-top (Saarbrücken, Germany), respectively. Diclofenac (DC), IP and SA were purchased from MikroMol GmbH (Karlsruhe, Germany). Flunixin (FLU) and naproxen (NP) were provided by Riedel-de Haën (Seelze, Germany). Witega (Berlin, Germany) supplied acetylaminoantipyrine (AAA), firocoxib (FCX), vedaprofen (VDP), AA-D_3, AAA-D_3, CPF-D_3, DC-$^{13}C_6$, FCX-D_6, FFA-$^{13}C_6$, FLU-D_3, FLUOH-D_3, IPAA-D_6, KTP-D_3, MAA-D_3, MCA-$^{13}C_6$, MFA-$^{13}C_6$, MLX-D_3, NFA-$^{13}C_6$, NP-D_3, OPB-$^{13}C_6$, PBZ-$^{13}C_{12}$, PRX-D_3, TFA-$^{13}C_6$ and VDP-D_3.

The solvents water, methanol (MeOH) and acetonitrile (ACN) were purchased from Biosolve (Valkenswaard, The Netherlands). MilliQ water was supplied by a Milli-Q Advantage A10 system. Acetic acid, ammonium acetate, magnesium sulphate, sodium citrate tribasic dehydrate and sodium citrate dibasic sesquihydrate were provided by Sigma Aldrich (Taufkirchen, Germany). Sodium chloride was purchased from VWR (Leuven, Belgium). Merck (Darmstadt, Germany) provided sodium acetate and β-glucuronidase/aryl sulfatase (from *Helix pomatia*; stabilised aqueous solution; β-glucuronidase 30 U mL^{-1}, pH 3.8, 38°C; aryl sulfatase 60 U mL^{-1}, pH 6.2, 38°C).

Preparation of Analyte Solutions

The analyte stock solutions were prepared in MeOH:ACN (1:9, v/v). The working solution contained a mixture of analytes and was freshly prepared in ACN:water (1:9, v/v). The analyte concentrations in the mix corresponded to 2.5 µg mL^{-1} for TFA, MAA, OH-A, AA, 2.0 µg L^{-1} for AAA, FLUOH, SA, 0.75 µg mL^{-1} for MLX, VDP, FIPF, RCX, 0.005 µg L^{-1} for DC, 0.1 µg L^{-1} for MFA, PBZ, PRX, IDP, OPB, NP, FLU, FFA, CPF, KTP, A, DMAA, FAA, IPAA and 0.5 µg L^{-1} for IP, FCX, CLX, MCA, NFA. The working solutions for the internal standards (ISTD) were prepared correspondingly.

Sample Preparation

For the spiking experiments, 2 g of fresh raw milk or 0.25 g of lyophilised raw milk reconstituted with 0.175 g of milliQ water was fortified with the ISTD mix and the analyte mix, vortexed and equilibrated for at least 15 min. Subsequently, 200 µL of 3.3 M sodium acetate buffer (pH 6) and 20 µL β-glucuronidase/aryl sulfatase were added and the sample was hydrolysed for 60 min at 37°C in a water bath. Then, 2 mL ACN were added to the sample. Pre-mixed QuEChERS salts (Chromabond QuEChERS Mix 1, Macherey-Nagel, Düren, Germany) or self-weighed QuEChERS salts (0.8 g $MgSO_4$, 0.2 g NaCl, 0.1 g sodium citrate dibasic sesquihydrate and 0.2 g sodium citrate tribasic dehydrate) were added and the mixture was immediately vortexed. Then the mixture was shaken for 15 min using an overhead shaker and centrifuged (2,840 *g*, 5 min, RT). The upper layer (ACN layer) was almost quantitatively transferred to a centrifuge tube containing 250 mg of Sepra C18-E SPE bulk material (Phenomenex, Aschaffenburg, Germany). For clean-up the sample was shaken for 5 min using an overhead shaker and centrifuged (2,840 *g*, 5 min, RT). An aliquot of 1.00 mL was evaporated to dryness at 40°C in a gentle nitrogen stream of max. 300 mL min^{-1} (evaporation station, Barkey, Bielefeld, Germany). Subsequently, the sample was reconstituted with 250 µL of ACN:water (1:9, v/v) and centrifuged (21,500 *g*, 15 min, RT). If appropriate, an aliquot of the extract was diluted (1:19, v/v) using ACN:water (1:9, v/v). Finally, the extract was transferred into HPLC vials.

Liquid Chromatography Tandem Mass Spectrometry

Analyses were performed using a 1290 Infinity UPLC system from Agilent Technologies (Waldbronn, Germany). For chromatography, a 2.1 x 100 mm, particle size 1.8 µm Acquity UPLC HSS C18 column with guard protection (Waters, Eschborn, Germany) was used. Eluent A was 100% water and eluent B 5% water and 95% ACN, both eluents containing 5 mM ammonium acetate and 0.05% acetic acid (v/v). A gradient elution was used, starting with 95% of eluent A, holding eluent A for 1.5 min, decreasing eluent A first to 45% within 13.5 min and second to 5% within 1 min, and then holding 5% A for 3 min followed by equilibration of the column with eluent A for 3.5 min. A flow rate of 0.5 mL min^{-1} was applied and the injection volume was 10 µL.

Electrospray ionisation (ESI) MS/MS data were acquired on an API 6500 QTrap MS/MS system (Sciex, Foster City, USA). The source parameters were +5,000 V or -4,500 V ion spray voltage, 400 °C source temperature, 45 psi curtain gas and 70 psi GS1 as well as GS2.The mass spectrometer operated in fast polarity switching mode and the data were acquired in advanced scheduled multiple reaction monitoring (sMRM) mode for the SRM transitions listed in Table 1 using the Analyst 1.6.2 software (Sciex, Foster City, USA).

Calibration and Quantification

Eight-point solvent calibration curves with a final volume of 500 µL were prepared in water:ACN (9:1, v/v) by pipetting 15, 20, 25, 35, 55, 85, 130, 200 µL of the analyte working solution and 40 µL of the ISTD working solution. Matrix calibration curves were prepared using the same volumes of the analyte mix and the ISTD mix and following the same procedure as described for sample preparation.

The quantification of the analytes was realised by calculating the peak area ratio of the analyte compared to the ISTD and using calibration curves obtained by simple linear regression. The specific ISTD which is applied for the quantification of a defined analyte is listed in Table 2. Data evaluation was carried out with MultiQuant 3.0.1 (Sciex, Foster City, USA).

Table 1. Compound-dependent parameters for the monitored analytes. The quantifier is indicated by an asterisk.

Analyte	Precursor ion (*m/z*)		Fragment ion (*m/z*)	Declustering potential [V]	Collision energy [V]	Cell exit potential [V]	Retention time [min]
A	$[M+H]^+$	189.0	56.1/ 76.9*	81	51/ 51	10/ 12	5.9
A-D_3	$[M+H]^+$	192.1	59.1	96	55	10	5.8
AA	$[M+H]^+$	204.0	56.0*/ 159	46	41/ 17	8/ 12	6.0
AA-D_3	$[M+H]^+$	207.0	58.9	46	55	8	6.0
AAA	$[M+H]^+$	246.1	104/ 228.1*	51	29/ 19	8/ 12	4.6
AAA-D_3	$[M+H]^+$	249.1	231.1	71	19	10	4.6
CPF	$[M-H]^-$	271.9	225.9/ 227.9*	-21	-38/ -14	-13/ -13	14.0
CPF-D_3	$[M-H]^-$	275.0	231.1	-51	-16	-10	14.0
DC	$[M+H]^+$	296.1	214.0*/ 250.0	41	45/ 17	12/ 10	13.9
DC-$^{13}C_6$	$[M+H]^+$	302.0	256.0	41	17	10	13.9
DMAA	$[M+H]^+$	232.1	111.1/ 113.0*	31	19/ 17	8/ 14	6.5
DMAA-$^{13}C_2$	$[M+H]^+$	234.1	115.1	41	17	8	6.5
FAA	$[M+H]^+$	232.1	82.9/ 214.1*	51	25/ 19	12/ 10	4.5
FCX	$[M+H]^+$	337.1	236.9/ 283.1*	61	21/ 13	10/ 12	13.1
FCX-D_6	$[M+H]^+$	343.1	288.9	61	13	16	13.0
FFA	$[M-H]^-$	280.1	215/ 236.0*	-51	-40/ -24	-13/ -8	14.8
FFA-$^{13}C_6$	$[M-H]^-$	285.9	221.0	-53	-42	-13	14.8
FLU	$[M-H]^-$	295.0	209/ 251.1*	-30	-38/ -22	-11/ -11	11.4
FLU-D_3	$[M-H]^-$	298.0	254.2	-65	-24	-11	11.4
FLUOH	$[M-H]^-$	310.9	227/ 267.1*	-40	-32/ -24	-9/ -11	10.4
FLUOH-D_3	$[M-H]^-$	313.9	270.0	-40	-24	-13	10.4
IP	$[M-H]^-$	205.0	159/ 161.1*	-26	-10/ -12	-9/ -9	15.1
IP-$^{13}C_6$	$[M-H]^-$	211.0	167.1	-30	-10	-7	15.1
IPAA	$[M+H]^+$	246.1	56.0*/ 96.0	66	47/ 23	8/ 14	7.5
IPAA-D_6	$[M+H]^+$	252.1	56.0	46	49	8	7.4
KTP	$[M+H]^+$	255.0	77/ 209.1*	81	65/ 19	10/ 14	11.5
KTP-D_3	$[M+H]^+$	258.0	212.0	81	19	14	11.5
MAA	$[M+H]^+$	218.1	56*/ 97.0	26	41/ 17	8/ 12	5.9
MAA-D_3	$[M+H]^+$	221.1	55.9	51	43	8	5.8
MCA	$[M-H]^-$	294.0	214.2/ 258.1*	-51	-28/ -20	-13/ -15	15.6
MCA-$^{13}C_6$	$[M-H]^-$	300.0	264.0	-51	-20	-15	15.6
MFA	$[M-H]^-$	239.9	191.9/ 195.9*	-23	-36/ -24	-11/ -11	15.6
MFA-$^{13}C_6$	$[M-H]^-$	245.9	202.0	-51	-24	-11	15.6
MLX	$[M-H]^-$	349.9	146.0*/ 285.9	-31	-28/ -20	-9/ -7	10.0
MLX-D_3	$[M-H]^-$	353.0	289.0	-31	-20	-7	10.0
NFA	$[M-H]^-$	281.0	176.9/ 237.0*	-31	-44/ -24	-11/ -13	11.9
NFA-$^{13}C_6$	$[M-H]^-$	286.9	243.1	-60	-22	-11	11.9
NP	$[M-H]^-$	229.0	169/ 185.0*	-35	-40/ -10	-9/ -7	11.9
NP-D_3	$[M-H]^-$	232.0	169.0	-30	-38	-7	11.8
OH-A	$[M+H]^+$	205.0	56.0*/ 146.0	56	39/ 19	8/ 8	6.8
OPB	$[M-H]^-$	323.1	134/ 295.2*	-41	-36/-28	-8/ -15	12.5
OPB-$^{13}C_6$	$[M-H]^-$	328.9	301.0	-56	-26	-9	12.5
PBZ	$[M-H]^-$	307.1	130.8/ 279.0*	-41	-32/ -26	-7/ -15	15.1
PBZ-$^{13}C_{12}$	$[M-H]^-$	319.0	291.2	-56	-26	-13	15.1
PRX	$[M-H]^-$	330.1	146.0*/ 266.0	-46	-26/ -18	-7/ -7	9.9
PRX-D_3	$[M-H]^-$	333.0	269.0	-35	-18	-13	9.9
RCX	$[M+H]^+$	315.0	189.1/ 269.0*	71	73/ 27	18/ 12	11.2
SA	$[M-H]^-$	136.9	64.9/ 92.9*	-15	-38/ -22	-9/ -11	3.0
SA-$^{13}C_6$	$[M-H]^-$	142.9	98.9	-30	-24	-7	3.0
TFA	$[M+H]^+$	262.0	179.9/ 209.1*	21	55/ 39	22/ 6	15.7
TFA-$^{13}C_6$	$[M+H]^+$	268.2	215.1	46	39	10	15.7
VDP	$[M+NH_4]^+$	300.2	155.1/ 201.1*	56	27/ 15	10/ 10	16.6
VDP-D_3	$[M+NH_4]^+$	303.1	158.1	46	29	18	16.6

Table 2: Validation parameters according to Commission Decision 657/2002/EC calculated with the InterVal software.

Analyte/ ISTD	Target NSAID mass fractions [µg kg^{-1}]	Limit [µg kg^{-1}]		CCα [µg kg^{-1}]	CCβ [µg kg^{-1}]	Apparent recovery at CCα [%]	RSD_r [%]	RSD_{wR} [%]
A/ D_3-A	1 - 2 - 3 - 4	-	-	1.30	1.52	92.1	4.7	8.1
AA/ D_3-AA	25 - 50 - 75 - 100	-	-	41.25	56.93	95.2	7.7	16.3
AAA/ D_3-AAA	20 - 40 - 60 - 80	-	-	23.17	25.61	100.4	2.0	4.8
CLX/ $^{13}C_6$-IP	5 - 10 - 15 - 20	-	-	7.63	10.83	100.4	14.2	16.5
CPF/ D_3-CPF	1 - 2 - 3 - 4	-	-	1.26	1.48	99.1	7.5	8.6
DC/ $^{13}C_6$-DC	0.05 - 0.1 - 0.15 - 0.2	MRL	0.1	0.12	0.14	101.3	7.2	9.0
DMAA/ $^{13}C_2$-DMAA	1 - 2 - 3 - 4	-	-	1.15	1.27	100.1	1.9	4.8
FAA/ D_3-A	1 - 2 - 3 - 4	-	-	1.33	1.57	107.5	5.4	8.0
FCX/ D_6-FCX	5 - 10 - 15 - 20	-	-	6.95	8.44	97.9	8.7	10.7
FFA/ $^{13}C_6$-FFA	1 - 2 - 3 - 4	-	-	1.42	1.74	100.6	8.2	10.8
FIPF/ D_3-CPF	7.5 - 15 - 22.5 - 30	-	-	10.44	13.49	98.1	9.7	14.7
FLU/ D_3-FLU	1 - 2 - 3 - 4	-	-	1.18	1.32	101.1	3.8	5.4
FLUOH/ D_3-FLUOH	20 - 40 - 60 - 80	MRL	40	43.52	47.24	99.3	2.6	4.5
IDP/ D_6-FCX	1 - 2 - 3 - 4	-	-	1.35	1.69	112.1	10.7	11.6
IP/ $^{13}C_6$-IP	5 - 10 - 15 - 20	RC	10	6.09	6.91	100.2	5.4	6.4
IPAA/ D_6-IPAA	1 - 2 - 3 - 4	-	-	1.25	1.45	100.8	4.4	7.5
KTP/ D_3-KTP	1 - 2 - 3 - 4	-	-	1.14	1.25	99.4	2.5	4.6
MAA/ D_3-MAA	25 - 50 - 75 - 100	MRL	50	57.64	67.65	92.6	6.7	8.8
MCA/ $^{13}C_6$-MCA	5 - 10 - 15 - 20		-	6.64	8.22	101.7	9.3	10.6
MFA/ $^{13}C_6$-MFA	1 - 2 - 3 - 4	RC	10	1.43	1.73	100.7	9.4	10.3
MLX/ D_3-MLX	7.5 - 15 - 22.5 - 30	MRL	15	16.35	17.82	101.2	3.3	4.8
NFA/ $^{13}C_6$-NFA	5 - 10 - 15 - 20	-	-	5.93	6.68	101.8	4.2	5.6
NP/ D_3-NP	1 - 2 - 3 - 4	RC	10	1.32	1.59	101.1	8.8	9.6
OH-A	25 - 50 - 75 - 100	-	-	-	-	-	-	-
OPB/ $^{13}C_6$-OPB	1 - 2 - 3 - 4	RC	5	1.19	1.34	101.4	2.9	5.6
PBZ/ $^{13}C_{12}$-PBZ	1 - 2 - 3 - 4	RC	5	1.24	1.42	97.5	4.3	7.0
PRX/ D_3-PRX	1 - 2 - 3 - 4	-	-	1.32	1.55	100.2	6.0	8.3
RCX/ D_3-KTP	7.5 - 15 - 22.5 - 30	-	-	9.91	11.88	100.2	8.0	9.6
SA/ $^{13}C_6$-SA	20 - 40 - 60 - 80	-	-	23.14	25.55	100.0	1.9	4.9
TFA/ $^{13}C_6$-TFA	25 - 50 - 75 - 100	MRL	50	59.44	69.47	102.0	6.5	8.7
VDP/ D_3-VDP	7.5 - 15 - 22.5 - 30	-	-	10.00	11.86	101.0	6.3	9.1

MRL: maximum residue limit, RC: recommended concentration, RSD_r: repeatability, RSD_{wR}: within-laboratory reproducibility

Validation

The validated NSAIDs include substances with MRL and substances which are not authorised. The non-authorised NSAIDs PBZ, OPB, IP NP and MFA have recommended concentrations. The MRLs and recommended concentrations of the NSAIDs validated are shown in Table 2. Commission Decision 2002/657/EC requires a validation around the MRL covering at least a range from 0.5x MRL to 1.5x MRL for authorised drugs, and at concentrations as low as possible for substances without MRL (European Commission, 2002). The four target concentration levels validated for each NSAID are given in Table 2.

The validation study was conducted on the basis of the alternative approach (European Commission, 2002). With the help of the InterVAL Plus software, version 3.4.0.0 (QuoData, Dresden, Germany), the study was designed and evaluated considering changes that may occur during routine analysis. In the course of the validation experiment, milk of in total five cows was used for the spiking experiments and seven factors were systematically varied on two levels in order to demonstrate the ruggedness of the method (Table 3).

Table 3: Description of factors and factor levels for validation of the analytical method.

Factor	Factor level (-)	Factor level (+)
Kind of matrix	Fresh	lyophilised
Storage of extract	no storage	2-3 days at -20°C
Removal of SPE material	15 min after extraction	immediately after extraction
Operator	occasional	routine
QuEChERS salt mix	bought	weighed
Sepra C18-E SPE bulk material	batch A	batch B
UPLC column	column A	column B

Results and Discussion

Analytical Method

A method for the simultaneous analysis of acidic and basic NSAIDs in milk has been developed. The method includes a hydrolysis step, a QuEChERS-based extraction step and a dispersive SPE for clean-up. The final extracts are analysed by UPLC-MS/MS.

As can be seen from Figure 1, most of the 31 analytes are well separated when using an Acquity UPLC HSS C18 column (2.1 x 100 mm, particle size 1.8 µm) under the chromatographic conditions described above. Since some of the NSAIDs analysed share the same SRM transitions, *e.g*. VDP and NFA (*m/z* 281 → *m/z* 237) or MCA and DC (*m/z* 294 → *m/z* 214), a base line separation is required for those analytes in order to guarantee reliable results. The chromatographic method developed meets this requirement for all analytes which potentially interfere with each other due to the same mass transitions. Even though the application of columns from different batches led to retention time shifts for some analytes, no selectivity losses were observed.

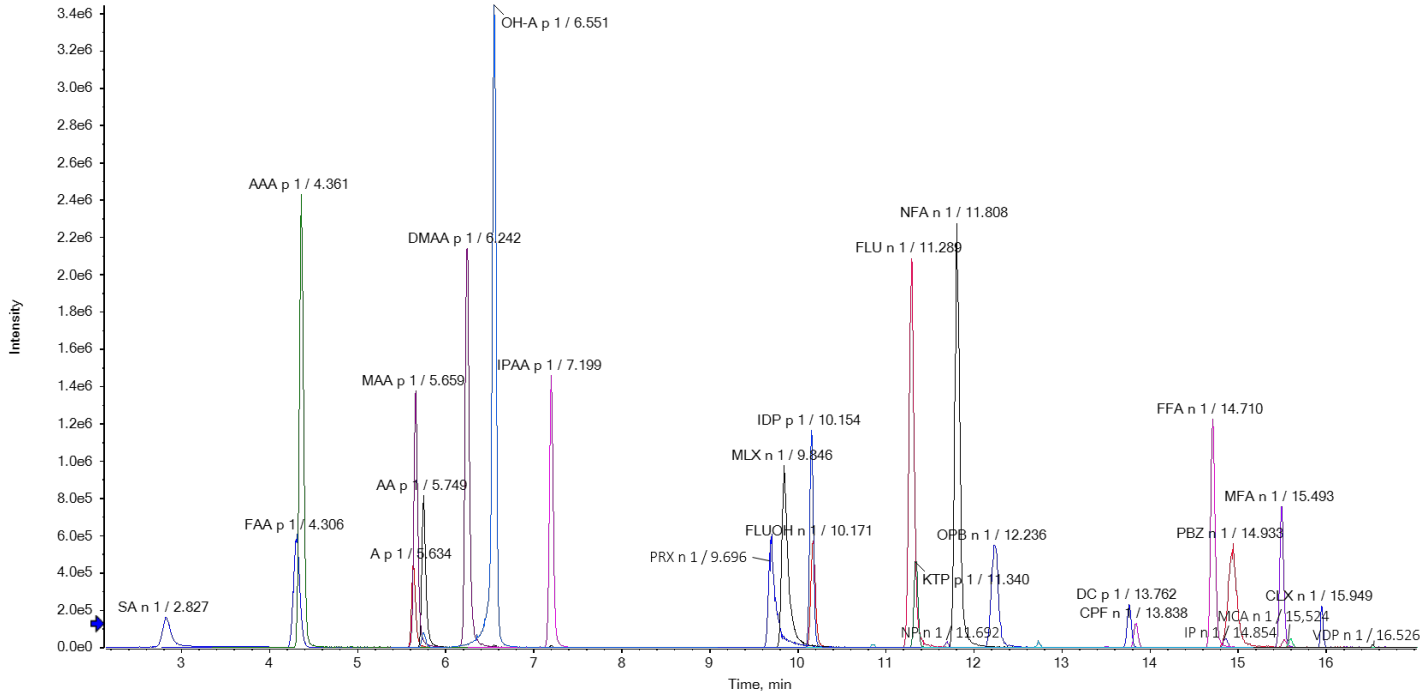

Figure 1: UPLC-ESI-MS/MS chromatogram for the basic and acidic NSAIDs included in the method developed with an amount of 50 pg on column in ACN/H_2O (1/9, v:v) for the SRM transition of the quantifier (n: negative ionisation mode, p: positive ionisation mode).

A compound optimisation for NSAIDs revealed that both the positive and the negative ionisation mode are needed for the measurement of NSAIDs. At least two SRM transitions for each analyte and one SRM transition for each ISTD are recorded. Due to the number of SRM transitions and the necessity of both polarities, the advanced sMRM mode is applied.

In order to improve the recovery of the target analyte a hydrolysis step is included. The aim of the enzymatic hydrolysis step is the deconjugation of sulphates and glucuronides. As can be understood from the EuroResidue VIII proceeding entitled "Investigations on the influence of hydrolysis on the total amount of marker residue and the consequences" the hydrolysis step had an impact on some analyte recoveries (O18, this series). Even the quantifiable amount of marker residue can be increased due to the hydrolysis step in some cases.

The unspecific QuEChERS extraction in combination with a clean-up via dispersive SPE was applied for the extraction of acidic and basic NSAIDs in milk samples. Due to the different chemical characteristics of the analytes the extraction efficiency varied significantly (data not shown). However, the isotopically labelled ISTDs compensated for analyte losses due to sample preparation, but also for matrix effects, and led to recoveries of about 100% (Table 2).

Method Validation

In accordance with Commission Decision 657/2002/EC the method has been fully validated for 30 NSAIDs in milk by applying the alternative validation approach. The validation data in Table 2 show that the method is fit for purpose. Due to the insuffi-

cient stability of OH-A, the method presented is not suited for the determination of this NSAID. The mean apparent recoveries for the other NSAIDs are within the limits defined in 657/2002/EC except for IDP. For this compound no stable isotopically labelled ISTD is available.

In addition, the method's ruggedness was confirmed by low within-laboratory reproducibilities between 4.8 and 16.5 % leading to CCα values well below the allowed CCα max. The method proved to be rugged against changes with regard to the operator, the extraction procedure (where self-weighed and bought QuEChERS salts were used), the clean-up procedure (different batches of material, duration of clean-up), the storage of the final extracts and the application of HPLC columns from different batches.

The specificity was shown by analysing a total of 24 blank milk samples from five different cows. However, few restrictions are to be emphasised. SA is known for its ubiquitous presence (Hignite & Azarnoff, 1977; Paterson *et al.*, 2008), which could also be confirmed for the blank milk analysed. For that reason a validation in the lower µg kg^{-1} range is challenging due to the lack of suitable blank material. The provisional MRL for SA in milk of 9 µg kg^{-1} was therefore not covered in this study and higher concentration levels were validated. In addition, the specificity was evaluated for NSAIDs in matrix after the sample preparation procedure. In consequence of sample preparation the analyte MAA is degraded to AA, which might lead to an overestimation of AA. This aspect can be regarded as of minor importance because MAA is the marker residue for the treatment of animals with metamizole. Recoveries for MAA ranged between 90 and 100%. Therefore, it seems that MAA and the isotopically labelled standard D_3-MAA are degraded to the same extent. Apart from these limitations the method can be regarded as specific.

The results for the recoveries and within-laboratory reproducibilities of the analysis of NSAIDs in milk presented here are in line with data published recently (Van Pamel & Daeseleire, 2015). However, a method improvement was achieved by including an enzymatic hydrolysis step covering also some conjugated residues and a clean-up ensuring a less frequent contamination of the MS instrument.

Conclusions

Taken all together, an LC-MS/MS-based method for the simultaneous analysis of acidic and basic NSAIDs in milk was successfully developed for 31 NSAIDs and fully validated for 30 NSAIDs in accordance with 657/2002/EC. The method comprises a hydrolysis step, an extraction by QuEChERS in combination with dispersive SPE and allows a reliable quantification of the NSAIDs validated.

Acknowledgements

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LC-MS/MS DETERMINATION OF CORTICOSTEROIDS AND NON-STEROIDAL ANTI-INFLAMMATORY DRUG RESIDUES IN FOOD, DEVELOPMENT AND COMPARISON WITH LC-HRMS

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Abstract

The aim of the present work is the development of a fast and reliable analytical multi-residue method for the confirmation of corticosteroids and non-steroidal anti-inflammatory drugs residues in food. Samples were extracted with organic solvent and purified on a SPE cartridge. The purified extract was evaporated to dryness and reconstituted in a methanol/water mixture. Determination was carried out by LC–MS/MS and LC-HRMS. The method was validated in accordance with the criteria defined in Commission Decision 2002/657/EC, decision limit (CCα) values and detection capability (CCβ) values have been established for each compound. Under the Official Control Plan activity, several samples were found positive for dexamethasone in liver and flunixin, meloxicam and carprofen residues in meat and milk. The method is used for confirmatory purposes in the research of corticosteroids and non-steroidal anti-inflammatory drugs residues in food; it is suitable for laboratories involved in official controls.

Introduction

Glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) are extensively used in veterinary medicine because of their main properties anti-inflammatory, analgesic and antipyretic. The use of these drug in veterinary medicines on farm animals is controlled under European law [1]. The legislation aims to protect consumers by stopping unacceptable levels or concentrations of residues from veterinary medicines getting into the food-chain. To prevent the presence of potentially harmful residues in animal derived products, withdrawal periods between treatment and slaughtering and maximal residue limits (MRLs) have been established for several compounds [2]. For example, NSAIDs in *bovine* muscle, MRL have been set only for carprofen, diclofenac, flunixin, meloxicam, metamizole, tolfenamic acid and vedaprofen, while for the glucocorticoids in *bovine* milk, MRLs have been set only for dexamethasone, betamethasone and prednisolone. Unfortunately, these substances are also used illegally as growth-promoters in association with other molecules like β-agonists or anabolic steroids [3].

To detect glucocorticoids and NSAIDs residues in food, different analytical approaches have been used, ELISA, gas-chromatography mass-spectrometry, liquid-chromatography with DAD and mass spectrometry detector [4-6]. In this work we present a multi-residue method, developed and validated according to Commission Decision 2002/657/EC for screening and confirmation of glucocorticoids and NSAIDs in different matrices.

Materials and Methods

Standard and stock solution

Standards of corticosteroid and NSAIDs were obtained from Sigma–Aldrich (Sigma Chemical Company, St. Louis, MO, USA) and Witega (Berlin, Germany). Formic acid, acetic acid, EDTA (ethylendiaminotetracetic acid), methanol, acetonitrile, ammonium acetate and ethyl acetate each of analytical grade, were purchased from Carlo Erba (Milan, Italy). Deionised water for the preparation of aqueous solutions was dispensed from a Milli-Q water system (Millipore, Milford, MA, USA).

Sample preparation

Two g homogenized sample (milk, meat, liver) was spiked with IS. All samples were mixed with 2.5 mL acetate buffer solution 2 M, pH 5.2, 1 mL EDTA 0.1 M, 50 µL glucuronidase/arylsulphatase (*Helix pomatia* - Merck, Darmstadt, Germany) and incubated for 2.0 h at 37°C. After cooling to room temperature samples were extracted twice with ethyl acetate. The organic phase was then evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol/water 10/90 and purified on a SPE cartridge activated with 3.0 mL of methanol and 3.0 mL of deionised water prior to use. The SPE cartridge was washed with 3.0 mL deionised water, 2.0 mL of methanol-water 5:95 v/v. Analytes were eluted with 3 mL methanol containing 0.1% of formic acid, the eluted samples were evaporated to dryness under nitrogen stream. The extracts were then dissolved in 250 µL methanol and 5 µL were automatically injected into the LC-MS system.

LC-MS/MS equipment and conditions

An API 5500 QTrap tandem mass spectrometer detector (Applied Biosystem, Foster City, CA, USA) equipped with a 1260 Infinity high performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) and an autosampler, Agilent G1367A (Agilent Technologies, Santa Clara, CA, USA) was used to analyse the extracted and purified samples.

Analysis was performed in negative electrospray ionisation mode with a capillary voltage of -4.2 kV and a source temperature of 500°C. Ultra-pure air was used as nebulizer gas, while ultra-pure nitrogen was both curtain gas and collision gas. Positive ions were acquired in multiple reaction monitoring (MRM) mode, acquiring two or more diagnostic product ions from the chosen precursors to obtain high specificity and sensitivity.

Analytical separation of drugs was achieved on a kinetex XB column (100 mm x 3.0 mm, 2.6 μm) with a mobile phase consisted of 0.1% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.450 mL min^{-1}, in gradient mode. Initial condition was 20% B, which was held for 1 min followed by a linear increase to 90% B after 5 min. During the next 2 min the column was kept at 90% B; then solvent composition returning to the initial conditions in 1 min. The analytical column was maintained at room temperature, and the injected volume was 5 μL.

LC-HRMS equipment and conditions

A quadrupole–orbitrap hybrid (Q Exactive) mass spectrometer (Thermo, Bremen, Germany) was used. A Thermo Scientific Dionex Ultimate 3000 RS pump, column compartments and autosampler were coupled and controlled by Chromeleon 6.80 Software (Thermo Fisher Scientific, Waltham, MA, USA and Dionex Softron GMbH, Germany) for analysis. The chromatographic system was attached to the MS through a heated electrospray ionization source II (HESI II) interface. The same column and separation conditions as for the LC–MS/MS were used. HESI II conditions were: spray voltage, -3.2 kV; sheath gas flow rate (N2), 35 units; capillary temperature, 300°C; S lens RF level, 52; heater temperature, 300°C. Mass calibration for Orbitrap was performed daily to ensure a working mass accuracy lower than or equal to 5 ppm. The acquisition was achieved in full scan/dd-MS^2. Mass range in full scan was within *m/z* 50–500. Xcalibur 3.0.63 software (Thermo Fisher Scientific) was used for instrument control and data processing.

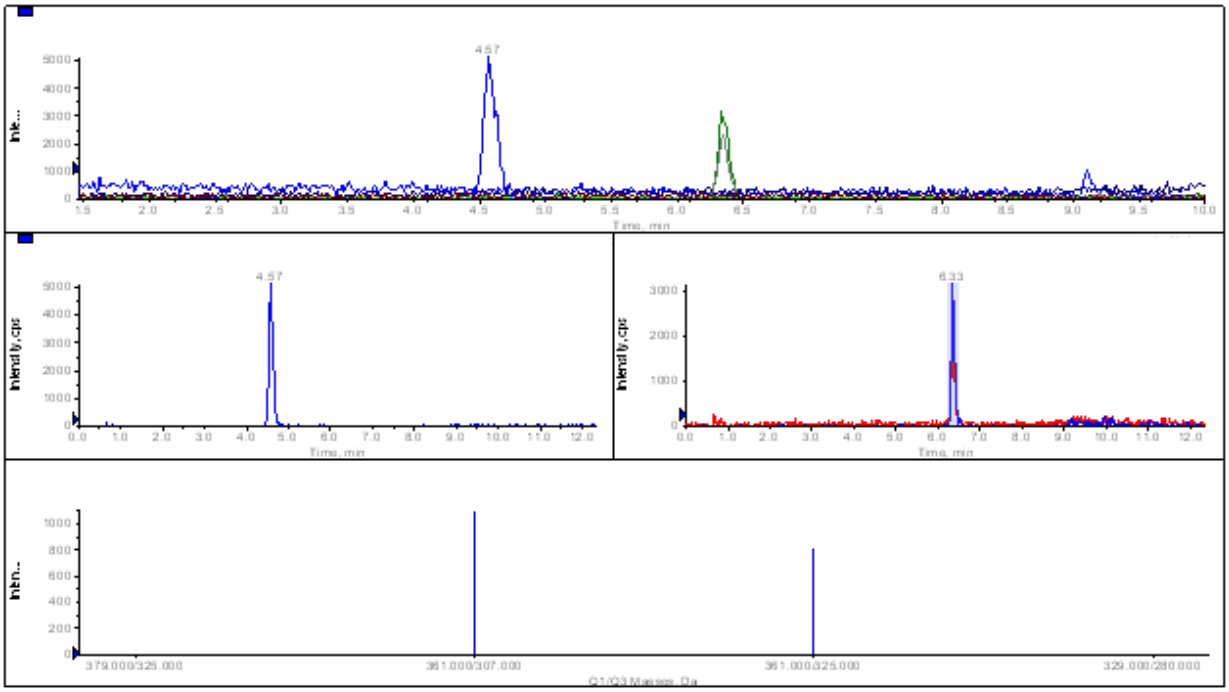

Figure 1. LC-MS/MS chromatograms of a non-compliant liver sample.

Results and discussion

The specificity of the method was demonstrated by analysis of 20 blank samples for each: muscle, milk and liver. No interfering peaks were observed. Method performance was evaluated by fortifying all matrices samples at different levels 0.5, 1.0 and 1.5 MRL for authorized veterinary drugs and 1, 2 and 5 μg kg^{-1} for not licensed veterinary drugs. Good recoveries were observed for all analytes (> 70%), the precision values (RDS %) were below 16.5 and 15.1 and 14.2 for liver, muscle and milk, respectively. Limit of decision (CCα) was satisfying for the analysis of all analytes in the three matrices considered.

The developed method was applied to monitoring the glucocorticoids and NSAIDs in food samples collected as part of the Italian National Plan of Residue Control. A small number of muscle samples were found to be non-compliance for flunixin, meloxicam carprofen and dexamethasone; a greater number of liver samples were non-compliant for dexamethasone.
LC MS-MS and LC-HRMS analyses of one of the positive liver sample (dexamethasone) are shown in Figures 1 and 2, respectively. Comparison of the LC-MS/MS and LC-HRMS methods revealed similar performances in terms of detection limits and specificity.

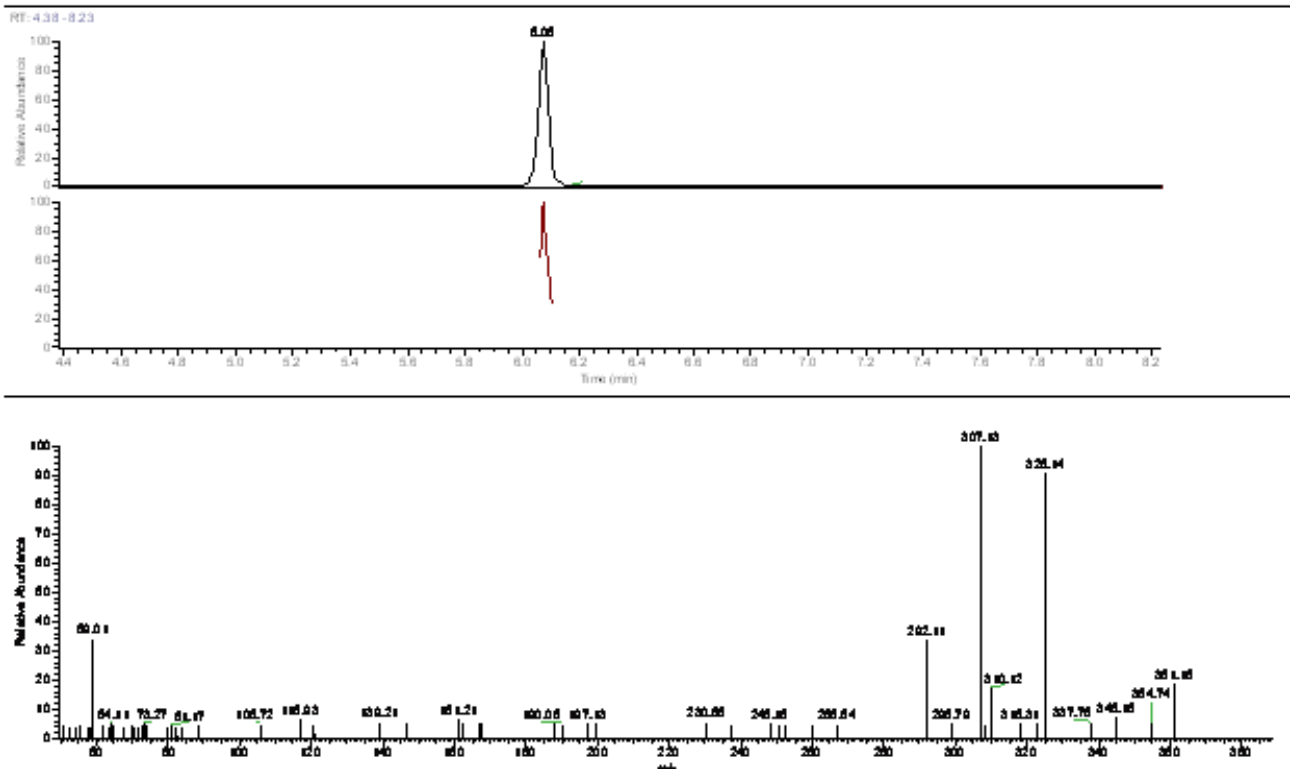

Figure 2. LC-HRMS chromatograms of a non-compliant liver sample.

Conclusions

The method of extraction and purification of analytes allows to obtain a good reproducibility and recovery, and is adequate for all investigated matrices. The CCα values are in accordance to compounds without an MRL.

Comparable performance was obtained with both LC-MS procedures. For some of the analytes, the analysis by LC-MS/MS showed the best performance in terms of linearity range. The LC-HRMS is a reliable and robust alternative tool for routine analysis of anti-inflammatory drugs in foods, with the additional advantage of possibility to perform retrospective analysis without the need to re-analyse the sample.

The proposed methods are suitable and are currently used for official inspection for the determination of NSAIDs and corticosteroid residues in food.

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COMPARING THE PERFORMANCES OF MS/MS AND HRMS ANALYSERS IN THE FAST ANALYSIS OF MULTI-CLASS ANTIBIOTIC RESIDUES IN MILK

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Abstract

High-resolution mass-spectrometry (HRMS) is used increasingly in the field of residue analysis. However, traditional triple quadrupole instruments based on tandem mass-spectrometry (MS/MS) proved to be fast, sensitive and robust for long time and for many different classes of compounds. Thus, why should a laboratory move to an HRMS approach?

The aim of this work is to compare qualitative and quantitative results acquired by both analysers: HRMS (based on orbitrap technique) operating in full scan mode at a resolution of 70,000 FWHM combined with data-dependent MSMS and triple quadrupole operating in MRM mode, to report benefits and drawbacks associated to each technology.

A fast multi-class multi-residue screening method for the determination of 41 antibiotics including tetracyclines, sulfonamides, macrolides and β-lactams was used to prepare complex raw milk extracts to be analysed by both instruments under investigation.

HRMS performance characteristics proved to be comparable to those of MS/MS in terms of accuracy and sensitivity for the majority of the investigated analytes. However, data collected indicate that fast UPLC chromatographic separation is more suitable for MRM detection to achieve an adequate number of sampling points per peak, whereas HRMS analysers represent the new state-of-the-art for their unmatched identification capacity and their applicability for untargeted or retrospective analyses.

Introduction

Triple quadrupole instruments and the application of multiple reaction monitoring (MRM) have been considered as the gold standard for quantitative analysis of drug residues. In recent years, high resolution mass spectrometry (HRMS) has become increasingly popular in the field of veterinary control. Initially used only for the development of screening methods, HRMS was recently recognized as a versatile and promising technology also for confirmation purposes (Gomez-Ramos *et al*., 2013; Kaufmann *et al*., 2011).

Indeed, the sensitivity of triple quadrupole instruments has increased over the last decade, but no relevant improvements in selectivity has been achieved. This means that very little amounts of compound can be detected in complex matrices, but still, the presence of interfering compounds can negatively affect the unambiguous identification required for confirmatory purposes. On the other hand, the resolving power provided by HRMS instruments have significantly improved the selectivity of detection (Boix *et al*., 2014; Kaufmann *et al*., 2010; Stoev *et al*., 2012), with the possibility to confirm the identification of a given compound thanks to the acquisition of fragmentation spectra. Thus HRMS instruments might provide significant advantages over triple quadrupoles, given that the high selectivity is capable to dramatically improve the signal-to-noise ratio (S/N) of analyte peaks in complex sample matrices, making this technology a strong competitor for quantitative and confirmative work.

To test these assumptions, the qualitative and quantitative performance of HRMS using a Q-Exactive (Thermo Fisher Scientific) instrument have been compared to MRM using an API 4000 (AB Sciex) instrument. Comparative tests were performed by analysing 41 compounds belonging to four classes of antibiotics (tetracyclines, sulfonamides, macrolides and β-lactams) in milk samples.

Materials and Methods

Sample preparation

Milk samples (10 g) were spiked with 20 ng of deuterated β-lactams (penicillin-d7, cloxacillin-^{13}C4, amoxicillin-d4, ampicillin-d5, cefalexin-d5) and 25 ng of deuterated sulfonamides (solfadoxin-d3, sulfadimetoxin-d6) as internal standards. Samples were centrifuged to remove fat (6,000 × g, 5 min, 4°C) and 5 g of milk serum were transferred in a new tube. The extraction of analytes was performed by adding 9.5 mL acetonitrile and 0.5 mL of 500 mM EDTA (pH 8.0). Samples were vortex-mixed for 10 min on a shaker, and sonicated in an ultrasound bath for 10 min, then centrifuged again (6,000 × g, 5 min, 4°C). The supernatant was collected (10 mL) and fat were removed again by adding twice 3.5 mL *n*-hexane that was discarded after phase separation. Milk extracts were centrifuged again (6,000 × g, 5 min, 4°C) and 3 mL of supernatant were collected and evaporated until dryness under a liquid nitrogen stream at 40°C. The complete sample preparation procedure was performed while keeping samples protected from light. Before LC-MS analysis, samples were reconstituted with 0.5 mL of a solution

containing water/acetonitrile (90/10, v/v), sonicated for 5 min in an ultrasound bath, and centrifuged again (18,000 × g, 5 min, 4°C). Supernatants were transferred in HPLC vials for subsequent analysis.

Liquid Chromatography and Mass Spectrometry

A Prominence UFLC (Shimadzu) interfaced with an API 4000 triple quadrupole (AB Sciex) was used for LC-MS/MS analyses. Two transitions per molecule were monitored using a scheduled MRM method setting a cycle time of 0.5 s and detection windows of 60 s. The separation was achieved using 0.1% formic acid (A) and acetonitrile (B) using a flow rate of 0.25 mL min^{-1}, and a 15 min gradient generated by mixing A:B as follows: initial conditions using 90:10, kept unchanged until 0.5 min, then 70:30 at 1.5 min, 50:50 at 9 min, 5:95 from 10 to 12 min to wash the system, and finally 90:10 from 12.5 to 15 min to re-equilibrate the column.

An Ultimate 3000 (Thermo Fisher Scientific) coupled with a Q-Exactive instrument (Thermo Fisher Scientific) was used for LC-HRMS analyses. The acquisition was performed using a Full scan at 70,000 resolution FWHM (from 200 to 650 *m/z*) and injection time to 300 ms, followed by MSMS scans at 17,500 resolution FWHM and injection time of 100 ms. An inclusion list (10 ppm tolerance) with a defined time window (2 min) for the acquisition of fragmentation spectra was used. The separation was achieved using 0.1% formic acid (A) and acetonitrile/methanol (50/50, v/v, B) as mobile phases and the following gradient (A:B) at a flow rate of 0.25 mL min^{-1}: initial conditions setting 95:5 kept unchanged for 1 min, then 5:95 at 19 min, then the column was washed until 22 min, and from 22.5 to 27 min the system was re-equilibrated to 5:95.

The column used for both chromatographic separations was a Poroshell, 120 EC-C18 (2.1 × 100 mm, 2.7 µm, Agilent Technologies), used at a temperature of 35°C.

Results and Discussion

Method validation

The method was validated for screening purposes following the guidelines of Commission Decision 2002/657/EC using both MRM and HRMS techniques. The analysed antibiotics were: amoxicillin, ampicillin, penicillin G, penicillin V (all spiked at 2 µg kg^{-1}), cephalexin, cephacetrile, cephapirin, cefazolin, cefoperazone, cefquinome, cefuroxime, ceftiofur, cefalonium, nafcillin, cloxacillin, dicloxacillin, oxacillin (spiked at 20 µg kg^{-1}) for β-lactams; spiramycin, tylosin, tilmicosin, erythromycin (spiked at 25 µg kg^{-1}) for macrolides; tetracycline, epi-tetracycline, oxytetracycline, epi-oxytetracycline, doxycycline, chlortetracycline, epi-chlortetracycline (spiked at 25 µg kg^{-1}) for tetracyclines; sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisoxazole (spiked at 25 µg kg^{-1}) for sulfonamides. Such fortification levels, successfully verified as detection capabilities (CCβ), correspond to concentrations lower or equal to the MRL of each molecule. The average recovery was found to be 97.7 and 97.1 % for penicillins, 86.7 and 89.5% for cephalosporins, 106 and 99.1% for macrolides, 68.6 and 60.0% for tetracyclines, 100.2 and 99.6% for sulfonamides for MRM and HRMS analyses, respectively.

The robustness of the method was also tested and critical variables were found to be the temperature of centrifugation (4°C performs better than room temperature) and the temperature of evaporation prior to LC-MS analysis (to be < 40°C). Intermediate precision of the method in terms of CV % was estimated using 20 fortified samples prepared by two different operators in four distinct working sessions. The average CV % calculated for all the molecules considered in the method was 12.3% for MRM and 8.0% for HRMS. The CV % values calculated for each antibiotic group for the different instruments tested is reported in Table 1. The most relevant differences observed between MRM and HRMS are mainly due to the contribution of some specific compounds which are characterised by lower precision in MRM in absence of the corresponding internal standard.

Table 1. Relative standard deviation (CV%) of the measurements obtained for the different antibiotic groups using the two instrumental platforms under comparison: MRM and HRMS.

Antibiotic family	MRM	HRMS
Penicillins	8.7	8.0
Cephalosporins	13.0	6.6
Macrolides	15.2	9.5
Tetracyclines	15.8	11.2
Sulfonamides	10.1	7.3

The specificity was determined by analysing 20 uncontaminated milk samples and verifying the absence of signal interferences around the expected retention time of each molecule. Both instruments performed well on average, however, MRM suffered of more pronounced signal interference for the confirmatory (secondary) transition of three molecules (*i.e.* amoxicillin, ampicillin and cephacetrile). An example of signal interference is reported in Figure 1. Method selectivity was assessed in

terms of signal to noise ratio (S/N). This parameter was comparable between the two instruments tested for the large majority of the antibiotics considered. However, amoxicillin, ampicillin and cephacetrile showed an S/N value that was barely acceptable when experiments were performed by MRM, but it was highly above 10 when HRMS was used (Figure 1).

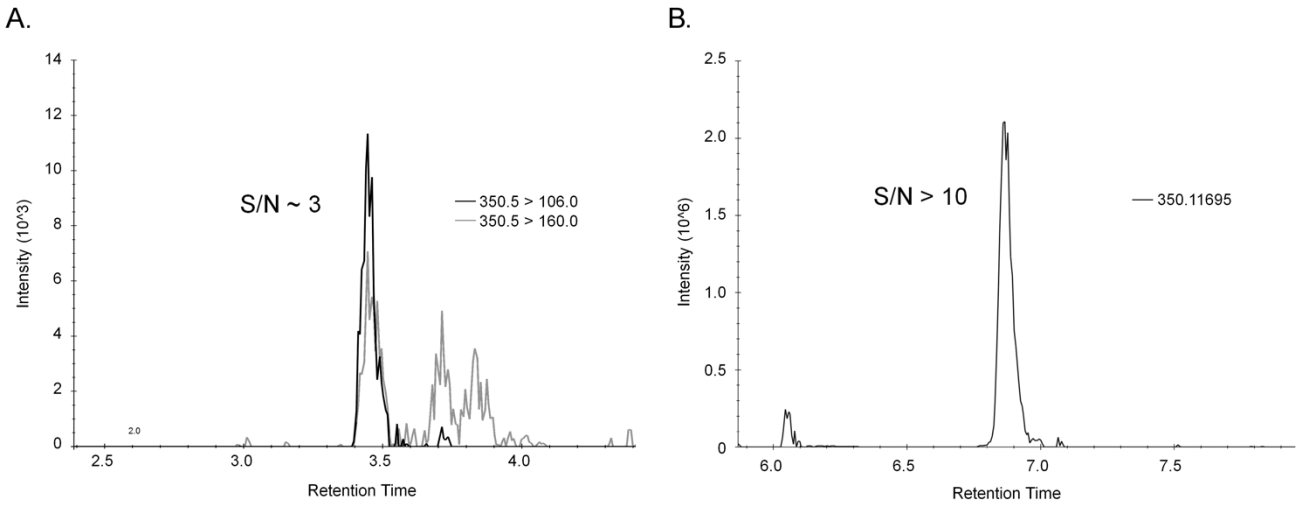

Figure 1. Signal-to-noise ratio comparison between MRM (A) and HRMS (B) for ampicillin.

A qualitative assessment of the chromatographic peaks was carried out counting the number of sampling points recorded across each chromatographic peak. Using a fast chromatographic separation of 15 min, typically generated using UPLC instruments, the MRM gave better performance with respect to HRMS especially for narrow time windows where several compounds co-elute. This is probably due to the slower cycle time of Q-Exactive instrument when full scan-data dependent MS/MS experiments are performed in high resolution. In fact, the fragmentation of several compounds co-eluting from the chromatographic column leads to a reduced frequency of acquisition of the full scan MS spectrum. To achieve sampling frequencies by HRMS comparable to those achieved using a triple quadrupole instrument, the separation of compounds must be modified using a slower gradient (Figure 2). In particular, for analyses performed by HRMS, a slower gradient combined with the use of a mixed organic mobile phase composed of acetonitrile/methanol (50/50, v/v) revealed to be successful leading to increased or unchanged signal intensity and enhanced separation of target compounds, if compared with acetonitrile or methanol alone. Unfortunately, this doubles the total chromatographic run time.

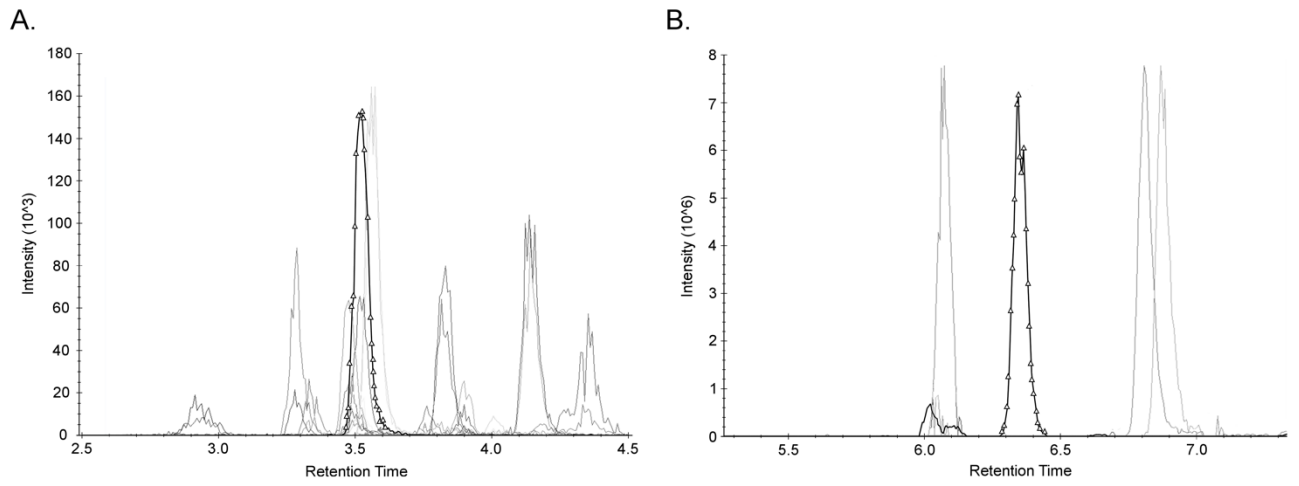

Figure 2. Number of acquisition points across the peak of cefalonium for MRM (A) and HRMS (B). Compounds eluting around the expected retention time of cefalonium are reported in light grey in the extracted ion chromatograms, while triangles represent each single acquisition point across the chromatographic peak of the considered molecule.

Analysis of incurred samples

The method was developed for screening purposes, nevertheless it was also preliminary tested for its potential application for (semi)-quantitative analyses. To this end a four levels calibration curve was built in processed milk extracts at the following concentrations: 0, ¼ MRL, ½ MRL, MRL. The performances of the developed method were tested by means of analysis of incurred milk samples that were also analysed by another accredited laboratory adopting a different analytical method, and by participating to a proficiency test. Quantitative data achieved analysing three incurred milk samples gave satisfactory results for both MRM and HRMS techniques. In particular, for the first incurred milk sample, the amount of cefalonium was found to be 34.5 µg kg^{-1} and 34.7 µg kg^{-1}, by MRM and HRMS respectively, for the second incurred milk sample the amount of cephalexin was found to be 4.6 µg $kg^{-1}$and 4.6 µg kg^{-1}, by MRM and HRMS respectively, and for the third incurred milk sample the amount of cefazolin was found to be 65.0 µg $kg^{-1}$and 64.3 µg kg^{-1}, by MRM and HRMS respectively. These results were in agreement to those achieved on the same samples by the independent laboratory.

In the milk sample analysed for the Proficiency test penicillin G and cefoperazone were detected: the concentration of penicillin G was 6.1 µg kg^{-1} and 6.4 µg kg^{-1} by MRM and HRMS, respectively (z-score = 0.2 by MRM and 0.4 by HRMS), the concentration of cefoperazone was 34.7 µg kg^{-1} and 37.8 µg kg^{-1} by MRM and HRMS, respectively (z-score = -0.7 by MRM and -0.3 by HRMS). In all cases results were completely satisfactory.

Conclusions

When analysing a number of different compounds with a common sample preparation procedure, multi residue methods require compromises. The use of EDTA during sample extraction enabled to increase the recovery of tetracyclines, but interfered with LC separation of amoxicillin that showed a large chromatographic peak.

Both instruments are suitable for quantitative analysis of drug residues when accuracy is compared, however HRMS shows a better performance in terms of selectivity and S/N ratio, while MRM seems to be the best choice in terms of speed of analysis. On the other hand, the possibility to link the fragmentation spectrum to a given precursor mass acquired in high resolution improves the identification and confirmation of compounds thanks to the use of spectral libraries that can be implemented within laboratory.

In addition, the feasibility of the full scan acquisition for retrospective analyses aimed at verifying the presence of a given compound in previously analysed samples represents a unique prerogative of HRMS technologies with respect to MRM. This notwithstanding, HRMS suffers from a legislative gap considering that Commission Decision 657/2002 does not include precise guidelines for these novel kinds of instrument set-up. It is therefore important to have well-defined criteria which ensure an equal reliable confirmation as currently available for MRM technique.

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DEVELOPMENT AND VALIDATION OF A MULTI-RESIDUE LC-MS/MS ANALYSIS FOR THE DETECTION OF AMINOGLYCOSIDES IN MILK

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Abstract
Aminoglycosides are a wide family of antibiotics with bactericidal activity that are especially active against aerobic Gram-negative bacteria. They are classified as group B substances by the European Council, and MRLs have been set for some. These legislative limits necessitate the need for sensitive analytical methods to detect, identify and quantify the absence or presence of such residues.
The aim of this study was to develop and validate a multi-residue method for the simultaneous analysis of ten aminoglycosides by means of a sensitive, selective and fast LC-MS/MS analysis. The mass spectrometric parameters were optimized on a Xevo TQ-S® (Waters) in order to allow the detection of minimum two product ions per precursor ion for each compound. Subsequently, the chromatographic conditions were optimized on the Acquity™ UPLC® (Waters) system equipped with an Obelisc R (Sielc®) (2.1x100 mm, 5 µm) column protected by a guard column. The extraction was performed using 20% trichloroacetic acid followed by a clean-up on OASIS HLB columns. The final optimized method was validated according to Commission Decision 2002/657/EC. Performance characteristics such as specificity, linearity (R^2), recovery (R), repeatability (RSD_r), intra-laboratory reproducibility (RSD_R) and the decision limit (CCα) were determined. It can be concluded that this method can be used in routine to check on the compliance of milk samples for aminoglycosides.

Introduction
Aminoglycosides are a wide family of antibiotics with bactericidal activity that are especially active against aerobic Gram-negative bacteria. They are substances with a narrow therapeutic-toxicological margin showing ototoxicity and nephrotoxicity. They are administered orally or parenterally. After parental administration, their distribution is limited to the extracellular fluids (1). Due to their high affinity to tissue, their use results in a high level of tissue residues and in prolonged withdrawal times (2). However due to the fact that they hardly pass the biological barriers, low concentrations in milk, bronchial secretions and cerebrospinal fluid are obtained (1). They are classified as group B substances by the European Council and MRLs have been set for some (3). These legislative limits necessitate the need for sensitive analytical methods to detect, identify and quantify the absences/presence of such residues.

Aminoglycosides are rather large molecules containing two or more amino sugars coupled to a central hexose/pentose. They are very polar compounds that result in high aqueous solubility and a poor retention on reversed-phase columns in the pH range commonly used in LC. Therefore, they cannot be included in the typical multi-class multi-residue methods for veterinary drugs (4).

The aim of this work was to develop and validate a multi-residue LC-MS/MS analysis for the detection of aminoglycosides in milk so that our laboratory is able to analyse all groups of antibiotics in this matrix.

Materials and Methods

Reagents and materials
Apramycin (APRA), dihydrostreptomycin (DIHYDRO), neomycin B/C (NEO), tobramycin (TOBRA) and ribostamycin (RIBO, IS) were from Sigma-Aldrich (Diegem, Belgium). Gentamycin C1/C1a/C2/C2a (GENTA), kanamycin A (KANA) and spectinomycin (SPECTINO) were from Fluka Vetranal (Sigma-Aldrich). Streptomycin (STREPTO) was from Sial (Sigma-Aldrich). Hygromycin (HYGRO) was from Dr. Ehrenstorfer (LGC standards, Molsheim, France) and paromomycin (PAROMO) was from TRC (Toronto Research Chemicals, Toronto, Canada). Trichloroacetic acid was from Sigma-Aldrich and OASIS HLB columns were from Waters (Milford, MA, USA). Acetonitrile (ACN), methanol (MeOH) and formic acid (FA) were from Biosolve (Valkenswaard, The Netherlands). Water was HPLC grade (generated by a Milli-Q Gradient purification system, Millipore, Brussels, Belgium).

Polyvinylidene fluoride (PVDF) filters were purchased from Merck-Millipore (Carrigtwohill, Ireland), polypropylene (PP) tubes and PP inserts were procured by Novolab (Geraardsbergen, Belgium) and Grace Alltech associates inc. (Lokeren, Belgium), respectively.

Individual stock solutions at a concentration of 1 mg mL^{-1} were prepared in water in plastic tubes, after correction for stock purity, and stored at -20°C. For screening experiments, fresh working solutions (in ACN/H_2O, 50/50) of 100 ng μL^{-1} were prepared for STREPTO and NEO, of 10 ng μL^{-1} for KANA, GENTA, SPECTINO, DIHYDRO and RIBO and of 1 ng μL^{-1} for TOBRA,

HYGRO, APRA and PAROMO. For confirmatory experiments a pool solution for all aminoglycosides enabling to spike at 0.5 x MRL, 1 x MRL and 1.5 x MRL was prepared. An overview of the legislation of aminoglycosides in milk is given in Table 1.

Table 1. Overview of current European legislation (MRLs) and internal MRPS for aminoglycosides in milk (Regulation 37/2010).

compound	marker	MRL or internal MRPL ($\mu g\ L^{-1}$)
kanamycin	kanamycin A	150
gentamycin	sum of gentamycin C_1, C_{1a}, C_2 and C_{2a}	100
spectinomycin		200
streptomycin		200
dihydrostreptomycin		200
neomycin (inclusive framycetin)	neomycin B	1500
apramycin		not for use in animals producing milk (20)
tobramycin		not in legislation (2.5)
hygromycin B		not in legislation (20)
paromomycin		not for use in animals producing milk (20)

Liquid chromatography and mass spectrometry

The liquid chromatographic system consisted of an Acquity UPLC® system (Waters, Milford, MA, USA). Separation was achieved on a Obelisc R (Sielc®) 2.1 x 100 mm, 5 µm column protected by a guard column of the same material. The column was held at 40°C, the injection volume was 10 µL and the eluent flow was at 500 µL min^{-1}. The elution was performed gradually with changing amounts of H_2O + 1% FA and ACN + 1% FA.

The mass spectrometric equipment consisted of a Xevo TQ-S® (Waters) equipped with a Z-spray system. The analytes were determined with tandem electrospray positive mass spectrometry with one transition in screening mode and at least two transitions for the confirmation mode.

Sample preparation and extraction

Ten mL of milk was brought into a polypropylene tube. Internal standard, and eventually standards, solutions were added and the sample was equilibrated for 10 min. Twenty mL of 20% trichloroacetic acid was added and the tube was placed on a shaker during 5 min at 250 rpm. The tube was centrifuged during 10 min at 4,000 rpm. The supernatant was brought on an OASIS HLB SPE column that was conditioned with 4 mL methanol, 4 mL water and 4 mL 20% trichloroacetic acid. The column was washed with 4 mL water, dried under vacuum and the aminoglycosides were eluted with 3 mL methanol. The eluate was filtered through a 0.22 µm filter in a polypropylene insert and 10 µL was injected into the LC-MS/MS system.

Validation

In a first stage the method was validated as a screening method according to the guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer) provided by the EURLs: 20 different milk samples were spiked at 0.5 x MRL or 0.5 x internal MRPL and were analysed together with 20 different blank samples. It was checked if the compounds could be detected in 19 out of 20 samples. If this was not the case, concentration was increased implementing that also the number of samples had to be increased.

Afterwards the method was validated according to Commission Decision 2002/657/EC as a confirmatory method. Following characteristics were determined for MRL substances: specificity, linearity (R^2), recovery (R), repeatability (RSD_r), intra-laboratory reproducibility (RSD_R) and the decision limit (CCα). For forbidden substances it is important to be able to identify the compound, so only CCα was determined being the concentration at which in 95% of the cases the identification criteria of Commission Decision 2002/657 could be fulfilled.

Specificity was tested by injecting the aminoglycoside standards individually and checking for interference on the MRM transitions of the other compounds. Also the 20 different blank samples were checked for interferences in the area of the retention times of the aminoglycosides.

To assess linearity, three sets of calibration curves in matrix were analysed in a relevant concentration range, depending on the MRL status. For the recovery three sets of blank milk samples were spiked at 0.5 x, 1 x and 1.5 x MRL. The recovery percentages were calculated using TargetLynx software version 4.1 (Waters). The experiments for recovery and linearity were carried out on three different days. From these data the coefficients of variation for RSD_r and RSD_R could be calculated at the different concentration levels by means of a one-way ANOVA. CCα was calculated using the data obtained in the recovery experiments at MRL-level, being MRL + 1.64 times the standard deviation.

Results and discussion

Before starting this project, another method making use of separation on a HILIC column after extraction with 20 % TCA solution and clean-up on OASIS HLB was applied in the laboratory. However, problems with peak shapes were observed. In 2015, Díez *et al*. (5) published a method for aminoglycoside analysis in food of animal origin making use of a zwitterionic stationary phase. This chromatographic method was combined with 5 extraction methods: 1) application note Waters (extraction buffer with 10 mM $NHOOCH_3$, 0.4 mM Na_2EDTA, 1% NaCl, 2 % TCA; Oasis HLB clean-up; elution with 3 mL formic acid/isopropanol/H_2O (10/5/85)); 2) extraction method described in Díez *et al*. (extraction buffer as in 1); Oasis HLB clean-up; elution with 3 mL 175 mM ammonium formate pH3); 3) extraction with 20% TCA; Oasis HLB clean-up with conditioning step with 20% TCA; elution with 3 mL MeOH; 4) extraction with 20% TCA; Oasis HLB clean-up without 20% TCA; elution with 3 mL MeOH; 5) extraction with 20% TCA; Oasis HLB clean-up without 20% TCA, elution with 3 mL formic acid/ispropanol/H_2O (10/5/85). This comparison was done with milk samples spiked at MRL or internal MRPL level. It was concluded that the combination of extraction 3 with the Obelisc R column provided the best results in relation to signal intensity and peak shape. This method was used to perform the validation.

For the screening method, in which one transition per compound is measured, levels of 0.5 x MRL/internal MRPL can be used for all compounds, except for streptomycin which can be screened at 87.5% of MRL (n=40). An overview of the validation levels for screening are shown in Table 2.

Experiments for the confirmatory method are still ongoing and the results will be presented during the poster presentation.

Table 2. Overview of the different compounds with the regulatory limit (MRL or internal MRPL), validation level and number of samples used in validation and CCß.

Compound	Regulatory limit (MRL or internal MRPL) (µg L^{-1})	Validation level (VL) (µg L^{-1})	Number of samples	CCβ
kanamycin	150	75	20	≤ VL
gentamycin	100	50	20	≤ VL
spectinomycin	200	100	20	≤ VL
streptomycin	200	175	40	≤ VL
dihydrostreptomycin	200	100	20	≤ VL
neomycin (inclusive framycetin)	1500	750	20	≤ VL
apramycin	20	10	20	≤ VL
tobramycin	2.5	1.25	20	≤ VL
hygromycin	20	10	20	≤ VL
paromomycin	20	10	20	≤ VL

Conclusions

A specific multi-residue LC-MS/MS analysis was developed and validated for the detection of aminoglycosides in milk. The method can be used as a screening tool in monitoring programmes. For confirmatory purposes, the method is still in validation. The next step after the validation will be the use of this method in a proficiency test.

Acknowledgements

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ANALYSIS OF ANTHELMINTIC RESIDUES IN LIVER BY MULTIPLEX SCREENING APPROACH: COMPARISON OF A BIOCHIP ARRAY VERSUS LC-MSMS APPROACH

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Abstract
Two multi-class multi-residue analytical approaches, for the screening of anthelmintics in liver samples were developed, validated according to the guidelines laid down by Commission Decision 2002/657/EC and compared: one was based on biochip array technology and one based on liquid chromatography-triple quad mass spectrometry (LC-MSMS). Results, advantages and drawbacks, are presented and discussed.

Introduction
Anthelmintic drugs are used in clinical and veterinary practices for the treatment of infections caused by parasitic worms. These substances belong to different chemical classes: macrocyclic lactones (avermectins/milbemycin), benzimidazoles, imidazothiazoles (Porter *et al.*, 2012). According to the Italian Residue Control Plan, several hundreds of liver samples are yearly collected to be analysed for these three distinct classes. The official laboratories usually adopt three distinct class-specific analytical methods based on chromatographic techniques.

The emerging trend in residue analysis is the development of methods that are capable of monitoring in each sample, a wide variety of compounds, representative of different classes of veterinary drugs, by means of one single analytical protocol (Kinsella *et al.*, 2009 and 2010; Porter *et al.,* 2012). This new approach could represent the basis for an evolution both for future monitoring plans and for supporting producers to better investigate the critical points related to their production chain.

The goal of this project was to develop, validate and apply a multi-class method based on biochip array technology for the determination of anthelmintics residues in liver samples and to evaluate the potentiality of this technique as an alternative to LC-MSMS-based analytical methods in terms of applicability and productivity.

Materials and Methods

Chemical and reagents
All reagents and solvents were of analytical or HPLC grade quality. Ultrapure water was obtained by Sartorious Stedim Biotec system (Aubagne Cedex, France). Original QuEChERS method (non-buffered) extraction packets kit 5982-7550 and EN fatty dispersive-SPE 15 mL kit 5982-5156 were purchased from Agilent (Agilent Technologies, Inc., Wilmington, DE, USA).

Abamectin (ABA), albendazole (ALB), doramectin (DOR), emamectin (EMA), febantel (FEB), fenbendazole (FEN), fenbendazole sulfone (FEN-S), flubendazole (FLU), ivermectin (IVER), mebendazole (MEB), moxidectin (MXD), oxfendazole (OXF), oxibendazole (OXI), thiabendazole (TIA), tetramisole-d5 (TETRA-d5) were purchased from Sigma (Milan, Italy); albendazole-sulfone (ALB-S), albendazole-sulfoxide (ALB-SO), eprinomectin (EPRI), levamisole (LVM) was purchased from Dr. Ehrenstorfer (Augsburg, Germany); albendazole-2-amino-sulfone (ALB-2NH2-S), 2-amino-flubendazole (NH2-FLU), 2-aminomebendazole (NH2-MEB), closantel (CLO), 5-hydroxymebendazole (OH-MEB), 5-hydroxythiabendazole (OH-TIA), triclabendazole (TRICLA), triclabendazole-sulfone (TRICLA-S), triclabendazole sulfoxide (TRICLA-SO), albendazole-d3 (ALB-D3), albendazole-sulfoxide-d3 (ALB-SO-D3) albendazole-sulphone-d3 (ALB-S-D3), closantel-13C6 (CLO-13C6), febantel-d6 (FEB-D6), fenbendazole-d3 (FEN-D3), oxfendazole-d3 (OXF-D3) were purchased from Witega (Berlin, Germany). The standard stock solutions at a concentration of 1,000 mg L^{-1} were prepared in methanol for EMA, EPRI, ABA, MXD, DOR, IVER, LVM, TRICLA, TRICLA-S, TRICLA-SO and TETRA-D5. All other substances were solved in dimethylformamide. All solutions were stored at -20°C. Suitable working standard solutions were obtained by appropriate dilution in acetonitrile of the corresponding stock solution and stored at 4°C.

The biochip array technology approach was performed using a biochip anthelmintics array kit containing the biochips, assay diluent, conjugate diluent, calibration series, signal reagent, washing buffer, calibration disc and barcodes (EV3770, Randox Laboratories, Crumlin, UK).

Sample preparation for biochip array immunoassay (BAI)
Five g of homogenised liver were weighed in a 50-mL centrifuge tube. A volume of 3 mL water was added to all samples and mixed for 30 s, then 10 mL of acetonitrile were added and mixed for 30 s. The QuEChERS extraction salts were added and the tube was shaken vigorously at 1,500 rpm for 1 min by 2010 Geno/Grinder (SpexSamplePrep, Metuchen, NJ, USA). A volume

of 4 mL of n-hexane was added and mixed for 1 min. After centrifugation at 3,000 x g for 12 min, an aliquot of 2 mL of acetonitrile extract was transferred in glass tube. After adding 250 µL dimethyl sulfoxide, the organic extract was dried down at 50°C under nitrogen streaming. About 0.75 mL diluted wash buffer was added to the residue to reach a final volume of 1 mL.

BAI analysis

The simultaneous biochip-based immunoassay for the screening of anthelmintic drugs is competitive. The biochips were supplied in carriers (3x3 biochip per carrier). The experimental procedure was performed following the manufacturer's instructions. Briefly, 150 µL assay diluent was applied to the biochips, followed by 50 µL of the sample. After 30 min of incubation at 25°C and 370 rpm, 100 µL conjugate was added to each biochip. After 60 min of incubation at 25°C and 370 rpm, the biochips were washed (6 cycles). Signal reagent was added and the chemiluminescent signal output generated in the discrete test sites on the biochips was captured using digital imaging technology. The signal was expressed as relative light unit (RLU).

Sample preparation for LC-MSMS analysis

Five g of homogenised liver were weighed in a 50 mL centrifuge tube and the ISs were added in each sample at a concentration corresponding to 25 µg kg^{-1}. A volume of 3 mL water was added to all samples and mixed for 30 s, then 10 mL acetonitrile were added and mixed for 30 s. The QuEChERS extraction salts were added and the tube was shaken vigorously at 1,500 rpm for 1 min by 2010 Geno/Grinder (SpexSamplePrep, Metuchen, NJ, USA). After centrifugation at 5,000 x g for 10 min, an aliquot of organic extract (6 mL) was transferred into EN fatty dispersive-SPE 15 mL tube. The samples were mixed for 1 min and centrifuged at 5,000 x g for 5 min. A total of 4 mL of each purified extract was transferred into an empty tube and evaporated to dryness under nitrogen streaming. The residue was dissolved in 0.4 mL of ammonium formate 0.3 mM + 0.1% formic acid aqueous solution/acetonitrile (50/50 v/v) before LC-MSMS analysis.

LC-MSMS analysis

The analysis of the samples was performed by means of an LC-MSMS system consisting of an Acquity UPLC provided with a binary solvent delivery system, a column heater module and a sample cooling device coupled to a XE Premier triple quadrupole system (MSMS) provide with an ESI source by Waters (Waters, Milford, MA, USA).

Chromatographic separation of anthelmintic was achieved on an HSS T3 C18 column (1.8 µm, 100 x 2.1 mm i.d; Waters, Milford, MA, USA). The mobile phases were ammonium formate 0.3 mM + 0.1% aqueous formic acid (A) and acetonitrile (B). The gradient programme was as follows: 0-0.2 min 0% B, 6.0 min 50% B, 8.5-12.0 min 90% B, 12.5-15.0 min 100% B. The column was maintained at 40°C, the flow rate was 0.45 mL min^{-1} and injection volume was 5 µL.

Ionisation was achieved in an ESI source in the positive and negative mode; source temperature 140°C; desolvation temperature 400°C; cone gas flow 50 L h^{-1}; desolvation gas flow 900 L h^{-1}. Acquisition parameters in MRM mode: conditions for the optimisation of primary and secondary transitions are summarised in Table 1.

Validation

The developed methods were fully validated as qualitative screening methods as described in Commission Decision 2002/657/EC and in CRL Guidelines for the Validation of Screening Methods for Residues: specificity, ruggedness, and detection capability (CCβ) were evaluated.

For BAI validation, FEN-S, NH2-FLU, OH-TIA, TRICLA-SO, LVM, MXD, DOR were selected as reference compounds for the validation study since they exhibit the worst cross-reactivity compared to other anthelmintics. The mentioned reference compounds belong to benzimidazoles (BZS), amino benzimidazoles (ABZ), thiabendazole (TBZ), triclabendazole (TCBZ), levamisole (LVM), moxidectin (MXD) and avermectins (AVM) class respectively. The level of interest for each reference analyte was chosen considering maximum residue limits (MRLs), detection capabilities (CCßs) recommended in the Italian Residue Control Plan and preliminary results achieved during method development. This level was chosen as equal to 50 µg kg^{-1} for FEN-S (BZS class) and for TRICLA-SO (TCBZ class), 12.5 µg kg^{-1} for NH2-FLU (ABZ class), 25 µg kg^{-1} for OH-TIA (TBZ class), 60 µg kg^{-1} for LVM (LVM class), 15 µg kg^{-1} DOR (AVM class), 20 µg kg^{-1} MXD (MXD class).

For LC-MSMS method validation, analytes selected for avermectin/milbemycin class were: ABA, DOR, EMA, IVER, MXD; for benzimidazoles/salicylanilide class were: ALB, FEB, FEN, FEN-S, FLU, MEB, OXF, OXI, TIA, ALB-S, ALB-SO, ALB-2NH2-S, NH2-FLU, NH2-MEB, OH-MEB, OH-TIA, TRICLA, TRICLA-S, TRICLA-SO, and CLO. LVM was selected as representative of the imidazothiazole class. Internal standards (ISs; denoted in brackets) were used for determination of ALB (ALB-D3), ALB-S (ALB-S-D3), ALB-SO (ALB-SO-D3), FEB (FEB-D6), FEN (FEN-D3), OXF (OXF-D3), CLO (CLO-13C6) and LVM (TETRA-D5). The level of interest for each analyte was chosen considering maximum residue limits (MRLs), detection capabilities recommended in the Italian Residue Control Plan and preliminary results during method development. This level was established at 10 µg kg^{-1} for avermectin/milbemycin class and at 25 µg kg^{-1} for the benzimidazoles/salicylanilide and imidazothiazole classes.

Table 1. Acquisition parameters.

ESI source in positive mode				
Analyte	Precursor Ion [*m*/*z*]	Product Ion [*m*/*z*]	Cone [V]	En. Coll (eV)
OH-TIA	218 ± 0.5	191 ± 0.5	45	26
	218 ± 0.5	147 ± 0.5	45	34
LVM	205 ± 0.5	91 ± 0.5	40	34
	205 ± 0.5	123 ± 0.5	40	28
	205 ± 0.5	178 ± 0.5	40	20
TETRA-D5	210 ± 0.5	183 ± 0.5	40	22
ALB-2NH2-S	240 ± 0.5	133 ± 0.5	40	30
	240 ± 0.5	198 ± 0.5	40	20
TIA	202 ± 0.5	175 ± 0.5	42	25
	202 ± 0.5	131 ± 0.5	42	32
ALB-SO	282 ± 0.5	240 ± 0.5	25	14
	282 ± 0.5	159 ± 0.5	25	38
ALB-SO-D3	285 ± 0.5	243 ± 0.5	25	15
NH2-MEB	238 ± 0.5	105 ± 0.5	45	26
	238 ± 0.5	77 ± 0.5	45	35
OH-MEB	298 ± 0.5	79 ± 0.5	32	38
	298 ± 0.5	160 ± 0.5	32	35
NH2-FLU	256 ± 0.5	123 ± 0.5	45	28
	256 ± 0.5	95 ± 0.5	45	38
OXI	250 ± 0.5	218 ± 0.5	32	18
	250 ± 0.5	176 ± 0.5	32	28
ALB-S	298 ± 0.5	159 ± 0.5	36	38
	298 ± 0.5	224 ± 0.5	36	26
ALB-S-D3	301 ± 0.5	266 ± 0.5	35	20
OXF	316 ± 0.5	159 ± 0.5	35	35
	316 ± 0.5	191 ± 0.5	35	22
OSF-D3	319 ± 0.5	159 ± 0.5	35	35
FEN-S	332 ± 0.5	300 ± 0.5	38	23
	332 ± 0.5	159 ± 0.5	38	38
MEB	296 ± 0.5	264 ± 0.5	33	22
	296 ± 0.5	105 ± 0.5	33	33
ALB	266 ± 0.5	234 ± 0.5	30	20
	266 ± 0.5	192 ± 0.5	30	33
ALB-D3	269 ± 0.5	234 ± 0.5	35	20
FLU	314 ± 0.5	282 ± 0.5	32	22
	314 ± 0.5	123 ± 0.5	32	36
FEN	300 ± 0.5	268 ± 0.5	35	22
	300 ± 0.5	159 ± 0.5	35	35
FEN-D3	303 ± 0.5	268 ± 0.5	35	22

Table 1. Continued.

TRICLA-SO	377 ± 0.5	359 ± 0.5	30	19
	377 ± 0.5	362 ± 0.5	30	22
EMA	886 ± 0.5	158 ± 0.5	45	40
	886 ± 0.5	302 ± 0.5	45	30
TRICLA-S	391 ± 0.5	242 ± 0.5	45	40
	391 ± 0.5	312 ± 0.5	45	28
FEB	447 ± 0.5	415 ± 0.5	25	14
	447 ± 0.5	383 ± 0.5	25	19
FEB-D6	453 ± 0.5	383 ± 0.5	25	20
TRICLA	359 ± 0.5	344 ± 0.5	40	26
	359 ± 0.5	274 ± 0.5	40	38
EPRI	915 ± 0.5	186 ± 0.5	15	18
	915 ± 0.5	330 ± 0.5	15	15
ABA	890 ± 0.5	567 ± 0.5	16	12
	890 ± 0.5	305 ± 0.5	16	15
MXD	640 ± 0.5	528 ± 0.5	14	8
	640 ± 0.5	498 ± 0.5	14	12
DOR	916 ± 0.5	331 ± 0.5	12	25
	916 ± 0.5	593 ± 0.5	12	12
IVER	892 ± 0.5	569 ± 0.5	16	15
	892 ± 0.5	307 ± 0.5	16	22
ESI source in negative mode				
Analyte	Precursor Ion [*m/z*]	Product Ion [*m/z*]	Cone [V]	En. Coll (eV)
CLO	661 ± 0.5	127 ± 0.5	60	48
	661 ± 0.5	315 ± 0.5	60	35
CLO-13C6	667 ± 0.5	127 ± 0.5	60	46

Specificity and Detection capability (CCß)

A qualitative approach was used to determine the performance parameter CCß as described in the CRL Guidelines. For both procedures a set of at least twenty blank liver samples from different species (*bovine*, swine, poultry) were analysed for specificity test; the same samples spiked at the concentration of interest were analysed for ß error verification. The analyses were carried out in within-laboratory reproducibility conditions (different days and operators). A threshold value T and a "cut-off factor" Fm were calculated starting from signals results for BAI and from concentration results for LC-MSMS by following equations:

$T = B\text{-}1.64\ SD_b$ (in the signal domain, for BAI)

$T = B+1.64\ SD_b$ (in the concentration domain, for LC-MSMS)

where B is the mean signal or concentration calculated from blank liver samples and SD_b the calculated standard deviation.

$Fm = M + 1.64\ SD_s$ (in the signal domain, for BAI)

$Fm = M - 1.64\ SD_s$ (in the concentration domain, for LC-MSMS)

where M is the mean signal or concentration calculated from spiked liver samples and SD_s the calculated standard deviation.

According to CRL Guidelines for the Validation of Screening Methods for Residues the following equations were verified:

$Fm < B$; $Fm < T$ (in the signal domain, for BAI)

$Fm > B$; $Fm > T$ (in the concentration domain, for LC-MSMS)

Ruggedness (minor change).

This parameter was evaluated by Youden fractional factorial design: seven variables were chosen (for BAI: analytical balances for sample weight, QuEChERS batch, shaking modality, centrifugation time, processing time, evaporation time, evaporation temperature; for LC-MSMS: analytical balances for sample weight, QuEChERS and SPE batch, shaken modality, evaporation

temperature, organic percentage in reconstitution phase and centrifugation time) and deliberately altered. Eight experiments were carried out on fortified samples according to the Youden scheme.

Table 2. validation results:

BAI validation results							
Class	Reference analyte/Cross reactivity (%)	Level of interest (µg kg^{-1})	B (RLU/RLU_0) (%)	T (RLU/RLU_0) (%)	F_m (RLU/RLU_0) (%)	Lowest MRLs (µg kg^{-1})	ß error
BZS	FEN-S/ 14	50	88.0	77.7	25.7	200	≤ 5% for FEN-S, MEB, FLU, parbendazole, OXF, OXI, ALB-SO, ALB, ALB-S
ABZ	NH2-FLU/ 99	12.5	89.6	80.0	12.9	400	≤ 5% for NH2-FLU, ALB-2NH2-S, NH2-MEB
TBZ	OH-TIA/ 91	25	83.2	74.2	63.0	100	≤ 5% for OH-TIA, TIA, cambendazole
TCBZ	TRICLA-SO/ 40	50	97.7	77.0	69.2	250	≤ 5% for TRICLA-SO, TRICLA, keto-triclabendazole
LVM	LVM/ 100	60	77.5	68.4	51.6	100	≤ 5% for LLVM
MXD	MXD/ 100	20	103.5	93.4	77.0	100	≤ 5% for MXD
AVM	DOR/ 75	15	101.1	86.9	69.4	10	≤ 5% for DOR, IVER, ABA, EPRI, EMA

LC-MSMS results							
Class	Reference analyte	Level of interest (µg kg^{-1})	B (µg kg^{-1})	T (µg kg^{-1})	F_m (µg kg^{-1})	Lowest MRLs (µg kg^{-1})	ß-error
Benzimidazoles (corresponding to BZS, ABZ, TBZ, TCBZ class of BAI)	ALB	25	0.05	0.09	21.8	1000 as sum	≤ 5%
	ALB-SO	25	0.04	0.06	21.5		≤ 5%
	ALB-S	25	0.04	0.11	21.9		≤ 5%
	ALB-2NH2_S	25	0.02	0.04	13.8		≤ 5%
	FEN	25	0.11	0.56	22.2	500 as sum	≤ 5%
	FEN-S	25	0.01	0.08	20.7		≤ 5%
	FEB	25	0.03	0.06	22.0		≤ 5%
	OXF	25	0.10	0.24	21.9		≤ 5%
	OXI	25	0.01	0.02	20.0	200	≤ 5%
	MEB	25	0.02	0.14	18.9	400 as sum	≤ 5%
	OH-MEB	25	0.07	0.21	19.8		≤ 5%
	NH2-MEB	25	0.01	0.06	10.6		≤ 5%
	FLU	25	0.02	0.05	18.6	400 as sum	≤ 5%
	NH2-FLU	25	0.01	0.07	10.8		≤ 5%
	TIA	25	0.03	0.15	18.0	100 as sum	≤ 5%
	OH-TIA	25	0.004	0.01	12.0		≤ 5%
	TRICLA	25	0.04	0.12	18.5	250 as sum	≤ 5%
	TRICLA-SO	25	0.35	1.46	8.8		≤ 5%
	TRICLA-S	25	0.06	0.15	10.7		≤ 5%

Table 2. Continued.

Salicylanilides	CLO	25	0.63	2.48	19.2	1000	≤ 5%
Imidazothiazoles (corresponding to LVM class of BAI)	LVM	25	0.02	0.08	21.5	100	≤ 5%
Milbemycins (corresponding to MXD class of BAI)	MXD	10	0.01	0.04	5.5	100	≤ 5%
Avermectins (corresponding to AVM class of BAI)	ABA	10	0.01	0.03	7.6	10 (poultry, swine)	≤ 5%
	DOR	10	0.01	0.02	7.0	100	≤ 5%
	EMA	10	0.01	0.02	7.6	10 (poultry)	≤ 5%
	EPRI	10	0.01	0.02	7.5	1500	≤ 5%
	IVER	10	0.03	0.20	6.0	100	≤ 5%

Results and discussion

The results of validation are summarized in Table 2.

For benzimidazoles (corresponding to BZS, ABZ, TBZ, TCBZ class of BAI) and milbemycins for both procedures the level of interest chosen for ß-error verification was far below the MRLs in liver.

For imidazothiazole the molecule selected was LVM for both procedures and the level of interest was far below the MRL for LC-MSMS and equal to 60% of the MRL for BAI.

For avermectins, the level of interest was set to 10 µg kg^{-1} for LC-MSMS and to 15 µg kg^{-1} during BAI method development considering DOR as reference analyte. These values are well below the MRLs established for DOR, EPRI, IVER but the level of interest chosen for BAI procedure is higher the MRLs established for ABA in poultry and swine and for EMA in poultry. For this reason, 6 blank swine liver spiked with ABA at 10 µg kg^{-1}, 6 blank poultry liver spiked with ABA at 10 µg kg^{-1} and 6 blank poultry liver spiked with EMA at 10 µg kg^{-1} were analysed. All of samples were recognized as non-compliant giving a ratio RLU/RLU0 < Fm of AVM.

Conclusions

Concerning the comparison of the proposed analytical approaches, it can be concluded that: (1) the sensitivity of LC-MSMS is better than the sensitivity of Biochip Array when referring to the entire group of analytes. (2) sample preparation is similar, but Biochip Array enables to capture images of up to 9 samples per carrier, thus reducing the total time required for the analysis of a consistent batch of samples; (3) The major drawbacks related to Biochip Array are: *i*) CLO is not detectable, *ii*) the cost of the dedicated kit, *iii*) a false suspect ratio for TCBZ (18%) observed during the implementation of this approach on routine samples, and *iv*) subsequent confirmatory analysis needed to confirm a positive result.

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MULTIMETHOD FOR ANTIBIOTIC ANALYSIS USING 2-D LIQUID CHROMATOGRAPHY MASS-SPECTROMETRY

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Abstract

The possibilities of 2D-LC multi-class for the analysis all relevant tetracyclines, sulfonamides, macrolides, (fluoro)quinolones and aminoglycosides were explored. The challenge using 2-dimensional liquid chromatography was to interface two orthogonal separation principles, in order to set up an analysis method for the simultaneous analysis of these antibiotic groups. After a selection of described separation techniques, various analytical columns, gradients and mobile phases were tested. Here, the combination of zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) and reversed-phase chromatography for the separation of the antibiotics was investigated. In addition, experiments were conducted to determine the usability of weak cation-exchange and of an α-acid glycoprotein (AGP) column. The main challenge was the selection of analytical columns, mobile phases and the connection of the mobile phase gradients. A chiral analytical separation column (α-acid glycoprotein) in the first dimension combined with a reversed-phase column in the second dimension was promising.

Introduction

In the Dutch National Residue Control Plan (NP), a large variety of antibiotics have to be monitored in many different matrices. For all antibiotics included in the NP, instrumental multi-analyte or multi-class methods are operational (Berendsen *et al.,* 2014; Sylvestro *et al.,* 2014; Berradada *et al.,* 2010). No multi-class methods are available that include all relevant aminoglycosides. To obtain a more efficient and therefore more price-effective monitoring, a method in which all relevant tetracyclines, sulfonamides, macrolides, (fluoro)quinolones and aminoglycosides are combined in a single method for the analysis of muscle is needed. Development of a multi-method for these different antibiotic groups is considered very challenging because of the wide variety of chemical properties.

A general sample extraction and clean-up method is developed followed by a novel analysis method. After an acidic extraction, the extract was cleaned by ion-pair SPE before LC-MS/MS analysis. As reversed-phase LC is not able to retain the aminoglycosides a novel approach was applied. An LC system consisting of a HILIC column in line with a reversed phase column proved to retain and sufficiently separate all compounds of interest within a reasonable timeframe. This work will be published elsewhere.

The research presented here focuses on the development of a 2D-LC method because this will simplify the analysis. During method development, several chromatographic separation columns with different separation characteristics were tested. Considered technics are ion-chromatography, chiral separation, HILIC and different reversed phase columns.

Materials and Methods

Materials

Acetic acid, isopropanol, Ultra LC-MS grade water, methanol and acetonitrile, (Actu-all chemicals, Oss, The Netherlands), formic acid 98-100% LC-MS grade, HCL solution 37%, (Merck, Darmstadt, Germany), ammoniumformate, ammoniumsulfate, methanesulfonic acid, (Sigma-Aldrich, Saint Louis, MI, USA), ammoniumacetate (VWR, Radnor, Pennsylvania, United States) were used. Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18.2MΩ cm^{-1} (Millipore, Billerica,MA, USA). The reference standard of apramycin sulfate, paromomycin sulfate, streptomycin sulfate, dapson, sulfathiazole, sulfadimethoxine, sulfamethoxypyridazine, sulfamonomethoxine, chlortetracycline, doxycycline, oxytetracycline, tetracycline, flumequine, spectinomycin dihydrochloride pentahydrate, marbofloxacine were obtained from Sigma-Aldrich (Saint Louis, MI, USA), gentamycin sulfate (spectrum chemical, New Brunswick, NJ, United states), kanamycin A (LKT laboratories, St. Paul, MN, United States), neomycin B sulfate (FLUKA), sulfamonomethoxine (TCI chemicals, Portland, Oregon, United states), tildipyrosin (Intervet). Figure 1 is a schematic overview of a general LC-MS formation and 2D-LC formation.

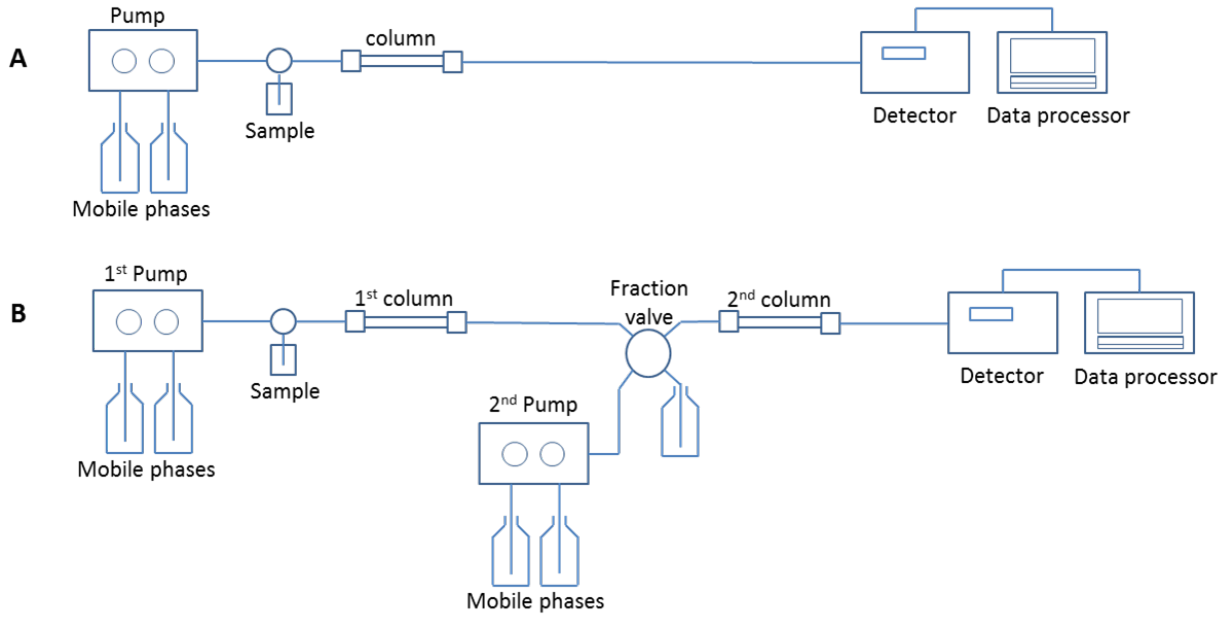

Figure 1: Illustration of method set-up. A: general LC-MS set-up, B: comprehensive 2D-LC set-up.

Table 1. Information about the used antibiotic groups and specific compounds with their precursor ion.

Antibiotic group	Component	Precursor ion (*m/z*)
Aminoglycosides	Spectinomycin	351.2
	Gentamycin C2a	450.3
	Gentamycin C2	464.3
	Gentamycin	478.3
	Kanamycin A	485.2
	Apramycin	540.3
	Streptomycin	582.3
	Dihydrostreptomycin	584.3
	Neomycin	615.3
	Paromomycine	616.3
Macrolides	Tildipyrosin	368.0
	Josamycin	828.1
Tetracyclines	Tetracycline	444.9
	Doxytetracycline	444.9
	Chlortetracycline	478.9
Sulfonamides	Dapson	249.0
	Sulfadiazine	251.0
	Sulfamethoxine	311.2
	Sulfadoxine	311.2
	Sulfathiazole	256.0
	Sulfamethoxypynidazine	281.2
	Sulfamonomethoxine	281.2
Quinolones	Flumequine	262.1
	Marbofloxacine	363.0
	Oxolinic acid	262.1

LC-MS analysis

The LC system used for comprehensive 2D-LC consisted of an Acuity column department (H13CHA4786), Acquity sample manager (FNT J13USM2216), two Acquity binary solvent managers (L06UPB425M and K13BUR756M), Acquity QDA detector, Rheodyne 2-position,10-port switching valve with two 200-µL loops. Different analytical columns were used during method development. For application in the first dimension the following columns were assessed: Chiralpak AGP 5µ 4 x 150 mm, a

Dionex IonPac CS19 4µ 2 x 250 mm, Sycronis HILIC 5µ 100 x 2.1 mm. For application in the second dimension, the following columns were used: Kinetex 2.6µ biphenyl 2.1 x 100 mm, Kinetex 5µ EVO C18 5 x 100mm, Sycronis HILIC 5µ 100 x 2.1 mm.

Model compounds were selected for use in method development and optimisation. In Table 1 these selected model compounds and corresponding precursor ion (*m/z)* are given. Each antibiotic group is represented by at least two compounds; all aminoglycosides are included because their retention behaviour is of special interest.

Results and Discussion

Different chromatographic separation principles are tested during development of the multi-method. The used chromatographic separation types were: HILIC, cation-exchange and α-acid glycoprotein combined with reversed-phase chromatography. Reversed-phase chromatography was chosen to be at least one of the separation techniques because it is widely applicable. Different types of analytical columns, eluents, gradient programs and loop switching options were investigated with regard to obtaining the best two-dimensional chromatographic resolution among the compounds and all compounds have some retention.

First, the combination of a reversed phase column in the first dimension and a HILIC column in the second dimension was tested because of the known orthogonal character of HILIC compared to reversed phase chromatography. It is observed that the maximum conditioning time in the second dimension was not long enough for HILIC to stabilize and therefore HILIC is considered unsuitable for use as a second dimension separation.

However, the combination of HILIC in the first dimension and reversed phase chromatography in the second was very promising. After optimisation of a separation using a HILIC column, primarily focusing on the aminoglycosides, retention is obtained for the aminoglycosides with satisfactory peak shapes. The other antibiotic compounds showed broad peaks and poor separation (no retention) using HILIC. It was concluded that these results could be sufficient for a 2D application, but only if a good separation can be obtained for the other antibiotic classes on the reversed phase column, within a minute. Unfortunately, experiments showed no satisfactory results for this combination. The main issue was incompatibility of the solvents of the two dimensions. The organic concentration of the HILIC gradient during elution of the compounds of interest was too high compared to the needed initial gradient of the 2-D RP gradient. After these findings it was considered to investigate other separation principles in which no high organic solvents are needed to overcome this problem.

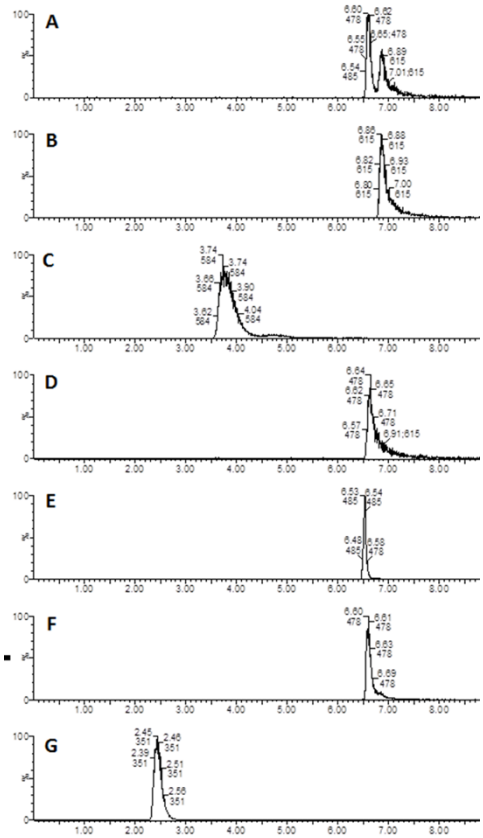

Figure 2: Chromatogram of aminoglycosides on an α-acid glycoprotein column. A, paromomycin; B, neomycin; C, dihydrostreptomycin; D, apramycin; E, kanamycin; F, gentamycin; G, spectinomycin.

Weak cation-exchange chromatographic separation was tested to investigate the possibility to apply it in combination with RP in 2D chromatography with respect to the selected compounds. After applying different types of gradient solvents and run times, it was concluded that a weak cation-exchange column was not suitable for the specific 2D combination. Some aminoglycoside compounds did not elute from the column, probably caused by their multiple charged character.

A chiral column (α-acid glycoprotein) was tested for its use in the first dimension. It was observed that all compounds can be retained using this column. During method development it was noticed that the pH of the mobile phases was very critical and thus an important parameter for gradient optimisation. Figure 2 shows a chromatogram of the aminoglycosides obtained on the α-acid glycoprotein chiral column. The generated separation of the aminoglycoside compounds was promising for use in a 2D chromatography setup. The used gradient solvents were fully compatible with the initial conditions of reversed-phase chromatography. Some peaks are relatively broad, however, peak sharpening will occur in the second dimension allowing broader peaks in the first dimension separation compared to traditional chromatography. For future research using 2D-LC for multi-class analysis of antibiotics, the combination of the chiral analytical column (α-acid glycoprotein) in the first dimension and a reversed phase column in the second dimension will be studied.

Conclusions

The possibilities of 2D-LC multi-class analysis that includes all relevant tetracyclines, sulfonamides, macrolides, (fluoro)quinolones and aminoglycosides were explored. Although no final method was developed yet, a lot of knowledge about 2D-LC applications was obtained. A chiral analytical separation column (α-acid glycoprotein) in the first dimension combined with a reversed phase column in the second dimension was promising.

Acknowledgements

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MULTI-RESIDUE AND MULTI-CLASS DETERMINATION OF ANTIBIOTICS AND ANTHELMINTICS IN FEED BY ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

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Abstract

The veterinary drugs most broadly used in medicated feed are antimicrobials and anthelmintics. Although these antimicrobials are authorized, traces are undesirable in non-medicated feed. As medicated and non-medicated feeds are often manufactured in the same production lines, carry-over of antimicrobials can occur when a feed miller switches from producing one feed to the next one. To decrease the level of cross-contamination in feed in Belgium, the Federal Agency for the Safety in agreement with Belgian feed producers, have decided to authorize a provisional carry-over at 1% until 31/12/2016.
An analytical strategy was developed for high-throughput analysis of multiple antibiotics and anthelmintics determination in feed. Because of the large differences in physicochemical properties of these compounds, sample preparation was a simple, fast and primarily focused on the extraction of colistin. After acidic extraction, samples were centrifuged, purified on SPE, and analysed by UHPLC-MS/MS in MRM mode. A quantitative validation was done for amoxicillin, chlortetracycline, colistin, doxycycline, fenbendazole, flubendazole, ivermectin, lincomycin, oxytetracycline, sulfadiazine, trimethoprim, tiamulin and tilmicosin and performances were in accordance with European Commission Decision 2002/657/CE. Matrix-matched calibration with internal standards were used to reduce the matrix effects. This method has been successfully used to routine monitoring residues in feeds since three years.

Introduction

Conventional livestock production systems make great use of veterinary drugs therapeutically, prophylactically and as growth-enhancers. Oral administration, especially through feeding stuff, remains the best way to administer veterinary drugs. Among the authorized veterinary drugs, the most widely used in medicated feed are tetracyclines, sulfonamides, trimethoprim, macrolides, β-lactams, aminoglycosides, pleuromutilins, lincosamides, benzimidazoles, and avermectins. Although the veterinary drugs listed here are authorized, traces are undesirable in non-medicated feed. As different types of feed (medicated and non-medicated) are often manufactured in the same production line, carry-over of veterinary drugs can occur when a feed miller switches production from one feed to the next. To decrease the level of cross-contamination in feed in Belgium and thus to protect animal health and minimize risk to consumers, the Federal Agency for the Safety of the Food Chain (FASFC), in agreement with the Belgian Compound Feed Industry Association, has decided to implement effective veterinary drug residue monitoring and to authorize provisionally a 1% carry-over at feed-producing plants.

Monitoring feed to ensure the absence of undesirable veterinary residues at low levels requires sensitive and selective methods. Here, such a method is presented. The adopted sample preparation procedure is simple, fast, cheap, and focused primarily on colistin extraction. Interactions between polypeptides and proteins present in the sample matrix have been already reported. A low pH of the extraction solution seems to be essential to ensure acceptable recoveries.

Materials and Methods

Chemicals

All chemicals were at least of analytical reagent grade. Dimethylsulfoxide was obtained from Sigma Aldrich (Bornem, Belgium). Methanol, formic acid, and acetonitrile were ULC-MS grade solvents purchased from Biosolve (Valkenswaard, the Netherlands). Water was purified with a Sartorius Arium ultra-pure laboratory water purification system (Sartorius, Göttingen, Germany). Oasis HLB SPE cartridges (200 mg, 6 ml) were obtained from Waters (Waters, Milford, MA, USA).

Chlortetracycline, doxycycline, ivermectin, lincomycin, tiamulin, and tilmicosin were purchased from LGC Standards (Molsheim, Germany). Amoxicillin, fenbendazole, flubendazole, oxytetracycline, sulfadiazine, trimethoprim and demeclocycline (internal standard, IS) were supplied by Sigma-Aldrich (Bornem, Belgium). Sulfadiazine-13C6 (IS), trimethoprim-d9 (IS) and triclabendazole-d3 (IS) were supplied by Witega (Berlin, Germany). Colistin was from TRC (Toronto, Canada).

Drugs were weighed to prepare stock solutions of individual compounds at 1 mg mL^{-1} in their appropriate solvent and at 0.1 mg mL^{-1} in water for colistin.

Two working solutions, namely an IS solution and a solution containing all the remaining compounds, were prepared by diluting aliquots of the appropriate stock solutions in methanol. The working standard solution contained all the analytes at concentrations appropriate for achieving the target concentration of each compound by spiking 5.0 g blank feed sample with 100 µL working standard solution (Table 1), 127.5 µL colistin, and 50 µL IS.

The compounds used as internal standards were demeclocycline (for all tetracyclines), trimethoprim-d_9 (for trimethoprim), triclabendazole-d_3 (for the two benzimidazoles and ivermectin), and sulfadiazine-$^{13}C_6$ for all other compounds. The working IS solution was a mixture of all the individual internal standards in 10 mL methanol (final volume). The concentrations used were 10 µg mL^{-1} for trimethoprim-d_9, 50 µg mL^{-1} sulfadiazine-$^{13}C_6$ and triclabendazole-d_3, and 25 µg mL^{-1} for demeclocycline. All stock and working solutions were stored for no more than one year at -18°C, except the ß-lactam stock and working solution, which were stored at -70°C and the colistin solution, which was freshly prepared before use.

Table 1. Analytical range of calibrations: from 0.25 to 4.0% carry-over level.

Compound	Lowest level 0.25%	Mid level 1.0%	Highest level 4.0%
	Concentration (µg kg^{-1})		
Amoxicillin	750	3,000	12,000
Chlortetracycline	750	3,000	12,000
Colistin	637.5	2,550	10,200
Doxycycline	625	2,500	10,000
Fenbendazole	82.5	330	1,320
Flubendazole	75	300	1,200
Ivermectin	25	100	400
Lincomycin	110	440	1,760
Oxytetracycline	500	2,000	8,000
Sulfadiazin	35	140	560
Tiamulin	100	400	1,600
Tilmicosin	1,250	5,000	20,000
Trimethoprim	87.5	350	1,400

Sample preparation

Representative samples were taken from previously analysed batches of three different types of feed (cattle, pig and poultry).

Sample extraction

To each 50-mL polypropylene tube containing 5 g of sample, 50 µL IS solution was added. To extract drug residues and precipitate proteins, 20 mL hydrochloric acid (0.5 M in aqueous solution) was added. The mixture was then shaken at room temperature for 15 min and centrifuged at 4,650 *g* for 10 min. The aqueous layer was collected and transferred to a clean Falcon tube. Then, 6 mL of feed sample extract was loaded on an Oasis HLB cartridge (preconditioned with 5 mL methanol and 5 mL water) under vacuum. The cartridges were then rinsed with 5 mL ultra-pure water and vacuum-dried. Finally, the retained components were eluted with 1.5 mL methanol/acetic acid (90/10; v/v) followed successively by 1.5 mL methanol/ water/formic acid (70/29.8/0.2; v/v/v) and 1.5 mL methanol.

The organic phase was evaporated to dryness under a stream of nitrogen in a water bath set at 40°C. The extract was reconstituted in 2 mL ACN/water (10/90, v/v). After centrifugation, the clear layer was transferred to a vial prior to UHPLC-MS/MS analysis.

Liquid chromatography/mass spectrometry

Chromatographic analyses were performed with an Acquity UPLC system (Waters, Milford, MA, USA) and separations were done on an Acquity UPLC HSS T3 column (150 × 2.1 mm, 1.7 µm particle size) from Waters. The column was equilibrated at 50°C and the injection volume was 20 µL. The mobile phases consisted of 0.05% formic acid in water (A) and ACN (B). The gradient used was as follows: 0-0.5 min: 10% B, 0.5-2.5 min: increase to 75% B, 2.5-4.0 min: 100% B; 4.0-5.0 min: 100% B) at a flow rate of 0.5 mL min^{-1} with post-column delivery of NH_4OH at a flow rate of 10 µL min^{-1} between 4.3 and 5.5 min. The column and autosampler were maintained, respectively, at 50°C and 15°C.

Mass-spectrometric analysis was carried out with a Waters Acquity TQ mass spectrometer (Waters, Manchester, UK). The instrument was operated with an electrospray ionization source in the positive (ESI+) and negative (ESI-) ionization modes. The ESI parameters were adjusted as follows: capillary voltage 3.0 kV, cone voltage 30 V, source temperature 150°C, desolvation temperature 600°C, cone gas (nitrogen) flow 50 L h^{-1}, desolvation gas (also nitrogen) flow 1,200 L h^{-1}. Collision-induced

dissociation was done with argon as the collision gas at 4 10^{-3} mbar pressure in the collision cell. The specific MS/MS transitions are shown in Table 2. Data acquisition was done with the MassLynx 4.1 software and the TargetLynx 4.1 software (Waters).

Table 2. MS/MS conditions for the MRM acquisitions and relative retention time (RRT) for each veterinary drug

Name	ESI	Quantification Trace	Confirmation Trace	RRT	IS
Amoxicillin	+	366.0 > 208.2	366.0 > 114.0	0.47	Sulfadiazine $^{13}C_6$
Chlortetracycline	+	478.9 > 443.9	478.9 > 97.9	1.05	Demeclocycline
Colistin A	+	390.9>379.0	390.9>385	0.93	Sulfadiazine $^{13}C_6$
Colistin B	+	386.0>380.0	386.0>374.0	0.91	Sulfadiazine $^{13}C_6$
Doxycycline	+	444.9 > 153.8	444.9 > 427.9	1.06	Demeclocycline
Fenbendazole	+	300.0 > 159.0	300.0 > 268.01	0.83	Triclabendazole-d_3
Flubendazole	+	314.1 > 282.2	314.1 > 123.2	0.76	Triclabendazole-d_3
Ivermectin	-	873.5 > 567.3	873.5 > 108.9	1.44	Triclabendazole-d_3
Lincomycin	+	407.0 > 126.0	407.0 > 159.2	0.94	Sulfadiazine $^{13}C_6$
Oxytetracycline	+	460.9 > 443.1	460.9>423.0	0.94	Demeclocycline
Sulfadiazine	+	251.0>156.0	251.0>91.9	1	Sulfadiazine $^{13}C_6$
Tiamulin	+	494.2 > 118.9	494.2 > 192.0	1.31	Sulfadiazine $^{13}C_6$
Timicosin	+	869.9 > 174.0	869.9 > 87.9	1.13	Sulfadiazine $^{13}C_6$
Trimethoprim	+	290.9 > 261.0	290.9 > 275.0	1	Trimethoprim-d_9
Demeclocycline	+	465.1>430.0			
Sulfadiazine $^{13}C_6$	+	257.0>162.2			
Triclabendazole-d_3	+	364.2 > 346.0			
Trimethoprim-d_9	+	300.0>234.1			

Results

In the absence of guidelines related to the validation of antimicrobials in feed by LC-MS/MS, a validation protocol was established to prove that the method performance was fit for the purpose, taking into account the requirements of Commission Decision 2002/657/EC for confirmatory and quantitative purposes. The evaluated parameters were linearity, selectivity, specificity, sensitivity, intra-day precision, inter-day precision, trueness (expressed as recovery) and uncertainty.

Matrix-matched calibration curves were constructed by plotting relative peak area versus the spiking concentration of each veterinary drug. The linearity of the method was determined by assaying eight levels (0, 0.25, 0.5, 0.75, 1.0, 2.0, 3.0 and 4.0% carry-over level) on each of the three validation days. The concentration ranges of the various compounds are presented in Table 1. The coefficients of determination (R^2) are shown in Table 3. Good linearity was achieved in all cases, with coefficients of determination higher than 0.986 (Table 3).

The selectivity and specificity of the method were evaluated by checking for the absence of interfering peaks at the expected relative retention times. This was done by analysing seven blank feed samples each day for three days. The chromatographic profiles obtained from a typical blank feed samples is presented in Figure 1A. No interfering peaks were detected in the region of interest for all analytes. This indicates that the method is selective.

The accuracy and precision of the method in terms of repeatability (% RSD_r, intra-day precision) and reproducibility (% RSD_R, inter-day precision) were determined using blank feed samples fortified with known amounts of the analytes (seven replicate measurements for each of three levels: 0.5%, 1.0% and 2.0% carry-over level on three days). The method was validated for three animal feed matrices: cattle (day 3), pig (day 2), and poultry (day 1) feed (one validation day per feed). Recovery, repeatability, and within-laboratory reproducibility data are given in Table 3. The mean recoveries for all of the target compounds were satisfactory. Repeatability and within-laboratory reproducibility values were calculated on seven replicates per level on the same day and on three different days, respectively. All results are presented in Table 3. RSD_r and RSD_R values were below 14.0% and did not exceed the limits calculated from Horwitz-Thompson equations. The recovery and % RSD values are satisfactory for all compounds at the three levels of fortification and are in agreement with European Decision 2002/657. The combined uncertainty was calculated at the 95% confidence level, taking into account the precision (within-laboratory reproducibility, RSD) and trueness (uncertainty of the bias) of the validation and QC data.

The method also meets the validation criteria for analyte identification and confirmation on the basis of retention times, two MRM transitions, and relative ion ratios of selected MRMs, as laid down by the European Decision 2002/657.

Table 3. Validation data of the analytical method with recovery rates, relative standard deviation (RSD), U and lowest R².

Coumpound	Fortification level (µg kg^{-1})	Recovery (%) (n=18, per level)	Within days RSD_r max (%) (n=6, per level)	Between days RSD_R (%) (n=18, per level)	Uncertainty (%)	Lowest R^2
Amoxicillin	1,500	99.1	3.4	5.1		
	3,000	93.0	1.9	9.7	28.2	0.986
	6,000	89.4	6.9	6.9		
Colistin (A+B)	1,275	95.5	12.4	14.0		
	2,550	95.4	8.7	11.8	36.6	0.997
	5,100	99.2	4.5	9.6		
Chlortetracycline	1,500	95.8	9.5	8.6		
	3,000	100.2	3.8	5.7	18.5	0.997
	6,000	94.9	8.0	9.4		
Doxycycline	1,250	96.9	10.2	10.0		
	2,500	94.0	3.9	6.6	21.2	0.996
	5,000	94.0	8.1	7.1		
Flubendazole	150	97.0	1.0	6.7		
	300	94.5	1.2	9.4	27.0	0.994
	600	99.6	0.9	6.3		
Fenbendazole	165	97.4	8.2	6.1		
	330	96.5	11.9	12.5	33.2	0.989
	660	106.7	8.2	11.4		
Ivermectin	50	103.5	6.6	9.3		
	100	96.0	9.1	7.1	35.4	0.998
	200	95.1	7.8	11.0		
Lincomycin	220	101.6	4.1	8.4		
	440	99.7	3.3	10.0	29.4	0.996
	880	93.7	4.3	8.0		
Oxytetracycline	1,000	101.7	7.0	9.3		
	2,000	100.1	5.9	7.7	18.6	0.998
	4,000	94.4	7.2	7.3		
Sulfadiazine	70	102.1	3.9	4.7		
	140	99.7	2.4	3.9	13.4	0.995
	280	101.5	2.4	4.1		
Tiamulin	200	92.3	7.3	4.7		
	400	93.5	11.7	7.1	32.8	0.999
	800	94.5	6.05	6.5		
Tilmicosin	2,500	104.4	5.0	6.2		
	5,000	95.6	8.2	5.6	33.2	0.990
	10,000	94.9	6.6	6.6		
Trimethoprim	175	103.9	7.8	9.1		
	350	92.9	8.8	8.2	23.8	0.998
	700	90.4	3.3	7.1		

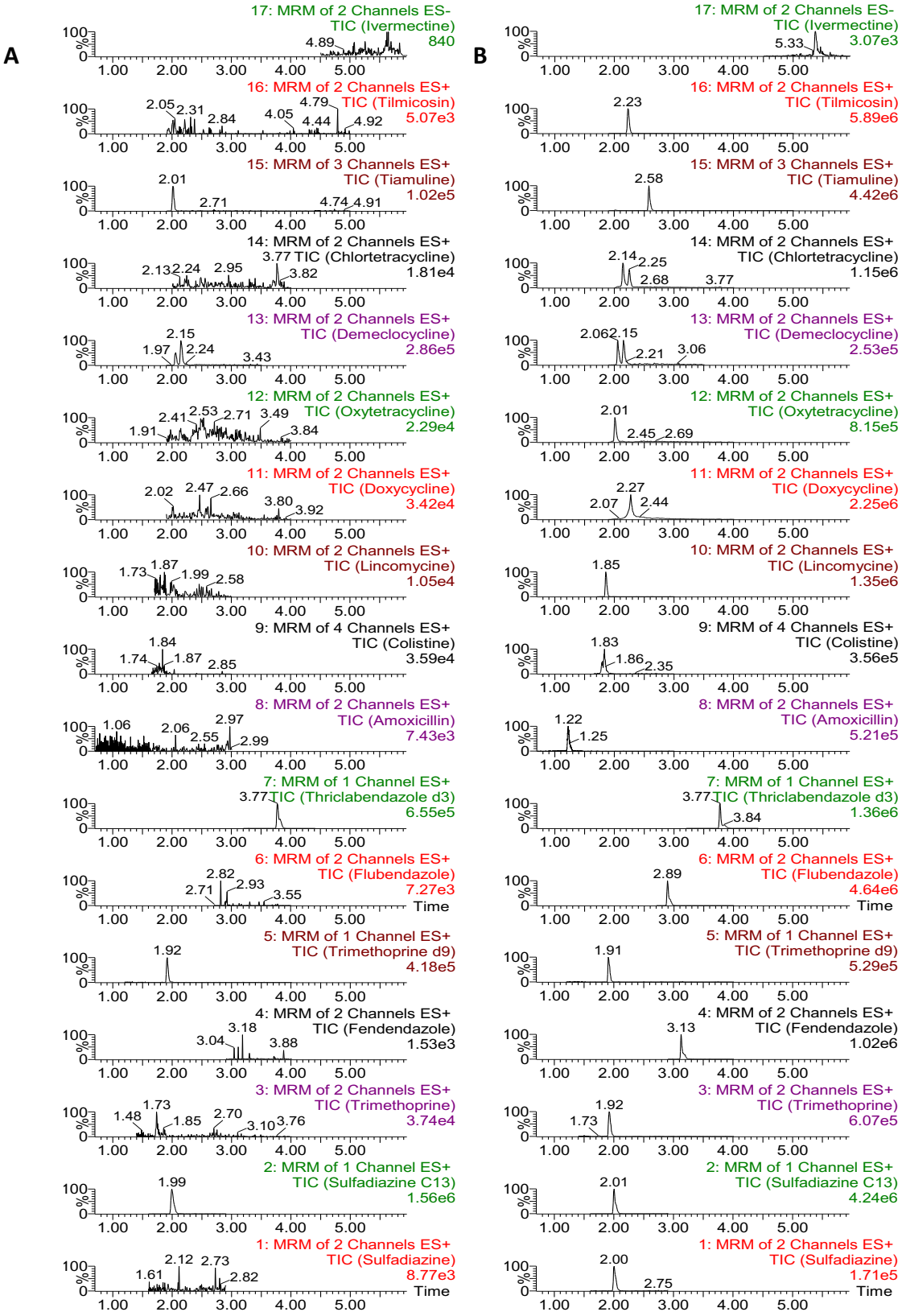

Figure 1. LC–MS–MS chromatograms of a (A) blank feed sample, and (B) fortified feed sample at the lowest calibration level corresponding to 0.25% carry over level.

Applicability to real samples

In addition to recovery studies, the trueness of the method was further demonstrated through participation in a proficiency test organized by Rikilt in 2013. Our laboratory characterized all samples correctly without any false negatives, and all z-scores were satisfactory for the detected compounds: chlortetracycline, oxytetracycline, and flubendazole. These good results also confirm that matrix effects are quite limited when matrix-matched calibration curves and appropriate internal standards are used.

Over the past three years, we have used our method successfully to analyse more than 200 real feed samples (including fish feed, liquid feed), notably feeds from the National Control Plan and auto-control samples from feed producers. More than one-half of the samples were found negative or the concentration was lower than the first calibration point, 26% of the samples were positive but the concentration was lower than the 1% authorized carry-over level and were declared compliant, and finally, 20% of the samples were positive with concentration of analyte higher than the 1% authorized carry-over level and were declared non-compliant.

Conclusions

Thanks to major advances in chromatographic separation and detection systems over the past decade, LC-MS/MS has become the most powerful technique for analysing food and feed matrices for drug residues. The compounds studied here possess a wide range of physicochemical properties. This paper presents an efficient determination method for veterinary residues in feed samples, combining a quick, simple extraction and purification procedure with the capabilities of UHPLC and mass spectrometry for the analysis of thirteen veterinary drugs from ten different families and including colistin, belonging to the fifth most sold group of antimicrobials. The novelty this method is the analysis of colistin A and colistin B together with selected pharmaceuticals in feed. The applicability of the method to the determination of veterinary residues was also verified by participating in a proficiency test. The developed method has been successfully used for the analysis of feed samples for more than three years.

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RAPID AND SPECIFIC EXTRACTION OF ANABOLIC STEROIDS (A1, A3, A4) AND CORTICOSTEROIDS IN URINE BEFORE DETECTION AND IDENTIFICATION BY UPLC-MS

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Abstract

A rapid and specific extraction method was developed for urine using an enzymatic hydrolysis, an SPE purification with OASIS HLB. The individual biological samples were further cleaned-up on self-made multi-immunoaffinity chromatography gel so as to decrease interferences due mainly to background signals.

A liquid-chromatography mass-spectrometry (UPLC-MS/MS) method was also developed using the ultra performance liquid chromatography (UPLC) technology, and electrospray ionization (ESI) in the positive and negative ion modes. Gradients UPLC separation conditions were optimized for a group of 44 analytes comprising anabolic steroids (stilbenes, steroids, resorcyclic acid lactones) and glucocorticosteroids including prednisolone. Validation was performed according to Decision 2002/657/EC criteria as a confirmation method: decision limit (CCα), detection capability (CCβ) and specificity were determined. Detection capability ranged between 0.05 and 4 µg L^{-1} depending on the compound. This method also allows the quantification of prednisolone.

Introduction

In the European Union, Council Directive 96/23/EC prohibits the use of anabolics as growth-promoters for meat production. The Veterinary Food Inspection of each Member State is required to comply with this legislation. To monitor biological specimens effectively for the presence of anabolic agents and their metabolites, it is essential to have analytical techniques for detecting trace amounts of these compounds in various biological matrices. The list of steroids liable to be abused in cattle fattening is ever lengthening and the analytical requirements are increasingly stringent. For this reason, specific immunoassays allowing the detection of a single molecule are now being replaced by multi-residue techniques such as mass-spectrometry (Delahaut *et al*., 1996, Leinonen *et al*., 2002) combined with chromatographic separations.

As the number of molecules abused has increased over the years, "anabolic cocktails" have made their appearance, *i.e.,* mixtures containing several different agents at low but still active concentrations. Because their effects are additive, the resulting mass increases recorded in cattle are similar to those produced by single compounds at higher concentration. Since smaller amounts of each agent are eliminated in biological specimens, they are much more difficult to trace. Detection requires highly sensitive techniques with low background interference. Immuno-affinity purification techniques (Hosogi *et al*., 2010, Moser *et al*, 2010, Thevis *et al*., 2005, 2006, Van Ginkel *et al*., 1991.) offer tremendous and decisive advantages over conventional methods used to pre-treat biological specimens. In this work, we combined multi-residue immune-affinity chromatography with efficient liquid chromatographic separation. This efficient method, validated for 44 compounds, is widely applicable to urine samples, *i.e.,* the biological specimen that veterinary inspectors usually take in slaughter houses and farms. It could provide a basis for a universal procedure for detecting multiple anabolic agents and their metabolites in such specimens.

Materials and Methods

Materials

Standards of the drugs and their metabolites were purchased from Sigma-Aldrich GmbH, Steraloids, LGC, Dr. Ehrenstorfer GmbH and Rikilt. Enzymatic hydrolysis was performed with *Helix pomatia* juice (Sigma-Aldrich). Solvents such as absolute ethanol, methanol, ethyl acetate, hexane, used in the various steps of the extraction procedure were of the highest quality available (pro analysis or HPLC grade).Other chemicals and reagents were of the highest purity available. Phosphate buffered saline (PBS, pH 7.4; 0.05 M) and sodium acetate buffer (pH 5.2; 2 M) were prepared weekly.

Multi-immuno-affinity chromatographic gel (MIAC) columns were used for the second purification step. They were prepared by mixing several individual gels, each of them being prepared with specific antibodies (anti-methyltestosterone, anti-nortestosterone, anti-fluoxymesterone, anti-zeranol, anti-clostebol, anti-ethynylestradiol, anti-diethylstilbestrol, anti-trenbolone, anti-norethandrolone, anti-stanozolol, anti-medroxyprogesterone acetate, ant-dexamethasone, anti-methylprednisolone, anti-triamcinolone acetonide and anti-beclomethasone). Antibodies (Erlanger *et al*., 1957) and gels (Moser *et al*., 2010) were produced by the Health Department (CER Groupe, Marloie, Belgium). They are commercially available, individually or collectively. The immunoglobulins (IgGs) were coupled to cyanogen bromide activated Sepharose 4B (GE Healthcare Biosciences AB, Uppsala, Sweden) according to the supplier's instructions.

Table 1. LC (retention time, RT) and MS (ionic species) characteristics of anabolic steroids in positive mode.

Name	Diagnostictic ions (*m/z*)			RT (min)
Methandriol	269.5	159	105	6.84
17β nortestosterone	275	109	83	4.97
17α nortestosterone	275	109	83	5.63
17β -boldenone	287	121.1	147.2	4.81
17α -boldenone	287	121.1	147.2	5.49
17β-testosterone	288.7	97	109	5.46
5α -androst-1-en-17α -ol-3-one	289.2	187.5	253.3	6.83
Methylboldenone	301.1	121.2	149	5.24
Methyltestosterone	303	96.9	109	6.1
Norethandrolone	303	109	267.1	7.01
Norgestrel	313	245	109	5.93
Progesterone	315	109	297	7.71
4-chloro-androst-4-ene 3,17 –dione (CLAD)	321.5	143	131	5.77
Stanozolol	329	95	121	6.82
16β-hydroxy-stanozolol	345.3	81.3	95.3	4.82
Fluoxymesterone	337.2	281.3	299.5	4.79
Medroxyprogesterone acetate	387.1	123	327.1	7.58
Trenbolone	271.3	253.3	107.2 *	4.84
Prednisone	359.3	313.3	323	2.83
Prednisolone	361.2	147	325.3	3.38
Methylprednisone	373	161	185	3.89
Methylprednisolone	375	339.3	161	4.31
Isoflupredone	379.3	359.3	341.3	2.99
Dexamethasone	393	373.3	355.3	4.18
Flumethasone	411.3	253.3	391.3	3.88
Clobetasol	411.3	373.3	147.3	5.11
Triamcinolone acetonide	435	415	397	4.45
Fluocinolone acetonide	453.5	356.8	375.2	4.35
Fluorometholone	377.5	339.4	357.3	4.60
Beclomethasone	409.3	373.3	391.3	4.37
Methenolone	503.4	205.1	187.1	6.17
Dexamethasone-d3	396.3	358.3		4.19
Prednisolone-d4	365.3	347.3		3.34
medroxyprogesterone acetate-d_3	390	330		7.55
Stanozolol-d3	332	81		6.79
17β-testosterone-d3(T-d3)	292	109.1		5.93

Preparation and characteristics of the multiple immunoaffinity (MIAC) gel

All polyclonal antibodies against anabolic agents were produced by hyperimmunization of rabbits. A range of bridging molecules (carboxymethyloxime, hemisuccinate, carboxypropyl ether) and carrier proteins (*bovine* serum albumin, keyhole limpet hemocyanin) acting as antigens were used. The protein content of the lgG fraction determined by Lowry's method after protein A chromatography was about 10 mg mL^{-1} serum. To each millilitre of gel, 3 mg isolated lgG protein were coupled. The elution programme and washing step applied to the gel columns were carefully optimized using mass-spectrometry. Under these experimental conditions, the capacity of each gel was tested on 1 mL aliquots. All capacities exceeded 300 ng mL^{-1} gel. The final MIAC gel was prepared by mixing adequate volumes of the individual gels. The gel slurry capable of recognising all tested anabolic steroids and corticosteroids was evenly distributed into 2 mL columns.

Routinely in the laboratory, the MIAC performance of each gel column is checked on a monthly basis by determining the recovery of each analyte. This is done by spiking samples with 20 ng of the individual steroids and corticosteroids and subjecting the eluates to UPLC-MS analysis.

Table 2: LC (retention time, RT) and MS (ionic species) characteristics of anabolic steroids in negative mode.

Name	Diagnostic ions (*m/z*)			RT (min)
Ethyloestranediol (EED)	271.3	161.2	109.2	4.45
Methyloestranediol (MeAD)	271.3	175.2	149.2	4.23
Dienestrol	265	92.9	147	3.82
Diethylstilbestrol	267	237	222	3.73
Hexestrol	269	134	119	3.86
17β estradiol	271	145	183	3.1
Ethynylestradiol	295.3	145.2	159.2	3.48
Zearalenone	317.3	175.2	131.2	3.70
Zeranol (α-Zeranol)	321	277	303	2.85
Taleranol (β-Zeranol)	321	277	303	2.30
Zearalanone	319.4	205	275	3.68
α-Zearalenol	319.4	160	275	2.97
β-Zearalenol	319.4	160	275	2.38
Diethylstilbestrol-d8	275	259		3.71
Zearalenone-d6	323.4	187		3.68
Zeranol-d4	325.2	281		2.84
Taleranol-d4	325.2	209.2		2.29
Zearalanone-d6	325.4	205		3.66
α-Zearalenol-d7	326.4	298		2.93
β-Zearalenol-d7	326.4	298		2.35
Ethynylestradiol-d4	299.3	147.2		2.51

Instrumentation and UPLC-MS/MS conditions

Chromatographic analyses were performed with an Acquity UPLC system (Waters, Milford, MA, USA) and separations were done on an Acquity UPLC HSS T3 column (150 × 2.1 mm, 1.8 µm particle size) from Waters. The column was equilibrated at 50°C and the injection volume was 20 µL.

Mass-spectrometry analysis was carried out with a Waters Acquity TQS mass spectrometer (Waters, Manchester, UK). The instrument was operated with an electrospray ionization source in the positive (ESI+) and negative (ESI-) ionization modes. The ESI parameters were adjusted as follows: capillary voltage 2.5 kV, source temperature 150°C, desolvation temperature 500°C, cone gas (nitrogen) flow 250 L h^{-1}, desolvation gas (also nitrogen) flow 1,200 L h^{-1}. Collision-induced dissociation was done with argon as the collision gas at 4×10^{-3} mbar pressure in the collision cell.

The complete sample analysis required two injections per sample. Samples were first analysed in ESI- with water (A) and ACN (B) as mobile phases and post-column delivery of NH_4OH at a flow rate of 20 µL min^{-1}. Then the samples were analysed in ESI+ with 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) as mobile phases. The following gradient was used for the first (ESI-) injection: 0-2.0 min 43.5% B; 2.0-4.5 min increase to 90% B; 4.5-6.0 min 90% B; 6.0-6.5 min decrease to 43.5% B; 6.5-8.5 min 43.5% B. The flow rate was 0.45 mL min^{-1}. At the same flow rate, the following gradient was used for the second (ESI+) injection: 0-2.0 min 50% B; 2.0-4.0 min linear increase to 67% B; 4.0-8.0 min 67% B; 8.0-13.0 min increase to 100% B; 13.0-14.0 decrease to 50% B.

The column and autosampler were maintained at 50°C and 15°C, respectively. A diverter valve leading the effluent to the mass-spectrometer was kept open for 1.2 to 8.0 min in the ESI – mode and for 2.3 to 9.7 min in the ESI + mode. The source was cleaned before each run to maintain sensitivity. The specific MS/MS transitions for each drug are listed in Table 1 and 2. Data acquisition was done with MassLynx 4.1 software, with the TargetLynx 4.1 program (Waters).

Extraction and clean up

To 2 mL of urine were added 0.5 mL of 2M acetate buffer (pH 5.2) and 20 µL of *Helix Pomatia* juice. The mixture was incubated for 2 h at 52°C and then centrifuged at 2700 g for 10 min. The solution was cleaned-up on an OASIS HLB column conditioning: 1 mL of methanol and 1mL water; washing: 1 mL of water, 1 mL of acetone/water(20/80) and 1 mL methanol/water(20/80); elution:1 mL ethylacetate]. After evaporation of the eluate, the residue was dissolved in 500 µL of ethanol followed by 4.5 mL of water and 4 mL of PBS buffer. The solution was applied to on a multi-immunoaffinity column. The column loaded with 2 mL of immunoaffinity gel was first conditioned with 5 mL of PBS buffer and 5 mL of water. The sample was loaded on to the column. After rinsing the column with 5 mL water, anabolic steroids and corticosteroids were eluted with 7 mL methanol/water (80/20, V/V). The extracts were evaporated to dryness under dry nitrogen. The residue was taken up in methanol/water (80/20, V/V) and 20 µL of the resulting solution were injected into the UPLC-MS/MS system.

Table 3: Decision limits (CCα) and detection capabilities (CCβ)

Compounds	CCα (µg L^{-1})	CCβ (µg L^{-1})
Ethyloestranediol	≤2.26	3
17β nortestosterone	0.11	0.125
17α nortestosterone	0.20	0.25
17β boldenone	0.22	0.25
17α boldenone	0.44	0.5
17β -testosterone	0.94	1
5α-androst-1-en-17α-ol-3-one	0.45	0.5
Methylboldenone	0.45	0.5
Methyltestosterone	0.22	0.25
Norethandrolone	0.11	0.125
Norgestrel	0.12	0.125
Progesterone	0.93	1
4-chloro-androst-4-en 3,17 -dione	0.22	0.25
Stanozolol	0.23	0.25
16β hydroxy-stanozolol	0.12	0.125
Fluoxymesterone	0.22	0.25
Medroxyprogesterone acetate	0.11	0.125
17α -trenbolone	0.46	0.5
17α-methyl-5β -androstane-3α,17β diol	2.29	3
Dienestrol	0.76	1
Diethylstilbestrol	0.41	0.5
Hexestrol	0.39	0.5
17β-estradiol	≤1.50	2
Ethynylestradiol	≤1.50	2
Methenolone	0.69	0.8
Zearalenone	0.91	1
Zeranol	0.38	0.5
Taleranol	0.38	0,5
Zearalanone	0.92	1
α-Zearalenol	0.91	1
β-Zearalenol	0.87	1
Prednisone	0.88	1
Prednisolone	0.23	0.25
Methylprednisone	0.43	0.5
Methylprednisolone	0.47	0.5
Isoflupredone	0.24	0.25
Dexamethasone	0.10	0.125
Flumethasone	0.12	0.125
Clobetasol	≤0.75	1
Triamcinolone acetonide	0.04	0.05
Fluocinolone acetonide	0.34	0.4
Fluorometholone	0.33	0.4
Beclomethasone	≤3.01	4

Validation study

The validation study was performed according to the Commission Decision 2002/657/EC criteria established for qualitative confirmatory methods. The specificity of the method was verified by analysing 20 independent blank urine samples (*bovine*, *porcine*, 10 each).

Bovine and *porcine* urine samples were spiked with standards of investigated steroids and corticosteroids at the concentration range of 0-6.0 µg L^{-1} and the concentration of 13 internal standards was 1 µg L^{-1} except for α and β-zearalenol (10 µg L^{-1}). Concentration levels 0.0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0 and 6.0 µg L^{-1} were used for all investigated analytes. The

CCβ limits were calculated from this dataset. The CCβ corresponds to a concentration giving a peak with a signal-to-noise ratio of 10. The determined CCβ was confirmed by the analysis of 20 samples spiked at the concentration close to the limit.

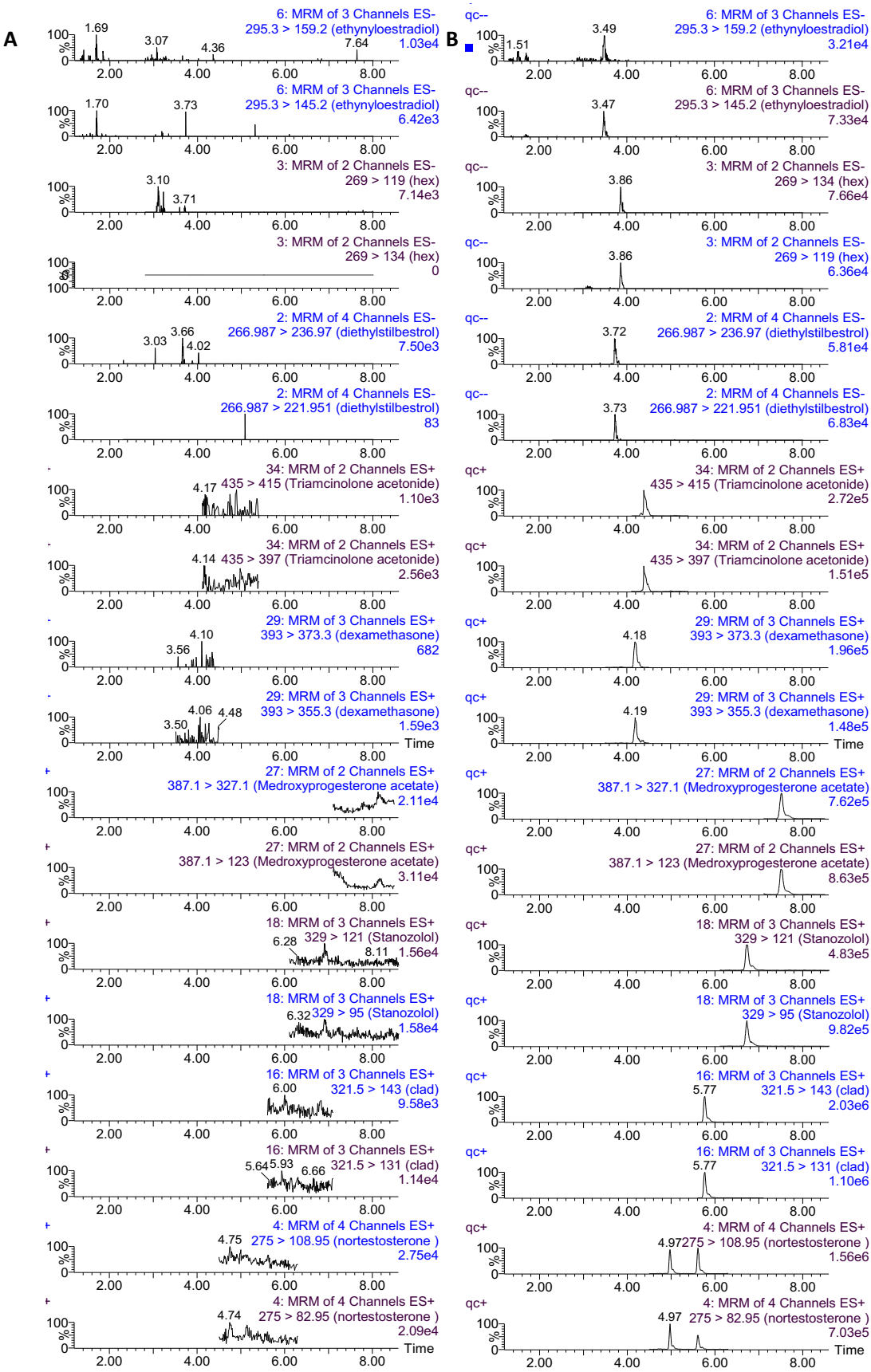

Figure 1: Reconstructed diagnostic ion traces recorded from (A) an MIAC-blank and (B) a treated sample spiked with 12 steroids.

Results and Discussion

The characteristics of the multiple-immuno-affinity gel used to purify samples containing anabolic steroids were described earlier. The efficacy of the MIAC purification step was tested on control samples prepared from blank urine samples spiked with pooled steroids and corticosteroids (each vial containing 44 products at a concentration range of 0.05- 10 µg L^{-1}). All samples underwent the entire extraction procedure and MIAC gel purification before analysis by UPLC-MS.

Figure 1 shows the reconstructed diagnostic ion traces recorded from an MIAC-blank and treated sample spiked with steroids (ethynylestradiol, hexestrol, diethylstilbestrol, medroxyprogesterone acetate, stanozolol, 4-chlorandrost-4-ene-3, 17 dione, 17β-nortestosterone) and corticosteroids (triamcinolone acetonide, dexamethasone). The efficacy of the MIAC purification step is illustrated by the heights of the analyte peaks as compared to the background. Standard MS data processing yielded a tremendously improved signal-to-noise ratio when the MIAC purification step was included. Sensitivity was also increased, which means that anabolic steroids can be detected at much lower levels.

All the chromatographic peaks of the different analytes were highly specific. Analysis of blank urine samples did not show interfering agents at the same retention time for any of the previously described steroids. No interfering peaks from endogenous compounds were found at the retention time of the target analytes. Detection capabilities were generally below 1 µg L^{-1} and ranged between 0.05 and 4 µg L^{-1} depending on the compound (Table 3).

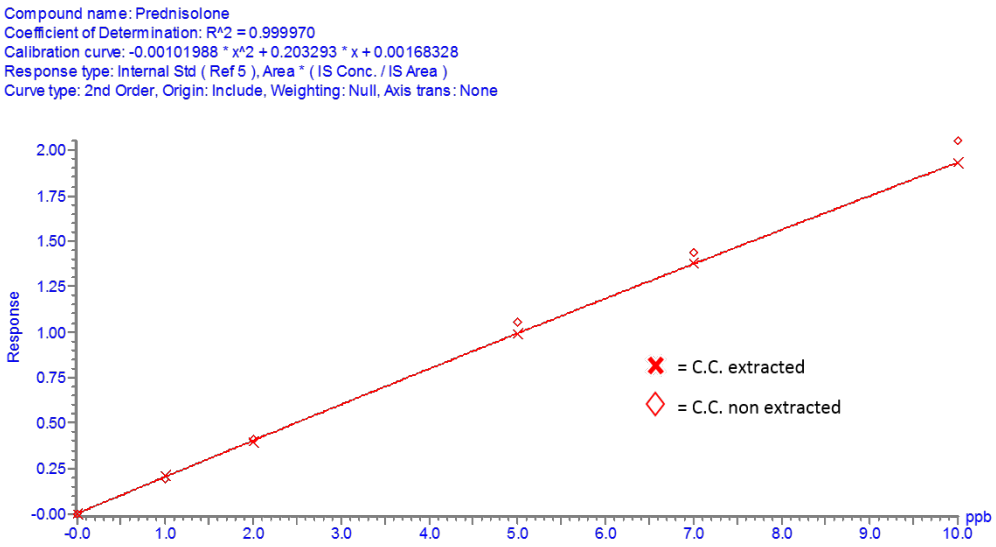

Figure 2: Extracted and non-extracted calibration curves for prednisolone

The method also meets the validation criteria for analyte identification and confirmation on the basis of retention times, two MRM transitions, and relative ion ratios of selected MRMs, as laid down by the European Decision 2002/657.

The method was found to be linear for prednisolone in the investigated interval (0-10 µg L^{-1}) with a correlation coefficient (r^2) of 0.99. Figure 2 shows that there is no matrix effect. Extracted and non-extracted calibration curves are identical.

The resorcyclic acid lactones (RALs), comprising zeranol, taleranol, α- and β-zearalenol, zearalanone and zearalenone, can also be detected and identified by this method. The ratio between the sum of zeranol and taleranol mass concentrations (indicating treatment) to the sum of the zearalenone and its two major metabolites, α- and β-zearalenol (indicating feed contamination), can be evaluated. This evaluation is possible until 10 µg L^{-1}. Zeranol can be formed *in vitro* after consumption of *Fusarium spp* contaminated feed. To determine if the source of an adverse finding of zeranol is "natural" or from abuse, a statistical tool was developed which can help deciding the origin of this adverse finding. The statistical model build is based on comparing the sum of zeranol and taleranol mass concentrations with the sum of the zearalenone and its two major metabolites, α- and β-zearalenol. This tool can be obtained from the EURL for residues. This model is fully validated for *bovines*, and it can probably be used for other species as well but for another purpose validation is needed. In case of a positive outcome, a follow-up study should be conducted, for example to analyse the feed the animals ate.

Conclusions

The approach presented here, *i.e*. combining purification by multi-immuno-affinity chromatography with UPLC-MS detection, has numerous, decisive advantages over conventional techniques currently used in most accredited laboratories.

All products initially present could be clearly detected in all processed samples. Application of the new method should considerably reduce time and labour, thereby reducing the cost of the analytical process and increasing sample throughput. This

amply justifies using the additional MIAC purification step, which additionally reduces background signals urine and increases detection sensitivity.

The capacity of the present gel mixture to bind multiple residues is linked to the specificity ranges of the Sepharose coupled antibodies. Our MIAC gel has been validated, to date, for 44 compounds among those routinely screened for in Belgium. Should new anabolic steroids emerge, they should be directly detectable provided that they cross-react with the gel. If not, the gel mixture must be adapted to include a gel binding the new product. This could be achieved easily by raising an antiserum against the agent, coupling the corresponding antibodies to the gel and incorporating the new gel into the gel mixture. As mentioned above, lAC purification affords a gain in sensitivity by improving the signal-to-noise ratio. The new technique is therefore perfectly applicable to routine qualitative confirmatory for anabolic compounds. It will be further validated for quantitative confirmatory determination of prednisolone.

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PASSIVE SAMPLERS, AS SURROGATES FOR BIOLOGICAL MONITORING, TO MEASURE EMERGING (MICRO) POLLUTANTS IN THE MARINE ENVIRONMENT

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Abstract

The extensive use of organic chemicals for different applications (industry, agriculture, pharmaceutical usage, etc.) leads to low-concentration but long-term exposure of the aquatic environment to their residues. The protection of our coasts and marine waters is a long-standing part of the European Community environmental policy, which is also broader internationally regulated. Various organisations involved (a.o. OSPAR, USEPA, etc.) stress the need for a more integrated, consistent and economically favourable strategy to meet legislative and international obligations. In the context of a recently started Belgian project, we are studying a relatively new approach making use of passive samplers and hybrid high-resolution mass-spectrometry coupled to liquid chromatography for analysing known and unknown emerging organic micro-pollutants in the marine environment. Amongst others, attention is given to endocrine disrupting compounds (EDCs) including steroidal hormones. In this particular study, we present the development and optimization of the analytical method to measure multiple steroidal EDCs. The analytes were separated on a 1.9 μm Hypersil Gold column (10 mm x 2 mm) and quantified in full-scan by a Q-Exactive benchtop™ mass spectrometer. Chromatographic variables like mobile phase flow, acidification, and column oven temperature were optimized by injecting analytical standards. By analysing sea extracts, the mass-spectrometric parameters (sheath gas, auxiliary gas, sweep gas, discharge current, capillary temperature, S-lens RF, and vaporizer temperature) were optimized. Next, 55 steroidal EDCs were successfully extracted from substitute ocean water. In a final step, this method will be validated and applied for the targeted analysis of steroidal EDCs in the marine environment.

Introduction

Endocrine disrupting compounds (EDCs) originating from industrial, rural and domestic activities are considered in an environmental context as emerging organic micro-pollutants (Garcia-Rodriguez *et al.* 2014, Laurenson *et al.* 2014). EDCs mainly include steroidal hormones and several non-steroidal synthetic compounds. The steroidal hormones can be subdivided in androgens, estrogens, gestagens and corticosteroids. Non-steroidal compounds are a.o. phthalates, phenols and pesticides. Most of these EDCs have been monitored in the aquatic environment with particular focus on riverine, ground, drinking and waste water. These four major water bodies are primarily limited to local anthropogenic activities and corresponding contamination (Tijani *et al.* 2013). By monitoring the marine environment, a broader view of EDC occurrence and contamination will be obtained. Since EDCs have barely been examined in the marine environment (Ronan and McHugh 2013), this work focuses on monitoring a broad range of steroidal EDCs in the North Sea environment.

Monitoring steroidal EDCs in the marine environment must deal with ultra-trace contamination levels as compared to river, ground, drinking and waste water (Zhang *et al.* 2016). Despite the expected low concentrations, the presence may have a significant effect on water organisms (Vlachogianni *et al.* 2013). As an example, EDC exposure to the Sea Urchin results in the inhibition of embryos development (Roepke *et al.* 2005). EDC studies with Zebra fish show disturbance of the sexual differentiation and reproduction (Segner 2009). Bioconcentration factors of EDCs (Arnot and Gobas 2006), listed in Table 1, show that bioaccumulation of these pollutants might be of main importance. Therefore, both the trace-level occurrence and the susceptibility of water organisms illustrate the complexity and need to develop a sensitive analytical method to monitor EDCs.

Beside a sensitive analytical method, there is also need to monitor the aquatic system over an extended period of time and obtain time weighted average concentrations. Promising tools to fulfil these needs are passive samplers. The most common passive samplers in literature are POCIS (Bartelt-Hunt *et al.* 2011, Vallejo *et al.* 2013), silicone rubbers (Naude *et al.* 2015) and Chemcatchers (Vrana *et al.* 2015). However, passive samplers have rarely been studied for steroidal EDCs in the marine environment. This work focuses on the development of a sensitive analytical method, which in the future will be implemented in monitoring using passive samplers. The analytical method is based on liquid-chromatography coupled to hybrid high-resolution mass spectrometry to monitor a broad range of steroidal EDC at ambient concentration levels.

Materials and Methods

Reagents and chemicals

The organic solvents were of optima UPLC-MS grade, obtained from Fisher Scientific (Loughborough, UK). The selected standards were obtained from Steraloids Inc (Newport, RI, USA) and Sigma Aldrich (St. Louis, MO, USA). In total, 55 steroidal hormones were included in this study. The main characteristics of the EDC subcategories can be found in Table 1. The androgens were 5α-androstan-17α-methyl-3α,17β-diol, 5α-dihydrotestosterone, 5β-androstan-3α-17β-diol, 5β-androstan-3α-ol-11,17-dione , 11β-hydroxyandrosterone, 17α-trenbolone, 17β-trenbolone, α-boldenone, α-testosterone, β-boldenone, β-nortestosterone, β-sitosterol, β-testosterone, 1,4-androstadieen-3,17-dione, 4-androsten-6a-ol-3,17-dione, 9-nortestosterone-17-decanoate, 11-ketotestosterone, androstenedione, androsterone, epi-androsterone, ethinyltstosterone, fluoxymesterone, formebolone, methandriol, methylboldenone, methyldihydrotestosterone, methyltestosterone, norethindron, norethandrolone, stanozolol, testosterone-acetate, testosterone-17β-cypionate and trenbolone-acetate. The estrogens were α-zeralenol, β-zeralenol, β-zeranol, 17α-estradiol, 17β-estradiol, 17α-ethinylestradiol, dien-diacetate, dienoestrol, diethylstilbestrol, estradiol-17-acetate, estradiol-17-glucosiduronate, estradiol-17-sulfate, estrone, estrone-3-sulfate, equilin, gestodene, hexoestrol and mestranol. The gestagens were 5α-pregnan-3α,20β-diol, 17α-acetoxyprogesterone, caproxyprogesterone, chlormadinon acetate, flugestone acetate, medroxyprogesterone, medroxyprogesterone acetate, megestrol, megestrol acetate, melengestrol acetate, norgestrel, pregnolone and progesterone. The corticosteroids were betamethasone, cortisol, cortisone, dexamethasone, prednisolone and prednisone. Internal standards were 17β-estradiol-d_3, hexestrol-d_4, methylprogesterone acetate-d_3 and methyltestosterone-d_3. Primary stock solutions and mixing standards were prepared in methanol ranging from 0.1 to 1,000 ng μL^{-1}. The solutions were stored in dark glass bottles at -20°C. The inorganic salts for making substitute ocean water were supplied by Sigma Aldrich.

Table 1. The main characteristics of the targeted EDCs divided in their sub-classes at 25°C based on the SciFinder database (ACD/Labs n.d.).

Sub-class	Molecular weight (g mol^{-1})	pKa	Log Kow	Solubility (mg L^{-1})	Bioconcentration factor (L kg^{-1})
Androgens	270.3 – 428.7	9.3 – 15.1	1.8 – 7.9	1.9 x 10^{-3} – 58	5.69 – 1.0 x10^6
Estrogens	270.4 – 446.7	-3.8 – 10.3	2.3 – 5.3	1.2 – 1900	1 – 6.6 x10^4
Gestagens	312.5 – 428.6	13.0 – 13.1	2.8 – 3.6	2.6 – 24	20.3 – 1.21 x10^4
Corticosteroids	358.4 – 392.5	12.1 – 12.5	1.4 – 2.0	35 – 140	7.23 – 20.7

Analytical method development: liquid chromatographic and mass spectrometric conditions

The EDCs were chromatographically separated using an ultra-high performance liquid chromatograph (U-HPLC) equipped with a degasser, autosampler, LC pump and column oven (Dionex Ultimate 300, Thermo Fisher). A Hypersil Gold column (1.9 μm, 10 mm x 2 mm, Intersciences, Louvain-La-Neuve, Belgium) was used to separate EDCs, as proven to be successful for anabolic steroids (Vanhaecke *et al*. 2011). Furthermore, the mobile phase consisted of methanol and water acidified by formic acid. The sample injection volume was 10 μL. The detection of EDCs was carried out using a Q-Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific), equipped with an atmospheric pressure chemical ionization source (APCI).

Liquid chromatographic and mass spectrometric optimization

The liquid chromatographic conditions were optimized by modifying the percentage formic acid addition, the column oven temperature, and the mobile phase flow between 0.00 – 0.10 % formic acid, 0.300 – 0.450 mL min^{-1}, and 30°C – 55°C, respectively. Subsequently, a linear L27 design was applied to statistically investigate the effect of 7 mass spectrometric parameters (Table 2) on the response of EDCs in real extracts. Each variable was assigned three levels, *i.e*., a low, central and high level. The reliability of the L27 design was increased by using 3 repetitive central experimental points and performing each test randomized.

Extraction

The development and optimization of the extraction was performed on substitute ocean water, prepared according to the ASTM-D1441 standard procedure. The substitute ocean water was spiked with 50 ng L^{-1} of each EDC. The samples were pretreated by filtering through a Whatman 0.45 μm filter. The obtained filtrate was adjusted to pH 8. Thereafter, the samples were spiked with 50 ng L^{-1} 17β-estradiol-d_3, hexestrol-d_4, methylprogesterone acetate-d_3 and methyltestosterone-d_3 as internal standards. Subsequently, a divinylbenzene sorbent was conditioned and rinsed by loading respectively with methanol and milli-Q water. Next, samples were loaded under vacuum, followed by a washing step with Milli-Q water. The elution was performed vacuum-free by the suitable extraction solvent, acidified with formic acid. The extracts were vaporized to dryness under nitrogen at a temperature of 60°C, and the EDCs were reconstituted in the starting conditions of the mobile phase. Finally, the samples were centrifuged prior to UHPLC-HRMS analysis.

Table 2. The ranges of the mass spectrometric parameters investigated by a linear L27 design on real marine extracts.

Variables	Unit	Lower Level	Central Level	High Level
Sheath gas	a.u.	15.0	32.5	50.0
Auxiliary gas	a.u.	5.0	15.0	25.0
Sweep gas	a.u.	2	4	6
Discharge current	kV	3	4	5
Capillary temperature	°C	250	300	350
S-lens RF	level	25	50	75
Vaporizer temperature	°C	250	375	500

Results and discussion

Liquid chromatographic optimization

The developed chromatographic method had a run time of 14 min. The best chromatographic separation was obtained by acidifying the mobile phase with 0.10 % formic acid, applying a flow rate of 0.45 mL min^{-1}, and setting the column oven temperature at 45°C. The results of the chromatographic optimization are depicted in Figure 1 for one of the steroid sub-classes, namely the androgens.

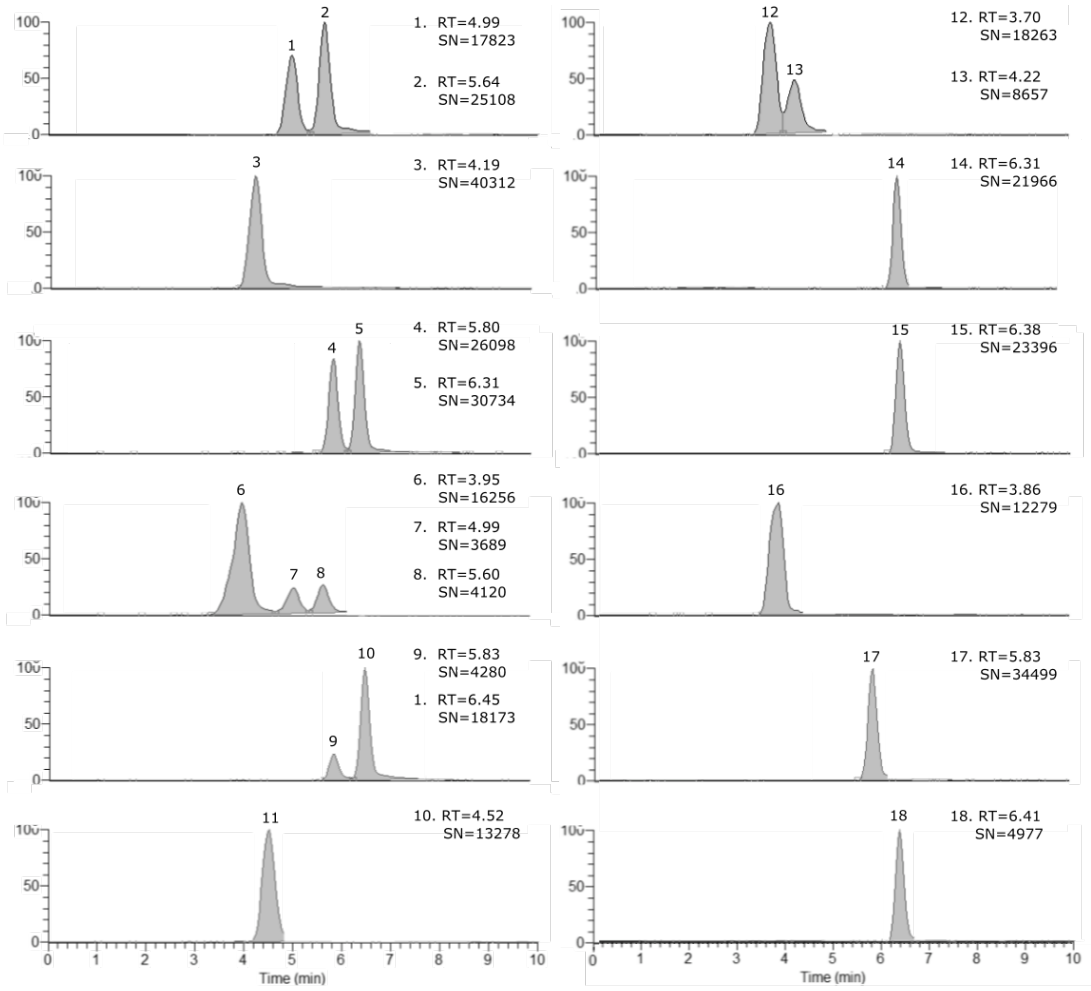

Figure 1. The result of the chromatographic optimisation for the androgens (1 ng injected on column). 1. 17β-testosterone, 2. 7α-testosterone, 3. 19- nortestosterone, 4. methyl testosterone, 5. norethandrolone, 6. formebolone, 7. androstenedione, 8. 1,4-andradien-17β-ol-3-one, 9. methandriol , 10. mestanolone, 11. methylboldenone, 12. trenbolone (α and β) , 13. testosterone acetate, 14. trenbolone actate, 15. stanozolol, 16. fluoxymesterone, 17. methandriol and 18. 5β-androstane-3α,17β-diol.

Mass spectrometric optimization

The Q-Exactive was operated in the full scan mode by alternating the polarity from negative to positive mode. Nevertheless, all the steroids were detected in the positive polarity mode. Subsequently, the EDC mass spectra – obtained with the APCI method – were mainly characterized by MH^+, MH^+-H_2O and MH^+-$2H_2O$. The loss of water in the positive APCI mode has been reported earlier for the analysis of steroids (Ma and Kim 1997). The detection was performed at a scan range of 60-900 Da and a resolution of 140,000 FWHM. The effects of other instrumental mass spectrometric variables were statistically evaluated by a L27 experimental design, making use of real extracts for analysis. The effect of each variable was evaluated at 95% (p=0.05) significance by an effect plot. To do so, the sum of all the normalized peak areas was taken into account as response parameter. The L27-design was performed with a determination coefficient of 0.845, which is acceptable for experimental designs. The significance of the mass spectrometric parameters is depicted in Figure 2. Five variables had a significant positive or negative effect on the total normalized chromatographic peak area, evaluated by the statistical p-value. The positive significant variables were sheath gas ($p= 4.88x10^{-8}$), auxiliary gas ($p= 2.57x10^{-1}$) and S-lens RF ($p= 5.49x10^{-6}$). The sweep gas ($p= 3.29x10^{-2}$) and capillary temperature ($p= 3.46x10^{-2}$) had a negative effect on the response. These significant parameters will be selected for future optimizing experiments, to increase the sensitivity and suppress matrix effects.

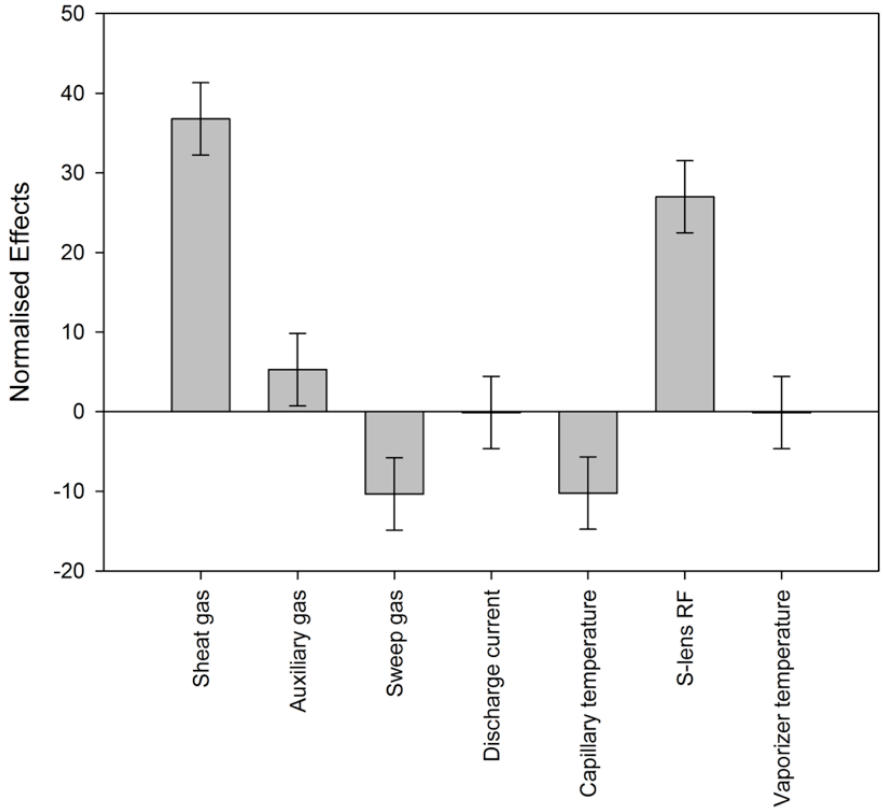

Figure 2. The experimental L27 design, used to statistically investigate the normalised effects on the MS variables at a 95% (p<0,05) significance level, show a significant effect of sheath gas, auxiliary gas, sweep gas, capillary temperature and S-lens RF.

Extraction

The newly optimised extraction method, developed for a broad range of EDCs, shows potential to extract 55 target EDCs from substitute ocean water (ASTM -D1441). The extraction must be further optimized in accordance to extract more EDCs and enhance the total signal of all detectable analytes.

Conclusions

Optimizing the chromatographic and mass spectrometric parameters lead to a sensitive and fast liquid chromatographic high-resolution mass-spectrometric (LC-HRMS) method for measuring a broad range of steroidal EDCs in the marine environment. Future perspectives are further optimizing the extraction method and validating the UHPLC-HRMS method according to CD 2002/657/EC. Finally, the developed method will be an important driver to different monitoring programs in the aquatic marine environment. This can lead to international regulations to protect our coast and marine environment.

Acknowledgements
This work fits within the NewSTHEPS project (BR/143/A2/NEWSTHEPS) supported by Belgian Science Policy (BELSPO). More information and future perspectives can be found on the NewSTHEPS website; www.newstheps.be. The authors would like to express their appreciation to the technical staff of the Laboratory of Chemical Analysis for their contribution to the analyses.

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PRODUCTION OF ANTISERA TO PHENYLBUTAZONE AND OXYPHENYLBUTAZONE FOR USE IN IMMUNOCHEMICAL DETECTION ASSAYS

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Abstract

Phenylbutazone is a non-steroidal anti-inflammatory drug licensed to treat musculoskeletal disorders in horses. It is not permitted for use in food-producing animals in the EU as it is known to produce adverse effects in humans. In recent years some non-compliant *bovine* and *equine* samples have been found. There are physiochemical methods available for sample analysis although less expensive, rapid screening methods such as immunoassays would be advantageous. This study details antiserum production to detect phenylbutazone and its metabolite, oxyphenylbutazone. Two of the haptens chosen for immunogen preparation, suxibuzone and γ-hydroxyphenylbutazone produced IC_{50}s (50% of maximal inhibitory concen-tration) of <5 ng mL^{-1} for phenylbutazone. IC_{50}s of 5.5 ng mL^{-1} and 5.6 ng mL^{-1} for phenylbutazone and oxyphenyl-butazone, respectively, were produced by antisera to the respective haptens. The figures suggest that the antisera could deliver detection capabilities below the concentration recommended by the Community Reference Laboratory (5 ng mL^{-1}).

Introduction

When assessed by the Committee for Veterinary Medicinal Products (CVMP) in 1997 it was found that the main health risks of phenylbutazone (PBZ) to the consumer were blood dyscrasias and the genotoxic/carcinogenic potential for which no thresholds could be identified and so no maximum residue limits could be established (European Medicines Agency, 1997). These risks were reaffirmed in 2013 by the European Food Safety Authority and the European Medicines agency. In the EU, PBZ is not permitted for use in any animal destined for human consumption, a stance also adopted by the USA, Canada and Japan. Suxibuzone (SBZ) is a pro-drug (Figure 1) of PBZ produced as an alternative NSAID in the 1970's to cause less gastrointestinal disturbances (Monreal *et al.*, 2004). It is metabolised to PBZ (Jaraiz *et al.*, 1999) and so is also unauthorised for use in food production animals. The European Food Safety Authority has published results showing that 5.4% of food producing horses tested for PBZ have delivered a non-compliant result from 2008 to 2012 (European Food Safety Authority and European Medicines Agency, 2014).

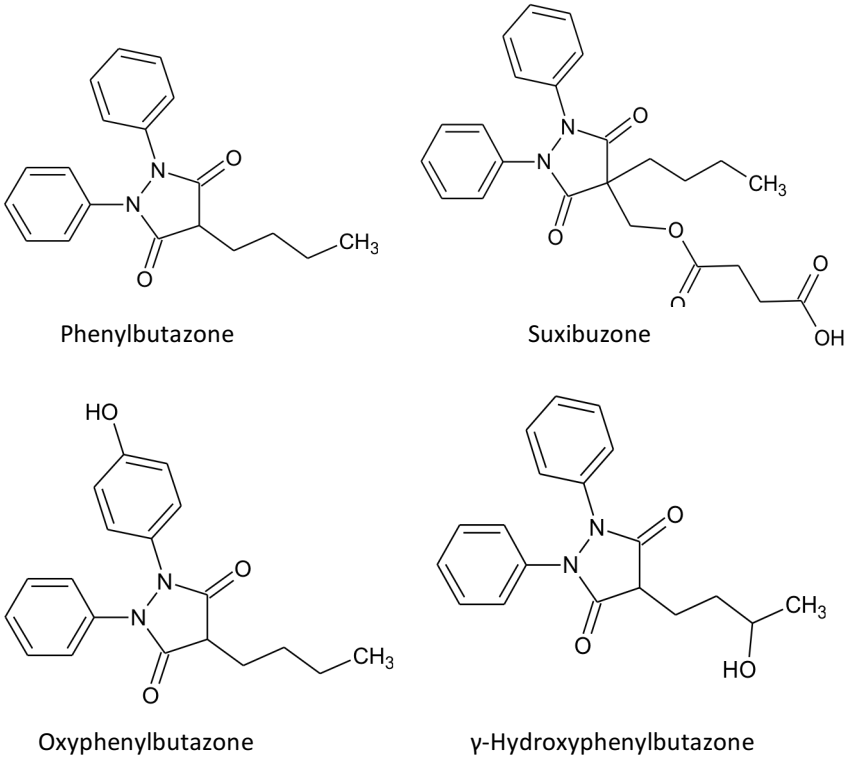

Figure 1. Chemical structures of PBZ, its prodrug SBZ and its metabolites OPBZ and HPBZ.

The use of the drug in food-producing animals is not limited to the *equine* species; *bovine* samples tested by EU member states from 2008-2012 produced a positive rate of 0.3% for the drug. The CVMP (1997) noted that PBZ is metabolised to oxyphenylbutazone (OPBZ) and γ-hydroxyphenylbutazone (HPBZ). Detection of both PBZ and its metabolites could be a useful tool for monitoring the illegal use of the drug.

Physicochemical methods of detection have been available for a decade now which can detect PBZ and OPBZ in plasma (Taylor *et al*., 1995), urine (Igualada *et al*., 2005), milk (Dowling *et al*., 2009) and muscle (Jedziniak *et al*., 2010).

As a less expensive and more rapid alternative to chromatographic methods, an electrochemical sensing technique was developed for PBZ detection in *equine* plasma (Meucci *et al*., 2013). The current study set out to develop antibodies capable of detecting both PBZ and OPBZ at concentrations below 5 ng mL^{-1}, the detection concentration recommended by the Community Reference Laboratory (CRL Guidance Paper, 2007). PBZ, SBZ, OPBZ and HPBZ were employed as haptens and various chemical coupling techniques were used to prepare immunogens and corresponding enzyme labels for use in ELISAs. The antisera produced were assessed for sensitivity and specificity to determine their potential as suitable reagents in an immunochemical-based analytical method.

Materials and Methods

Reagents and chemicals

Phenylbutazone (P8386), suxibuzone (S5521), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC; E1769), *N*-hydroxysuccinimide (NHS; 130672), human serum albumin (HSA; A1887), 2-(*N*-morpholino) ethanesulfonic acid (MES; M3671), 4-aminobenzoic acid (ABA; A9878), sodium nitrite (237213), 1,1'carbonyldiimidazole (CDI; 115533), *N,N'*-disuccininmidyl carbonate (DSC; 43720) and 4-(dimethylamino)pyridine (DMAP; 107700) were obtained from Sigma-Aldrich (Poole, Dorset, UK). Oxyphenylbutazone was obtained from LGC standards (Teddington, Middlesex, UK) and γ-hydroxyphenylbutazone (FH24086) was obtained from Carbosynth Ltd (Compton, Berkshire, UK). *N*-(*p*-Maleimidophenyl)isocyanate (PMPI; 28100), N-Succinimidyl-S-acetylthiopropionate (SATP; 26100), Hydroxylamine hydrochloride (26103) and Diaminoethanetetra acetic acid disodium salt (EDTA; D/0650/53) were purchased from Fisher Scientific UK (Loughborough, Leicestershire, UK); horseradish peroxidase (HRP; 10-814407-001) was purchased from Roche-Diagnostics Ltd. (Burgess Hill, West Sussex, UK); PD-10 desalting columns containing Sephadex G25 (17-0851-01) were obtained from GE Healthcare (Little Chalfont, UK) and Montanide ISA 50 V adjuvant was obtained from Seppic (Paris, France).

Preparation of PBZ-HSA immunogen:

Sodium nitrite (24 mg) was dissolved in 100 µL of water and added to 40 mg ABA, dissolved in 1 mL of water with 100 µL concentrated hydrochloric acid (HCl) and allowed to mix gently for 4 h at room temperature in darkness. PBZ (90 mg) was dissolved in 250 µL dimethylformamide (DMF) and added to the mixture then allowed to react overnight at room temperature before being freeze dried to remove the solvent. The residue was reconstituted in 1 mL of pyridine and 200 µL of this was added to 10 mg EDC and 5 mg NHS, dissolved in 100 µL MES buffer and allowed to react at room temperature for 10 min. The activated PBZ-ABA complex (200 µL) was then added to 20 mg HSA dissolved in 1 mL phosphate buffered saline (PBS) pH 7.2 and allowed to react for 3 h at room temperature with gentle mixing followed by purification by dialysis against saline (0.1 M NaCl).

Preparation of SBZ-HSA immunogen

SBZ (24 mg) and 52 mg CDI were dissolved in 12 mL of dry acetone and allowed to react in darkness for 4 h at room temperature with gentle stirring. The acetone was removed under a stream of nitrogen gas at 40˚C until dryness. The residue was reconstituted in 600 µL PBS pH 7.2 and 200 µL pyridine for complete solubility. HSA (20 mg) was dissolved in 2 mL PBS pH 7.2 and the activated SBZ was added with additional pyridine for solubility and the mixture was incubated for 72 h at 4˚C with gentle stirring before dialysis against saline (0.1 M NaCl).

Preparation of OPBZ-HSA immunogen

SATP (4 mg) was dissolved in dimethyl sulphoxide (DMSO) and added to 20 mg HSA, dissolved in 2 mL PBS pH 7.2 and allowed to react for 30 min at room temperature while stirring before purification with a sephadex 25 desalting column against PBS pH 7.2. Deacetylation solution(200 µL of 0.5 M hydroxylamine hydrochloride and 0.25 mM EDTA in PBS pH 7.2), was added to the activated HSA mixture and allowed to react for 3 h at room temperature while stirring before purification with a PD-10 column against PBS pH 7.2. OPBZ (4 mg) and 7 mg PMPI were dissolved in DMSO and allowed to react for 3 h at room temperature and added to the deacetylated HSA then incubated overnight at 4˚C while stirring. The mixture was then purified by dialysis against saline (0.1 M NaCl).

Preparation of HPBZ-HSA immunogen
HPBZ (2.5 mg), 5.5 mg DMAP and 12 mg DSC were dissolved in 3 mL dry acetone and allowed to react for 4 h at room temperature while stirring. The solvent was removed under a stream of nitrogen gas at 40°C and the residue reconstituted in 200 µL PBS (pH 7.2) and 200 µL pyridine. The activated HPBZ was added to 20 mg HSA dissolved in PBS (pH 7.2) and incubated overnight at 4°C while stirring before purification by gel filtration against saline (0.1 M NaCl).

Preparation of hapten-enzyme labels
Enzyme conjugates for each immunogen were prepared using the same respective techniques. The enzyme conjugates were employed in direct competitive ELISAs to assess the antisera for specific antibodies and to determine their relative sensitivity.

Immunisation and blood sampling protocol
To prepare the immunogenic emulsions, the immunogen (0.5 mg protein in 0.5 mL saline) was mixed with an equal volume of Montanide ISA 50 V adjuvant. Two New Zealand White rabbits were immunised every four weeks at four injection sites, and blood samples were collected 10 days after each immunisation. All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, UK.

Assessment of specific antibody response
A competitive assay format was employed for ELISA checkerboards to assess antisera samples throughout the immunisation schedule. Each enzyme conjugate was assessed against each of the hapten antisera samples to compare the homology and heterology of the assays. Microtitre plates (Falcon 353070) were coated (100 µL/well) with serial dilutions of rabbit serum in 1 mM sodium acetate solution, pH 6.2, and incubated overnight at room temperature. Analyte standard (50 µL) diluted to 200 ng mL^{-1} in 1 mM sodium acetate solution, pH 6.2 and horseradish peroxidase conjugate (50 µL) serially diluted in blocking buffer (2 mg mL^{-1}, w/v *bovine* serum albumin in 1 mM sodium acetate solution, pH 6.2) were added to positive control wells. Negative control wells containing all components except the analyte were also included. The plate was then incubated overnight at 4°C. After twelve washes with wash solution (0.9% sodium chloride, 0.1% Tween 20), antibody bound peroxidase was measured using 3,3',5,5'-tetramethylbenzidine (ELISA) (TMB/E; Chemicon International ES001), a colorimetric substrate. Absorbance was read at 450 nm on a Tecan Sunrise plate reader after 12 min colour development.

Determination of assay sensitivity and specificity in buffer by ELISA
Sensitivity and specificity of each antiserum was assessed against PBZ, OPBZ, SBZ and HPBZ using the SBZ-HRP conjugate as this performed best with each antiserum. Optimum antiserum and SBZ-HRP dilutions were determined by ELISA as described above. Standard concentration ranges (0, 1, 2.5, 5, 10, 25, 50 and 100 ng mL^{-1}) of PBZ, OPBZ, SBZ and HPBZ were prepared and applied in direct competition with SBZ-HRP to a microtitre plate previously coated with antisera. The ELISA technique employed has been described above. Optical densities of wells containing 0 ng mL^{-1} standard (0 STD) represent 100% binding and the remaining standards were normalised relative to the optical density of the zero standard. Half-maximal inhibitory concentration (IC_{50}) was used as an indicator of sensitivity and was calculated as the concentration of unlabelled analyte to give a 50% reduction in binding of the enzyme labelled analyte. Cross-reactivity values were determined as a ratio of the IC_{50} of the relevant hapten with the IC_{50} of the other competitors and given as a percentage.

Results and discussion
Immunochemical methods capable of detecting PBZ and its two metabolites, OPBZ and HPBZ, require antibodies proficient in binding these haptens. A method to detect SBZ is not necessary as it is a prodrug of PBZ, but was used as a hapten (along with PBZ, OPBZ and HPBZ) for immunogen preparation due to possessing a favourable functional group. PBZ lacks a functional group that can be reacted directly with primary amines on a carrier protein so a technique described (Kawashima *et al*., 1975) was employed to react *p*-aminobenzoic acid with the drug prior to coupling to the carrier protein via the introduced carboxylic acid group. The structures of the hapten-carrier protein conjugates for PBZ, OPBZ, HPBZ and SBZ are shown in Figure 2. A similar point of conjugation at one of the benzene rings was used for PBZ and OPBZ while conjugation occurs at the opposite end of the molecule for HPBZ and SBZ. It could be anticipated these two hapten pairs may produce similar antibody cross reactivity profiles. However, while HPBZ provides a functional hydroxyl group on the butyl chain, conjugation to the hemisuccinate chain on SBZ leaves the entire structure of PBZ free as an epitope for antibody production.

Each hapten was evaluated for effectiveness at producing antibodies towards the target compounds with respect to sensitivity and specificity. The four hapten-enzyme labels were employed in conjunction with each antisera and SBZ-HRP provided the optimum response so only this enzyme label was used for the determination of sensitivity and specificity. It was found that the heterologous assay format was more sensitive than the corresponding homologous ones for PBZ, OPBZ and HPBZ antisera. SBZ antisera performed better in a homologous format, indicating the reaction used to prepare SBZ-HRP is more efficient than the reactions used for the other three haptens.

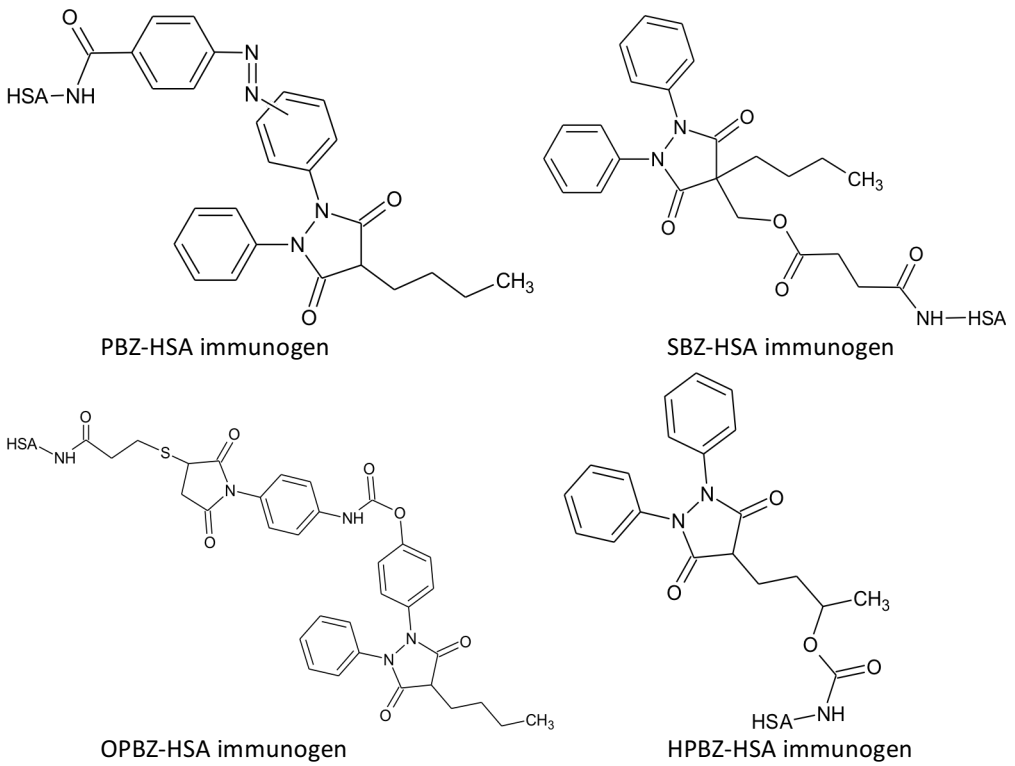

Figure 2. Hapten-carrier protein conjugates for PBZ, SBZ, OPBZ and HPBZ.

Table 1 displays the IC_{50} and % cross-reactivity (CR) for each antiserum in combination with the SBZ-HRP enzyme label. One rabbit, immunised with OPBZ-HSA did not produce an antiserum that could specifically bind any of the four haptens at concentrations lower than 100 ng mL^{-1}. However, the remaining seven rabbits did produce antisera that could specifically bind PBZ at concentrations of 18.3 ng mL^{-1} or less; the two SBZ and one of the HPBZ rabbits produced the three most sensitive antisera to PBZ with IC_{50}s ranging from 0.9 to 4.6 ng mL^{-1}. Although SBZ is a prodrug of PBZ and detection is not required, four antisera with IC_{50}s of less than 6 ng mL^{-1} were produced by one rabbit from each pair immunised with each of the haptens. The IC_{50}s for OPBZ were >50 ng mL^{-1} for six of the antisera but one of the rabbits, immunised with the OPBZ hapten, produced an antiserum that could be capable of detecting the metabolite at the required concentration, displaying an IC_{50} of 5.6 ng mL^{-1}, another rabbit immunised with the HPBZ produced antisera with an IC_{50} of 9.3 n mL^{-1} for OPBZ. In a similar way the IC_{50}s for the metabolite HPBZ were greater than 100 ng mL^{-1} for all haptens except HPBZ itself; the two rabbits immunised with this hapten delivered IC_{50}s of 3.9 and 12.7 ng mL^{-1}.

Table 1: The sensitivity (IC_{50}) and specificity (CR) of each antiserum in combination with SBZ-HRP in an ELISA.

	IC_{50}s (ng mL^{-1})				**CR (%)**			
Immunogen	**PBZ**	**OPBZ**	**HPBZ**	**SBZ**	**PBZ**	**OPBZ**	**HPBZ**	**SBZ**
PBZ-HSA	18.3	53.9	>100	94.6	100	34	ND	19
PBZ-HSA	5.5	57.2	>100	5.5	100	10	ND	100
SBZ-HSA	4.6	89.2	>100	9.1	198	10	ND	100
SBZ-HSA	3.6	>100	>100	1.4	39	ND	ND	100
OPBZ-HSA	5.8	5.6	>100	2.2	97	100	ND	255
OPBZ-HSA	>100	>100	>100	>100	ND	ND	ND	ND
γ-HPBZ-HSA	7.7	>100	12.7	7.3	165	ND	100	174
γ-HPBZ-HSA	0.9	9.3	3.9	3.7	433	42	100	105

Note: ND = Not determined

Analytical methods employed to detect veterinary drug residues for substances not licensed for use in food-producing animals are required to have limits of detection that are as low as possible. In the case of PBZ, a minimum required performance

limit (MRPL) of 5 ppb (ng mL^{-1}) in muscle, milk, liver, kidney and plasma for PBZ and OPBZ residues has been assigned by the Community Reference Laboratory (CRL Guidance Paper, 2007); there is no such figure recommended for HPBZ.

Pharmacokinetic studies have shown the elimination half-life of PBZ is approximately 10 times greater in cattle than in horses, explained by a relatively reduced metabolism of the drug (Lees *et al*., 1988). Consequently, PBZ predominates in *bovine* plasma, with less than 1% of the total drug present as the metabolites OPBZ or HPBZ (De Veau *et al*., 1998). In *equine* plasma OPBZ concentrations are approximately 20% of PBZ concentrations while HPBZ levels are very low (Lees *et al*., 2013). Concentrations of PBZ are significantly less in tissue than plasma and decline over time in line with plasma (Arifah *et al*., 2002), information on OPBZ concentrations in tissue are limited.

The studies above determined the depletion of PBZ from cattle by measuring levels in plasma by liquid chromatographic methods with detection capabilities down to 1 µg mL^{-1} (De Veau *et al*., 1998), 50 ng mL^{-1} (Arifah *et al*., 2002) or 10 ng mL^{-1} (Lees *et al*., 1988). These levels of detection are sufficient for pharmacokinetic studies but not for a food safety regulatory laboratory which is required to detect PBZ at levels below 5 ng mL^{-1} (CRL Guidance Paper, 2007). A concentration of 400 ng mL^{-1} may be considered low for OPBZ in *bovine* plasma with respect to biological activity (Arifah *et al*., 2002) but it is a substantial amount as a residue indicating the use of an unauthorised substance. Further pharmacokinetic studies would be beneficial to determine if tissue is a more sensitive target matrix for analysing samples from animals that may have been withdrawn from PBZ for some time before slaughter and therefore the majority of the drug could have been metabolised.

Sensitivity is paramount when developing an immunoassay to detect a drug which is not licensed for use in food-producing animals. Sample matrix can have a detrimental effect on the sensitivity but the limit of detection will often be lower than an IC_{50} obtained in buffer. Antisera produced by the SBZ and HPBZ haptens with respective IC_{50}s of 3.6 and 0.9 ng mL^{-1} for PBZ could provide sufficient sensitivity for a detection method for PBZ in compliance with the CRL Guidance Paper (CRL Guidance Paper, 2007). Antisera from one of the OPBZ rabbits would be adequately sensitive for a detection method of OPBZ. As far as we are aware this is the first report describing the production of antisera for PBZ and OPBZ that are capable of detecting the drugs in food samples of animal origin.

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ABUSE OF ANABOLIC AGENTS IN BEEF CATTLE: BILE AS A POSSIBLE ALTERNATIVE MATRIX FOR OFFICIAL CONTROL

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Abstract
The use of anabolic agents is prohibited in food-producing animals in the European Union since 1988. Efficient control of abuses depends on knowledge of metabolic pathways, tissues distribution and associated kinetics of elimination. To obtain data concerning metabolites production and discrimination criteria, two *in vivo* residual studies in *bovine* bile after typical growth-promoting treatments are presented. In the first study, sixteen beef cattle were implanted with Revalor-XS® (trenbolone acetate and estradiol) for 71 days. At the same time, sixteen beef cattle were kept as control group. In the second experimental study, three animals were implanted with zeranol pellets (Ralgro®) for 22 days before slaughter whereas three more animals received feed contaminated with zearalenone for 17 days. In both experiments, the results evidenced high concentrations of the administered drugs or their marker metabolites in bile. These concentrations were much higher than those found at the same time in "traditional" biological fluids and tissues, suggesting the possible use of bile to complement analytical controls at the slaughterhouse.

Introduction
Since 1988, the use of hormonal substances in food producing animals is banned in Europe (Council Directive 88/146/EEC). To enforce this prohibition, several effective screening and confirmatory methods have been developed to detect and quantify growth-promoters in urine and tissues. At present, highly sensitive analyses are routinely achieved by gas- or liquid-chromatography coupled to tandem mass-spectrometry (GC-MS/MS and LC-MS/MS). In the last few years, the emerging high-resolution mass-spectrometry analysers have been successfully applied for the analysis of anabolic agents (GC-HRMS and LC-HRMS) (Nielen *et al*., 2007; Vanhaecke *et al*., 2013).

Traditionally, urine has been the matrix of selection since it is relatively easy to collect and samples can be taken before slaughter avoiding contaminated meat to reach the market (Stolker *et al*., 2007). However, it is well-known that residues in urine are generally no longer detectable after two to five days after the last administration. The discovery of hormone abuses could be improved by research studies aiming at the application of the classical targeted methods to unconventional biological matrices in which, hopefully, matrix interferences are reduced and/or residue concentrations are increased. A successful example of alternative matrices has been the introduction in the nineties, namely hair and retina for the control of clenbuterol abuses in cattle.

Mainly at the end of the eighties and in the nineties, some *in vivo* studies pointed out that bile could concentrate some anabolic agents (Chichila *et al*., 1988; Kennedy *et al*., 1998; McEvoy *et al*., 1999). These observations were no longer investigated, but they suggested that the presence of some active principles could be found more easily in bile, than in the traditional matrices.

In this work, the biliary excretion of a selected package of substances after two different *in vivo* growth-promoting treatments (trenbolone/estradiol and zeranol) was studied in cattle. In the first experiment (A), a control group and a group implanted with a combination of trenbolone acetate and estradiol were involved. In the second one (B), one group was treated with zeranol and a second group received feed contaminated with zearalenone, the *Fusarium* mycotoxin that is metabolized to zeranol.

Suitable LC-MS/MS methods applying either ESI or APCI sources have been developed and validated to quantify the compounds of interest at trace levels. The final aim was to add new knowledge about the metabolic patterns and the associated kinetics of elimination in bile after growth-promoting practices.

Materials and Methods
The two animal experiments (A and B) were conducted within the Department of Veterinary Animal Health (Faculty of Veterinary Medicine, University of Bologna, Italy). The protocols were approved by the ethical committee of the same University. The analytical activities connected with the animal experiments were carried out by Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (Study A) and by Istituto Zooprofilattico Sperimentale delle Venezie (Study B).

Animal experiment A

Thirty-two male beef cattle (Charolaise) 10-14 months old were involved. After the acclimatization period, sixteen healthy cattle were randomly assigned to the treatment group and sixteen to the control group. Over the study period, each animal from the treatment group was treated with trenbolone acetate and ß-estradiol by a subcutaneous implant (REVALOR-XSR - kindly gifted by Merck Animal Health). Each implant, a device containing 200 mg trenbolone acetate and 40 mg 17ß-estradiol, allows a delayed, controlled and prolonged release of active principles over a period up to 250 days, taking advantage of the fact that the first dose is immediately available after implant, while the second dose, shielded by a polymer coating, is available after 70-80 days. The study considered only the first 70 days of efficacy of the implant. After 71 days both control and treated animals were sacrificed.

Chemical and reagents. All solvents were analytical grade if used for extraction and purification steps and HPLC grade if used for LC-MS/MS analysis. *Helix pomatia* β-glucuronidase type H-2, code G0876 (Sigma-Aldrich, St. Louis, MO, USA) was used for the enzymatic hydrolysis. SPE Isolute NH2 (500 mg, 6 mL) cartridges were obtained from Biotage (Uppsala, Sweden) and SPE Oasis HLB (200 mg, 6 mL) from Waters (Milford, MA, USA).

Reference standards. 17α-estradiol (α-E2), 17ß-estradiol (ß-E2), 17ß-trenbolone (β-TB) and 17ß-estradiol-d3 (s-E2-d3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 17α-trenbolone (α-TB), 17α-estradiol-d3 (α-E2-d3), 17α-trenbolone-d5 (α-TB-d5) and 17ß-trenbolone-d5 (β-TB-d5) were supplied by TRC (Toronto, Canada). The stock solutions were stored in amber vials at -20°C.

Sample preparation. Five grams of bile were spiked with 25 µL of the solution of the four internal standards (1 µg mL^{-1}) and, after about 15 min, 10 mL of acetate buffer 0.2 M (pH 5.2) were added. Hydrolysis was carried out by incubation at 37°C over night, with 100 µL of β-glucuronidase and the sample was cleaned up using solid phase extraction (Oasis HLB followed by NH_2 cartridge). The eluate was evaporated and the residue was dissolved in 300 µL of hydroxylamine 1.5 M and incubated at 90°C for 60 min (derivatisation step). Finally, 700 µL of water were added and a double extraction was carried out using 2 x 2 mL of TBME. The supernatant was dried, dissolved in 500 µL of a methanol/water (50/50 v/v) mixture and injected into the LC-MS/MS system.

LC-MS/MS Conditions. Chromatography was performed on a Thermo Electron Surveyor instrument (San Jose, CA, USA). Analytes were separated on a Kinetex C18 (2.1 mm x 100 mm i.d., 2.6 µm, Phenomenex, Torrance, CA, USA). Two separate runs were carried out in positive (ESI) and negative mode (APCI), respectively. The column temperature was 25°C and the sample temperature was kept at 12°C. The flow rate was 0.2 mL min^{-1} and the injection volume 15 µL. The MS equipment consisted of a Thermo Electron TSQ Quantum Ultra mass spectrometer (San Jose, CA, USA) controlled by the Xcalibur software. The capillary temperature was 320°C and the capillary voltage 4.0 kV for positive ESI. For negative APCI, discharge current and vaporizer temperature were set at 8.0 µA and 250°C, respectively. Sheath gas, auxiliary gas and ion sweep gas (nitrogen) pressures were set at 40, 25 and 0 arbitrary units for positive ESI run and 50, 40 and 8 arbitrary units for negative APCI, respectively. Collision gas (argon) pressure: 1.5 mtorr. More details are reported in Table 1.

Method validation. Method validation was carried out following Commission Decision 2002/657/EC. In order to study the method accuracy, about 1 kg of a bulk sample was prepared pooling bile of different animals and preliminarily analysed to establish the endogenous concentration of α-E2. Later, seven progressive validation levels were performed covering a wide concentration range (0.1 to 100 µg kg^{-1}). Four replicates (n=4) were carried out for each level and repeated on three separate days, varying operator and calibration status of LC-MS/MS equipment. The precision (repeatability and within-laboratory reproducibility) was calculated applying the Analysis of Variance (ANOVA) at each level. Limit of decision (CCα), and detection capability (CCβ) were estimated using the "surrogate analyte approach" (Li *et al*., 2003). This particular methodology was introduced for α-E2 for which there was a lack of free-analyte samples. It was then applied to all analytes in order to unify the validation process.

Animal experiment B

Six animals (*bovine* intact males – *Bos taurus*) about 24 months old were randomly divided in two separate groups (three animals each). Animals of group 1 were implanted with three 12 mg zeranol pellets (36 mg/animal) and were sacrificed after 22 days without any withdrawal period. The animals of the second group (group 2) were fed a diet containing 3 mg/animal/day of zearalenone for 17 consecutive days and after 5 days the animals were slaughtered. Bile samples were collected at the abattoir during post mortem examinations.

Chemical and reagents. All solvents were analytical grade if used for extraction and purification steps and HPLC grade if used for LC-MS/MS analysis. *Helix Pomatia* β-glucuronidase type H-2, code G0876 (Sigma-Aldrich, St. Louis, MO, USA) was used for the enzymatic hydrolysis. SPE Oasis HLB (200 mg, 6 mL) and SPE Sep-Pak NH2 (500 mg, 3 mL) cartridges were obtained from Waters (Milford, MA, USA).

Reference standards. Zeranol (ZER), taleranol (TAL), α-zearalenol (α-ZOL), ß-zearalenol (ß-ZOL), zearalenone (ZON), zearalanone (ZAN) were purchase from Sigma-Aldrich (St. Louis, MO, USA). ZER-d4 and TAL-d4 were purchased from Rikilt (Wageningen, The Netherlands), while α-ZOL-d7, ß-ZOL-d7 and ZON-d6 from TRC (Toronto, Canada). The stock solutions were stored in amber vials at -20°C.

Table 1. MS/MS parameters for estradiol and trenbolone in bile (animal experiment A).

Analyte	IS	Precursor ion (*m/z*)	Tube lens offset	Product ions (*m/z*)	Collision energy (eV)	Acquisition Mode
ß-TB	ß-TB-d5	286.2	106	197.2	26	Positive (ESI)
				236.2	28	
				251.2	22	
ß-TB-d5 (IS)	-	291.2	104	202.2	28	Positive (ESI)
				256.3	24	
α-TB	α-TB-d5	286.2	106	197.2	26	Positive (ESI)
				236.2	28	
				251.2	22	
α-TB-d5 (IS)	-	291.2	104	202.2	28	Positive (ESI)
				256.3	24	
ß-E2	ß-E2-d3	271.2	114	145.1	44	Negative (APCI)
				183.1	41	
				239.2	40	
				253.2	34	
ß-E2-d3 (IS)	-	274.2	125	145.0	32	Negative (APCI)
				185.2	43	
				242.5	38	
α-E2	α-E2-d3	271.2	114	145.1	44	Negative (APCI)
				183.1	41	
				239.2	40	
				253.2	34	
α-E2-d3 (IS)	-	274.2	125	145.0	32	Negative (APCI)
				185.2	43	
				242.5	38	

Sample preparation. Five grams of bile were spiked with 100 µL of a 0.1 µg mL^{-1} internal standards solution. Then, 10 mL 0.15 M acetate buffer (pH 5.0) were added and enzymatic hydrolysis was carried out by incubation at 37°C over night, with 100 µL of β-glucuronidase. pH 9.0 was reached by adding 1 M NaOH and, if necessary, HCl before clean-up step by solid-phase extraction (Oasis HLB followed by NH2 cartridge). The eluate was dried and the residue dissolved in 500 µL 5 mM ammonium acetate/acetonitrile (60/40 v/v). The sample was then injected into the LC-MS/MS system.

LC-MS/MS Conditions. The LC System consisted of a LC-20ADXR Shimadzu HPLC. Reversed-phase liquid chromatography separation with gradient elution was performed by Supelco Ascentis Express C18 column 100 mm x 2.1 mm, 2.7 µm (Sigma-Aldrich, St. Louis, MO, USA), kept at 40°C and the sample temperature was kept at 5°C. Flow rate was set to 0.3 mL min^{-1} and injection volume was 20 µL. Elution solvents were 5 mM ammonium acetate in water (A) and acetonitrile (B). The mass spectrometer was an API-4000 triple quadrupole (ABSciex, Framingham, MA, USA) operating in negative electrospray mode (ESI) and controlled by Analyst software. Air was used as nebulisation and desolvation gas, while nitrogen was used as curtain gas. Source temperature was set to 650°C with an ion spray voltage of -4,000 V. MS/MS experiments were performed using nitrogen as the collision gas at a pressure of 3.5 10^{-5} torr. More details are reported in Table 2.

Method validation. Method validation was carried out following Commission Decision 2002/657/EC requirements. For accuracy evaluation, three progressive validation levels were investigated covering different concentration ranges depending on the analyte. Six replicates (n=6) were carried out for each level and repeated on three separate days, varying operator and calibration status of LC-MS/MS equipment. The precision (repeatability and within-laboratory reproducibility) was calculated applying the Analysis of Variance (ANOVA) at each level. Decision limits (CCα) and Detection Capability (CCs) were determined by the calibration procedure according to ISO 11843 following the approach proposed by Lega *et al*. (2013).

Results and discussion

Animal experiment A

The bulk material used during the validation study was preliminarily characterized to define the incurred concentrations of endogenous steroids. Only α-estradiol was found at about 12 µg kg^{-1}. Accordingly, for this compound, only the validation data with spiking concentrations higher than 10 µg kg^{-1} were considered. Decision limits (CCα) were 0.05 µg kg^{-1} for α-TB and β-TB and 0.2 µg kg^{-1} for α-E2 and β-E2. Detection capabilities (CCβ) were 0.2 µg kg^{-1} for trenbolone isomers and 0.4 µg kg^{-1} for estradiol ones. Apparent recoveries ranged from 89 to 117%, repeatabilities (CV_r) between 2.6-13 % and intra-lab reproducibility (CV_{Rw}) between 3.1-23%. All these values agreed with the criteria stated in Commission Decision 2002/657/EC.

The mean, median, standard deviation, minimum and maximum values of E2 and TB isomers found in bile samples of each group are listed in Table 3. Trenbolone is a synthetic steroid and its presence was not found in the control group in which only α-estradiol (E2) was detected. This latter increased significantly in the animals belonging to the treated group. The administered estrogen, β-E2, was never detected. The most interesting observation is about the found levels of the marker metabolite of TB: in bile α-TB reached concentrations of two orders of magnitude higher than in other biological fluids and tissues measured within the same *in vivo* experiment at the slaughterhouse (data not shown).

Table 2. MS/MS parameters for resorcylic acid lactones in bile (animal experiment B).

Analyte	IS	Precursor ion (*m/z*)	Declustering potential (V)	Product ions (*m/z*)	Collision energy (eV)	Acquisition Mode
ZER	ZER-d4	321	108	277	32	Negative (ESI)
				303	30	
ZER-d4 (IS)	-	325	108	307	30	Negative (ESI)
TAL	TAL-d4	321	108	277	32	Negative (ESI)
				259	30	
TAL-d4 (IS)	-	325	108	281	32	Negative (ESI)
α-ZOL	α-ZOL-d7	319	120	275	29	Negative (ESI)
				160	36	
α-ZOL-d7 (IS)	-	326	119	282	29	Negative (ESI)
ß-ZOL	ß-ZOL-d7	319	120	275	29	Negative (ESI)
				174	36	
ß-ZOL-d7 (IS)	-	326	120	282	32	Negative (ESI)
ZON	ZON-d6	317	120	175	32	Negative (ESI)
				273	28	
ZON-d6 (IS)	-	323	120	279	28	Negative (ESI)
ZAN	-	319	120	257	30	Negative (ESI)
				205	34	

Animal experiment B

The widespread natural occurrence of ß-ZOL (ZON metabolite produced during the digestion in ruminants' stomach) and of ZON in *bovine* bile samples represented an obstacle to method validation. As a consequence, the validation ranges were chosen depending on the investigated RAL. The repeatabilities (CV_r) were between 4-15 % and the intra-lab reproducibilities (CV_{Rw}) between 5-21%. The observed recoveries ranged from 89% to 120%. Decision limits (CCα) and detection capabilities (CCß) were from 0.24 to 2.6 µg kg^{-1} and from 0.41 to 4.4 µg kg^{-1}, respectively.

Zeranol (α-zearalanol, ZER) might occur naturally in urine and bile from cattle following the metabolism of the *Fusarium* spp. toxins (in particular ZON and α-ZOL), which can contaminate animal feedstuffs (Erasmuson *et al*., 1994; Launay *et al*., 2004). Thus, the finding of zeranol, on its own, is not a sufficient proof of the occurrence of an illicit treatment with this substance. It is therefore necessary to establish quantitative criteria to distinguish zeranol abuse from environmental contamination with *Fusarium spp*. toxins. The results of analysis of bile samples from *in vivo* experiment B are reported in Table 4.

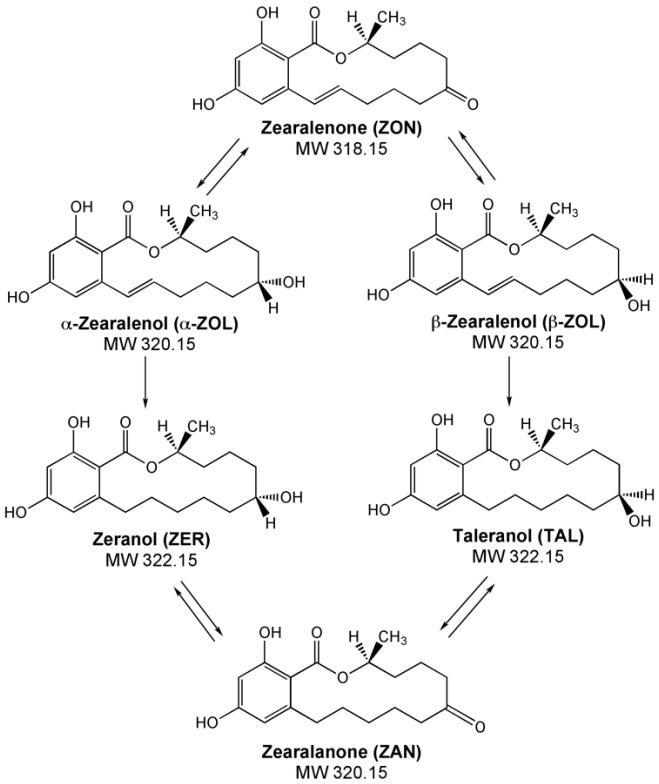

Figure 1. Chemical structures and metabolic relationships among RALs.

As expected, ZER and TAL were present in bile of both groups. However, their levels in implanted animals ("group 1") were definitely higher than in the animals belonging to the non-implanted group. Moreover, as already reported (Chichila *et al*., 1988), TAL seems to be the main metabolite of ZER in bile as its concentration is roughly twice that of ZER in "group 1" samples. The concurrent increase of ZAN seems to indicate the existence of a direct metabolic pathway from ZER to this substance, probably during the epimerization reaction between ZER and TAL as shown in Figure 1 (Kleinova *et al*., 2002). Therefore, the key-markers to prove zeranol-based illegal treatments seem to be ZER, TAL and ZAN, which were significantly higher in the animals belonging to "group 1".

Finally, as already observed in the Experiment A, for "group 1" the measured mean level of ZER in bile was about 2.5 and 17 times higher than in urine and in liver, respectively, of the same animals (data not shown). For TAL the concentration ratios were about 3 (urine) and 23 (liver).

Table 3. Animal experiment A: results in bile (µg kg^{-1}).

Group (animals)	Parameter	β-TB	α-TB	α-E2[a]
	Mean	<CCα	<CCα	6.82
	Median	<CCα	<CCα	7.04
Control (16)	Std dev	-	-	3.75
	Min	<CCα	<CCα	1.87
	Max	<CCα	<CCα	14.7
Treated (16)	Mean	0.33	38.2	16.0
	Median	0.29	30.4	15.1
	Std dev	0.16	20.0	6.18
	Min	0.08	14.7	4.73
	Max	0.77	93.1	30.7

[a] *The mean value of α-E2 in treated animals was significantly higher than in control group (Mann-Whitney test). β-E2 was not found neither in control nor in treated animals.*

Table 4. Animal experiment B: results in bile (µg kg^{-1})

Treatment	Animal	ZER	TAL	α-ZOL	ß-ZOL	ZON	ZAN
"Group 1"	1	12.7	20.7	6.3	54.1	9.8	10.0
Zeranol (ZER)	2	14.1	23.5	5.3	54.9	7.1	6.6
	3	14.2	21.4	3.2	42.4	5.2	8.6
Mean		13.7	21.9	4.9	50.5	7.4	8.4
"Group 2"	1	<CCα	0.6	2.4	30.0	3.5	<CCα
Zearalenone (ZON)	2	0.5	0.9	3.4	54.5	5.8	<CCα
	3	0.8	1.7	5.9	56.4	7.4	<CCα
Mean		0.4	1.1	3.9	47.0	5.6	<CCα

Conclusions

The high concentrations of the marker residues observed in bile in both *in vivo* experiments compared with levels detected in urine and liver collected at the same time, corroborate the previous studies about the capability of this fluid to bioconcentrate certain anabolic substances. This suggests a new possible scenario in the control of hormone abuse at the slaughterhouse. Its use as target matrix could therefore permit to detect illegal hormone administration for a longer period and might represent a further tool to disclose the practice of using cocktails of anabolic compounds at very low doses.

Acknowledgements

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WHICH ANALYTICAL TECHNIQUES CAN REDUCE MATRIX EFFECTS IN LC-MS ANALYSIS

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Abstract
Matrix effects might exert a detrimental impact on important method parameters (analytical limits, linearity, accuracy, and precision). Ion suppression or enhancement appears as a kind of matrix effect specifically linked to mass spectrometry that probably represents one of the main sources of pitfalls in LC-MS analysis. The negative influence of matrix effects from the testing material influences significantly the sensitivity and the selectivity of the measurements. Hence, matrix effects need to be evaluated and examined during method validation in order to achieve a consistent quantification. In this survey various analytical techniques are applied to investigate ways of eliminating these disturbing matrix effects. The following compound-matrix combinations were examined:

- Coccidiostats in compound feed;
- β-Agonists in hair (*bovine*);
- (oxy)-Phenylbutazone in *equine* muscle.

Extracts from above matrices were analysed using the following analytical techniques:

- Accurate mass using Exactive and Q-Exactive mass spectrometer;
- Ion mobility using Selexion from QTRAP6500 mass spectrometer;
- MS^3 measurements using QTRAP6500 mass spectrometer.

Applying different analytical techniques resulted in some cases to an improvement of the signal-to-noise ratio and selectivity retrieving the performance of the LC-MS and reducing the negative effects of these phenomena.

Introduction
Liquid-chromatography in combination with atmospheric-pressure ionization mass-spectrometry (LC–API-MS) has well proved to be an efficient analytical tool in the field of multi-residue analysis, achieving identification and quantitation of analytes in complex matrices due to its high selectivity and sensitivity. However, the high selectivity of LC-MS does not guarantee the efficient elimination of interferences from endogenous co-eluting residual matrix components which influence the ionization of the analyte of interest causing ion-suppression or enhancement. Such matrix effects (ME) are responsible for poor and inaccurate data in quantitative analysis causing significant effects to reproducibility, linearity, and accuracy of the method. Several strategies have been proposed in order to minimize or eliminate the interferences of co-eluting matrix compounds, such as improved chromatographic selectivity to avoid co-elution, use of mobile phase modifiers, improvement of sample preparation to reduce the presence of interfering components in the final extract by using efficient solid phase extraction (SPE), stable isotope-labelled internal standards (IS), and changing ionization modes (positive or negative), source designs and ionization techniques (ESI, APCI or APPI) for the mass spectrometric conditions (Hoff *et al*., 2015; Liu *et al*. 2013; Trufelli *et al*. 2011; Gosetti *et al*. 2010).

Although these approaches were claimed to be effective for model analytes chosen, there are essential drawbacks depending on the complexity of the matrix. In the recent years, rapid developments in the introduction of new mass spectrometers and in the advancement and augmentation of the technology (ion-detection and mass-analyzers) have offered more capabilities in solving challenging analytical tasks. In the present work, our aim was to apply different analytical techniques in order to evaluate the occurrence of ME in LC-MS methods for the analysis of coccidiostats in compound feed, the analysis of β-agonists in *bovine* hair and phenylbutazone in *equine* muscle. Mass analyzers enabling high resolution mass spectrometry (HRMS), such as, Orbitrap technology and low resolution, such as, triple stage quadrupole together with ion mobility spectrometry (IMS) were the main analytical platforms used for this experimental design.

Materials and Methods

Chemicals and Reagents
All standards and internal standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents used were of LC-MS grade and obtained from Merck (Darmstadt, Germany). Salts, basis and acids were of analytical grade and provided from Fluka (Steinheim, Germany).

An appropriate amount in mg of each analyte was accurately weighed and dissolved in the appropriate organic solvent in order to produce stock standard solutions. The intermediate working solutions were prepared by dilutions of the stock solution in the organic solvent. All standard solutions were stored at –20°C in the dark.

Samples
Samples of animal muscle, feed and hair were provided through the National Residue Control Program (NRCP) by the NVWA (Dutch Food and Consumer Safety Authority). After analysis, these samples were used as blank samples for further fortification with the analytes throughout the study. Sample extracts after applying sample preparation were stored at -80^{o}C prior analysis.

Instrumentation
Liquid-chromatography mass-spectrometry analysis was performed on the following instruments: a QTRAP 6500 system with SelexION Differential Ion Mobility Technology (AB Sciex, Framingham, MA, USA), an Exactive and Q-Exactive (ThermoScientific, San Jose, CA, USA) and a Xevo TQS system (Waters, Milford, MA, USA).

Analysis of coccidiostats in feed
The pre-treatment and analysis of animal feed samples for coccidiostats is described in an in-house validated and accredited RIKILT standard operating procedure. The compounds included were lasalocid, maduramycin, monensin, narasin, semduramycin and salinomycin. Briefly, the sample preparation includes extraction with a mixture of solvents (methanol / acetonitrile / sodium hydroxide, pH 6-7), dilution with water and final analysis on the LC-MS/MS system. An Acquity UPLC HSS T3, 1.8 µm, 100 x 2.1 mm analytical column (Waters) was used and the MS was operated in MRM (+)-ESI mode.

Analysis of β-agonists in hair
The pre-treatment and analysis of hair samples for β-agonists is described in an in-house validated and accredited RIKILT standard operating procedure. The compounds included were fenoterol, terbutaline, metaproterenol, cimaterol, salbutamol, clenbuterol and salmeterol. The sample preparation includes extraction with water by adjusting the pH value, SPE with Varian Bond Elut certify, evaporation, re-dissolving and final analysis on the LC-MS/MS system. An Acquity UPLC BEH C18, 1.7 µm, 100 x 2.1 mm analytical column (Waters) was used and the MS was operated in MRM (+)-ESI mode.

Analysis of phenylbutazone in muscle
The pre-treatment and analysis of muscle samples for phenylbutazone (FBZ) and its main metabolite oxy- phenylbutazone (oxy-FBZ) are described in an in-house validated and accredited RIKILT standard operating procedure. Briefly, the sample preparation includes extraction with acetonitrile, clean-up with Primary Secondary Amine (PSA) material, evaporation, re-dissolving and final analysis on the LC-MS/MS system. An Acquity UPLC HSS T3, 1.8 µm, 100 x 2.1 mm analytical column (Waters) was used and the MS was operated in MRM (+) and (-)-ESI mode.

Experimental design
The selection of the compounds and matrices analysed was based on already observed variation in the analysis. Sample preparation and LC conditions remain the same in order to compare the data mainly based only on the different detection technique of each instrument. The detection techniques applied were QTRAP 6500 in MS/MS mode (Q2 MS^2), QTRAP 6500 in MS^3 mode (Q2 MS^3), QTRAP 6500 with Selexion Differential Ion Mobility (SI) with (m md) and without modifier (z md), Q-Exactive in full scan (FS) accurate mass at different resolutions (17.5K, 35K, 70K and 140K), Q-Exactive (Qexa) in MS/MS (TMS^2) at different resolutions (17.5K, 35K, 70K and 140K) and TQS in MS/MS (TQS MS^2).

Assessment of matrix effects
Replicates of ten different samples were analysed as such and including fortification with the analytes before and after the extraction. Based on these results the matrix effects (ME), recovery (RE), signal-to-noise ratio (S/N), response factor (RF) and ion ratio (R) were evaluated if applicable.

ME (%) = B/A*100%, where A represents the average peak area of the analyte of the standard solution and B the average peak area of the sample extract fortified after extraction. A value of ME > 100% indicates ionization enhancement, whereas a value < 100% indicates ionization suppression.

RE (%) = C/B*100%, where C represents the average peak area of the sample extract fortified before extraction.

RF (%) = $A_{analyte}/A_{IS}$*100%, where $A_{analyte}$ represents the peak area of the analyte and A_{IS} the peak area of the internal standard. The repeatability (*R*) in this case should be less than 14.7% (*R* = 2.8**Sr,* where *Sr* is the within-day standard deviation of the repeated samples).

R (%) = A_{low}/A_{high}*100%, where A_{low} represents the area of the product ion with the lowest intensity and A_{high} the area of the product ion with the highest intensity. The repeatability in this case should also be less than 14.7%.

Results and Discussion

Selection of analytical techniques

Liquid-chromatography combined with tandem mass-spectrometry is the analytical technique mostly applied in routine analysis and that is why the TQS and QTRAP 6500 instruments were selected. MS^n experiments provide additional fragmentation of the analyte that can increase specificity and reduce interferences, which is why the MS^3 option of the QTRAP 6500 was applied. Ion mobility spectrometry (IMS), when coupled with mass-spectrometry, offers an added value achieving separation of isomers, isobars, and co-eluting interferences and increases selectivity. Based on the different mass-analyzers, high-resolution mass-spectrometry, such as, Orbitrap technology can be useful when mass interferences are detected in tandem MS. The analytes may co-elute with compounds from the matrix, or even with each other. In case of complex matrices, it is not unlikely that the exact mass of a co-eluting compound is close to that of an analyte, resulting in incorrect calculation of the target analyte. This issue can be controlled by the resolving power which is a key parameter affecting the correct assignment of the masses for the analytes. Narrowing mass extraction windows with the combination of the required resolving power can have significant impact to the quantitative results.

Application for the analysis of coccidiostats in feed

MS^3 experiments and SI did not provide satisfactory results for the analysis of coccidiostats in feed. The ME is generally not significantly influenced by applying different analytical techniques, except for maduramycin and semduramycin, for which the MS^2 experiments with the QTRAP 6500 and Q-Exactive at higher resolution can decrease ME (Figure 1).

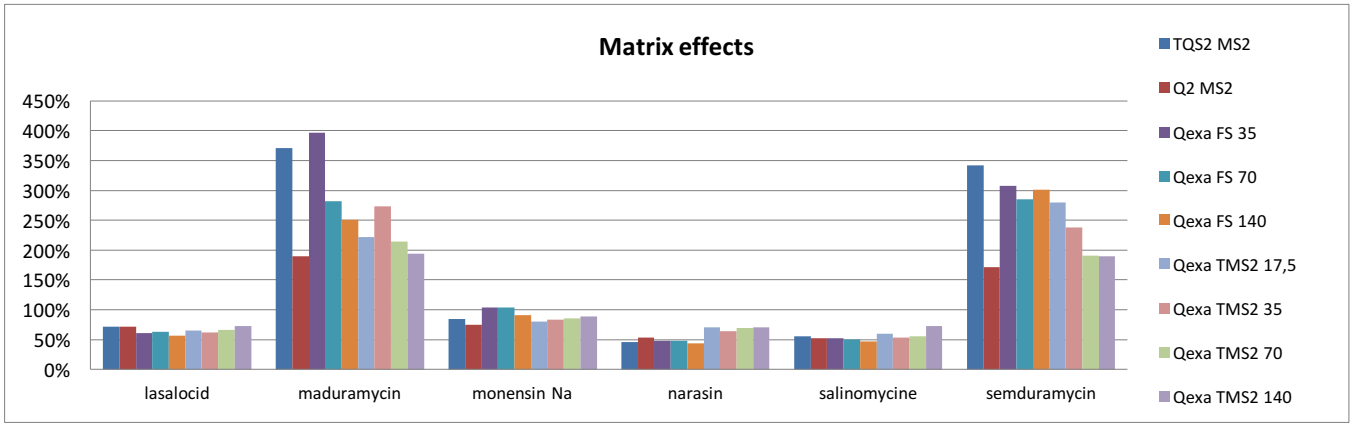

Figure 1. Matrix effect for the analytes based on the different analytical techniques tested.

Concerning the recoveries all measurements with a variance of higher than 20% were rejected and only four techniques were evaluated. In optimum conditions the recoveries should remain the same regardless of the method applied. This seems to appear but not exactly for maduramycin, salinomycin and semduramycin (Figure 2).

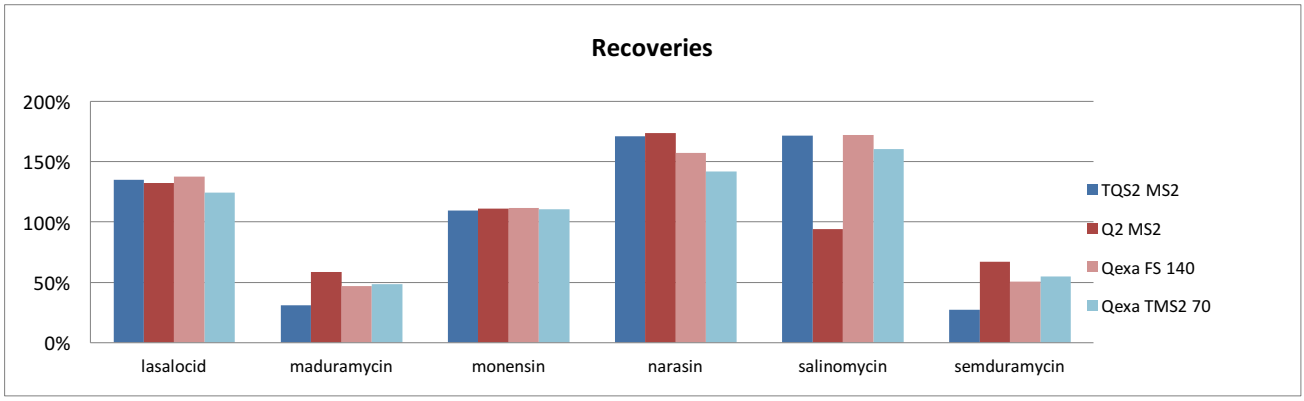

Figure 2. Recovery results for the analytes based on different analytical techniques tested.

The S/N ratios of the samples fortified with the analytes after the extraction provided higher values for TQS MS^2 followed by the Q2 MS^2, and worst values by SI and Q2 MS^3. Concerning the RF and the criterion of repeatability less than 14.7% only semduramycin meets the requirements set on Q2 MS^2 and Q-Exactive at 70K and monensin on the Q2 MS^2. Furthermore, the

majority of the ion ratios measured by all the MS^2 and Selexion techniques meet the needed tolerances according to the EU requirements. By evaluating also the ratio of the sodium and ammonium adducts of the coccidiostats Q-Exactive TMS^2 at 35K and 70K gave relatively low variance compared to the other techniques tested.

Application for the analysis of β-agonists in hair

TMS^2 experiments with Q-Exactive at resolution of 35K and 70K did not provide satisfactory results for the analysis of salmeterol in hair. The ME is generally not significantly influenced by applying a specific analytical technique (Figure 3). Experiments on Qexa FS at 17.5K showed high variability and are not included.

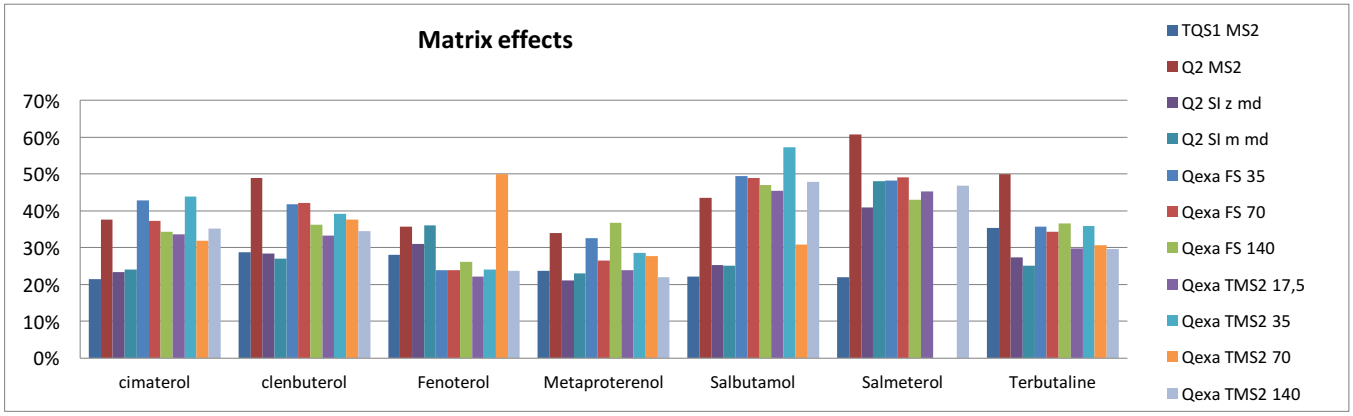

Figure 3. Matrix effect for the analytes based on the different analytical techniques tested.

Concerning the recoveries, all measurements with a variance of higher than 20% were rejected and only four techniques were evaluated. At optimum conditions, the recoveries should remain the same regardless of the method applied. This appears, but for metaproterenol and fenoterol the recoveries were very low and could not be calculated in some cases (Figure 4).

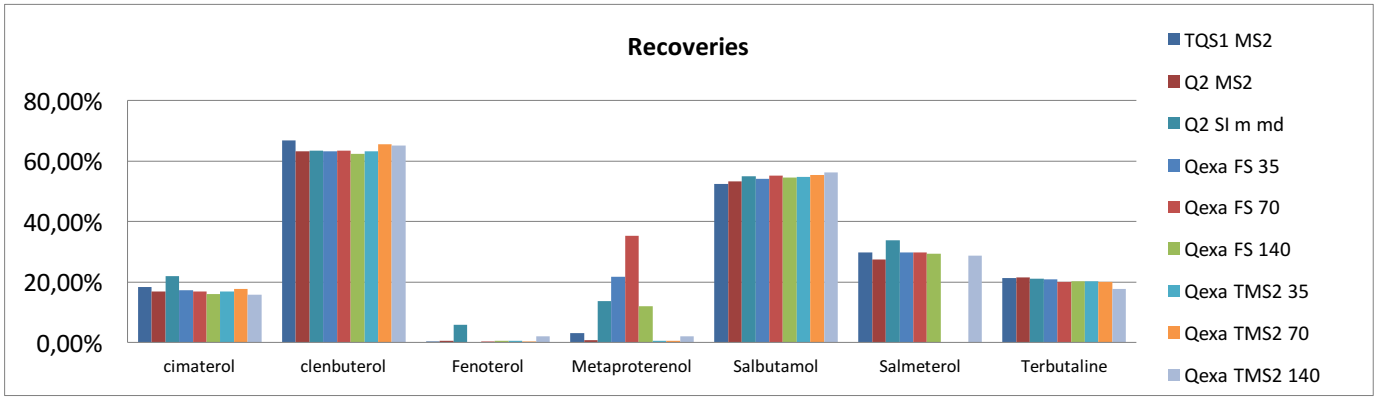

Figure 4. Recovery results for the analytes based on different analytical techniques tested.

The S/N ratios of the samples fortified with the analytes after the extraction provided higher values for TQS MS^2 followed by the Q2 MS^3, and comparable for the others. Concerning the RF and the criterion of repeatability less than 14.7%, clenbuterol meets the requirements set on TQS MS^2, Q2 MS^2, SI with and without modifier and Qexa FS at 35K and 70K and terbutalin on the TQS MS^2 and Q2 MS^2. Generally, MS^2 results provide the best results. For MS^3, Qexa TMS^2 at 35K, 70K and 140K no internal standard was used in order to have sufficient data points, so they were not included in the reprocessing. Furthermore, the majority of the ion ratios measured by all the MS^2 and Selexion techniques meet the needed tolerances according to the EU requirements.

Application for the analysis of phenylbutazone in muscle

For the analysis of phenylbutazone in muscle the analytical techniques applied were QTRAP 6500 MS^2, MS^3, SI, Exactive in full scan at 50K and Q-Exactive in full scan at 70K and TMS^2 at 17.5K. The ME was evaluated as 0% without any matrix effect (Figure 5). In addition, both negative and positive ionization modes were evaluated and also an additional dilution of the final extract to twenty times.

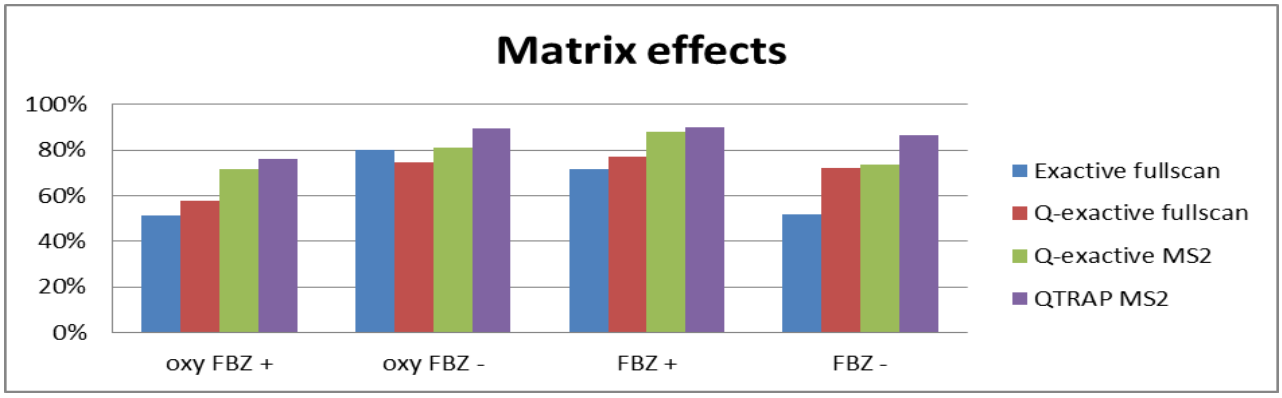

Figure 5. Matrix effect for the analytes based on the different analytical techniques tested.

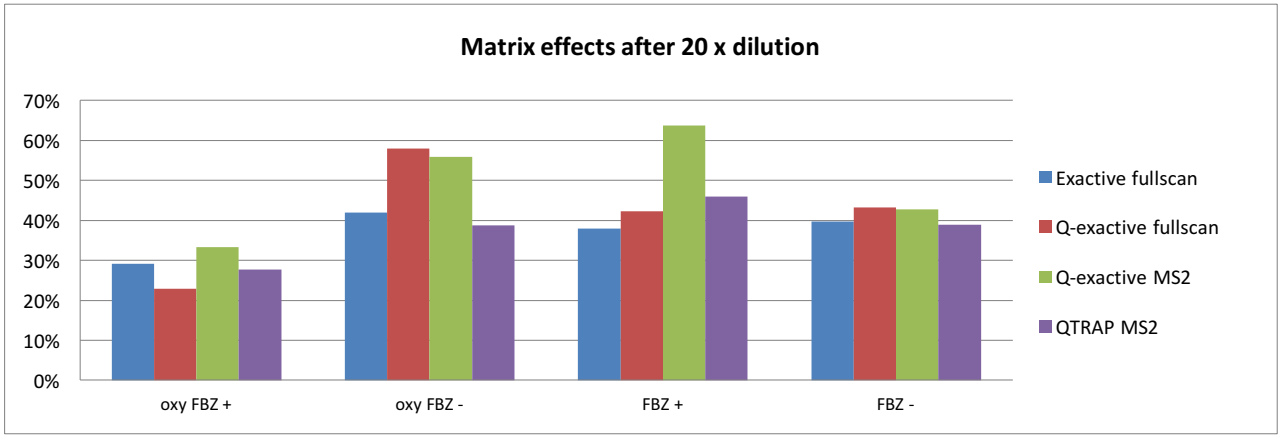

Figure 6. Matrix effect results for the 20 times diluted extracts of the analytes based on the different analytical techniques.

Diluting the sample extracts has a great influence on the decrease of the ion suppression and the increase in the sensitivity and selectivity of the measured components of the analysis (Figure 6). The S/N ratios also increased significantly by dilution as the interfering matrix components decreased.

Comparing Selexion measurements with a QTRAP resulted in some cases to reduce ME without changes in the S/N ratios. Additionally, comparing QTRAP MS^2 and MS^3 measurements increased the S/N ratio, especially in negative mode. The Q-Exactive showed increased sensitivity of a factor of 20 in positive mode and 10 in negative mode compared to Exactive. Recoveries were not able to be evaluated because of the very low values for FBZ and oxy-FBZ. The RF values did not show significant changes and the ion ratios fulfilled the needed tolerances.

Conclusions

Matrix effect is a very frequent issue in multi-analyte LC-MS based analysis and its magnitude is essential to be minimized. Thus, the application of different analytical techniques was evaluated. It seems that there is no exclusive analytical technique that can eliminate the matrix effects. Generally, more improved results appear by conducting the analysis in MS^2 mode and in some cases in HRMS MS^2. But also dilution steps and changing ionization modes can have an effective impact. Nevertheless, a strategy in order to avoid matrix effects is not straightforward and there is a strong correlation between the group of analytes and type of matrix selected for the analysis.

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IMPLEMENTATION OF AN ANALYTICAL METHODOLOGY FOR DETECTION OF SULFACHLORPYRIDAZINE (SCP) RESIDUES IN BROILER FEATHERS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Abstract

For the detection and quantification of sulfachloropyridazine (SCP) in broiler feathers, a reliable and accurate analytical method is needed. For this purpose, an analytical methodology by LC-MS/MS was implemented and optimized. Blank samples of feathers were spiked at five level concentrations (50-100-150-200-250 µg kg^{-1}) with certified analytical standards; 13C6-sulfamethazine was used as internal standard. Ethyl acetate and sodium hydroxide solutions were tested as extraction solvents. For the samples clean up, aromatic sulfonic acid (SCX) solid phase extraction cartridges (SPE) were used. Instrumental analysis was performed by LC-MS/MS.

The analytical methodology showing best performance was the extraction using both solvents (ethyl-acetate and sodium hydroxide solution). The use of SCX SPE did not improve the recovery or linearity of the method. This sample treatment showed an average recovery of 112%, explained by the matrix effect. The linearity was determined by calibration curves within a range of 50-250 µg kg^{-1}, and it presented a determination coefficient of 0.96. The recovery and linearity showed that this method is suitable to detect SCP antibiotics in feathers. The methodology allows the recovery and detection of SCP residues in chicken feathers in a range of 50-250 µg kg^{-1}. Therefore, it can be used for further studies using this matrix.

Introduction

In poultry production, antimicrobials are important for the treatment of bacterial diseases (Landoni and Albarellos, 2015). However, the presence of veterinary drug residues in products and by-products of animal origin may cause different adverse effects on human health, such as hypersensitivity, tissue damage, gastrointestinal alterations and neurological disorders (Babapour *et al.,* 2012; Mensah *et al.*, 2014). Additionally, the presence of residues contributes to the development of resistance in bacteria in treated animals. These resistant bacteria may be transferred to human hampering treatment of human infections (Lozano and Arias, 2008). Sulfonamides are authorized antibiotics for poultry use, due to their chemotherapeutic effect in bacterial and protozoarian infections. For this reason, they are used in feed and water, in order to treat poultry diseases, such as infections of the digestive and respiratory tract (Fang *et al*., 2006).

The denomination “sulpha” applies to every antibacterial drug containing a sulfonamide R-SO_2NH group derived from a para-amine-benzene-sulfonamide in which substituent R is an aniline derivative. Discovery of the sulphas gave rise to microbial chemotherapy, since these substances interfere with microbial metabolism stopping anabolism, instead of working as plain bactericides. Interest in sulfonamides has decreased due to the emergence of other antimicrobials, but they remain as the focus of attention in veterinary medicine, considering that some of them are occasionally irreplaceable. These drugs can be quantified in all matrices through several methods; the instrumental ones the most widely used (Guzmán, 2001).

Since they were synthesized in 1908, around 15,000 analogous compounds have derived from sulfonamides, particularly those related to the *p*-aminobenzoic acid. Currently, there are around 30 different types of commercial sulfonamides, some of them with great antimicrobial results (Guzmán, 2001). Currently, the European Union (EU) has set an MRL of 100 µg kg^{-1} for sulfonamides in all edible tissues (EU Regulation Commission N° 37/2010).

By-products derived from poultry production can be incorporated into the food-chain through feed, such as the case of feathers(Cornejo *et al.,* 2011). Feather meal can re-enter the productive chain in several ways, but mainly, as additive for poultry, swine, ruminants and fish feed, since they are considered a low-cost protein source (Divakala *et al.,* 2009). For example, in 2008 United States produced over 604,000 tons of feather meal, and74,000 tons were exported (Swisher, 2008).

Some studies have proven the bioaccumulation of antimicrobials in poultry feathers (San Martin *et al.,* 2007; Cornejo *et al.,* 2011). This, poses a risk for human and animal health, as it is an unrecognized path of re-entry of multiple drugs and contaminants into the food chain (Love *et al.*, 2012).

Love *et al.* (2012) carried out a study in which feather meals were sampled in different states from United States of America (Arkansas, North Carolina, Oregon, California, Idaho and Tennessee) and China. These researchers found the presence of 2 to 10 antimicrobials in each analysed sample. A number of seventeen different drugs in six antimicrobial families representing a total of 26 different drugs were detected.

Hitherto, there are no studies showing depletion of sulfonamides in feathers and its relationship to the concentrations found in edible tissues of treated birds. For studying the behaviour of these drugs in this matrix. an analytical methodology able to detect and quantify the analyte of interest in broiler feathers is needed. In the present study, an analytical methodology was implemented for the extraction and detection of sulfachloropyridazine in this matrix.

Materials and Methods

Implementation of analytical methodology
For the detection of sulfachloropyridazine (SCP) in feathers, an analytical method by LC MS-MS based on previous analytical methodologies published by Hindle (2003), Renew (2004), Shao *et al.* (2005), Pang *et al*. (2005), Stubbings and Bigwood (2009), Bedendo *et al.* (2010) and Yu *et al.* (2011) was implemented.

Sample processing
Commercial broiler feathers were used, treated with a food processor for a pre-grinding, and then an industrial food processor (Robot Coupe (4.5L)). Liquid nitrogen was added for a cryogenic treatment of the samples.

Standards, reagents and solvents
For the analysis and quantification, certified purity sulfachloropyridazine (SCP) from Sigma Aldrich was used. Sulfamethazine-phenyl-13C6 hemihydrate (SMZ 13 C6; Sigma Aldrich) was used as internal standard (IS). Reagents and solvents were HPLC-grade water (Millipore or similar), P.A.-grade ethyl acetate (JT Baker or similar), HPLC-grade methanol (JT Baker or similar), chlorohydric acid, P.A-grade sodium hydroxide (Merck or similar), HPLC-grade water (Merck or similar) and formic acid 98/100% (Merck or similar). Two solutions were used for extraction: A, 0.1% formic acid in methanol, pH 2.9 ± 0.3, and B, 0.1% formic acid in water, pH 2.7 ± 0.2.

Analyte extraction from biological matrix
Blank feathers fortified with sulfachloropyridazine at five concentrations (10, 20, 40, 80 and 100 µg kg^{-1}) were used. To each sample, 4.8 µg kg^{-1} IS was added. An amount of 2 ± 0.02 g of sample was weighed in a 50-mL polypropylene tube and 40 mL ethyl acetate were added. Samples were stirred for 15 min on a vortex-mixer and sonicated for 5 min. Subsequently, feathers were centrifuged at 1,800 g for 10 min. Supernatant was filtered through glass wool into a fresh 50-mL polypropylene tube. Samples were concentrated to 15 mL using water bath at 40-50° C under a mild nitrogen flow. For solid phase extraction (SPE) cationic-sulfonic acid (6 mL and 500 mg) columns were conditioned with 6 mL hexane and 6 mL ethyl acetate, and washed with water (2 mL) and methanol (2 mL). Elution was performed with 10 mL of a mixture of methanol (48.5 mL) and ammonia solution (1.5 mL). The eluate was evaporated using water bath at 40-50°C under a mild nitrogen flow. Samples were reconstituted in 300 µL mixture of solution A and phase B (15/85). The obtained sample was stirred on a vortex-mixer and sonicated for 5 min, transferred into an Eppendorf tube and centrifuged at 17,000 *g* for 10 min. Finally, samples were filtered through 33 mm millex filters with 0.22 µm polyvinylidene fluoride (PVDF) membranes. The filtrate was transferred into glass vials for LC MS-MS injection and analysis.

Instrumental analysis
For instrumental analysis a liquid-chromatograph was used (Agilent 1290 infinity series), coupled to a triple-quadrupole mass-spectrometer (API 3200, ABSCIEX). A Symetry C8 3.5µm 2.1 x 100mm Waters analytical column was used. Analyst 1.5 was used as integration software. Isocratic chromatographic separation and flow gradient at 200 µL min^{-1} was obtained with 45% phase A and 55% phase B as listed in Table 1, corresponding to extraction solutions A and B. Volume of injection was 20 µL and the column oven temperature was maintained at 35°C.

Table 1. LC gradient of mobile phases A and B (portion in volume percentages).

Minute	Flow rate µL min^{-1}.	Mobile phase A	Mobile phase B
0.0	200	45%	55%
4.0	200	45%	55%

Results
Using the presented method, the analyte of interest (SCP) was detected in the biological matrix through its retention time and specific masses. Data corresponding to six pure standard injections show that the coefficient of variation (CV) of the average retention time did not exceed 5% for SCP (Table 2).

Table 2. Parent ion mass, product ion mass, Average and CV (%) for retention times of the six SCP and SMZ pure standard injections.

Analyte	Parent ion mass	Product ion mass	Retention time Average	CV (%)
SCP	284.9	155.9	2.978 min.	0.25
		108.2		
SMZ 13 C6 (IS)	285.1	124.1	2.588 min.	0.29

After chromatographic detection of the analytes, calibration curves fortified at five work concentrations at 10, 20, 40, 80 and 100 µg kg^{-1} were used with the aim of determining the linearity of the analytical method. Concentrations were defined by considering the MRL for muscle matrix at 100 µg kg^{-1} (EU Commission Regulation No 37/2010). At a signal-to-noise ratio higher than 3:1, the limit of detection (LoD) was 10 µg kg^{-1}. Figure 1 shows chromatograms of pure standard and a control sample fortified at LoD. Calibration curves showed a coefficient of variance (R^2) above 0.96 with a CV of 1.61% for the three matrix-matched calibration curves (Figure 2).

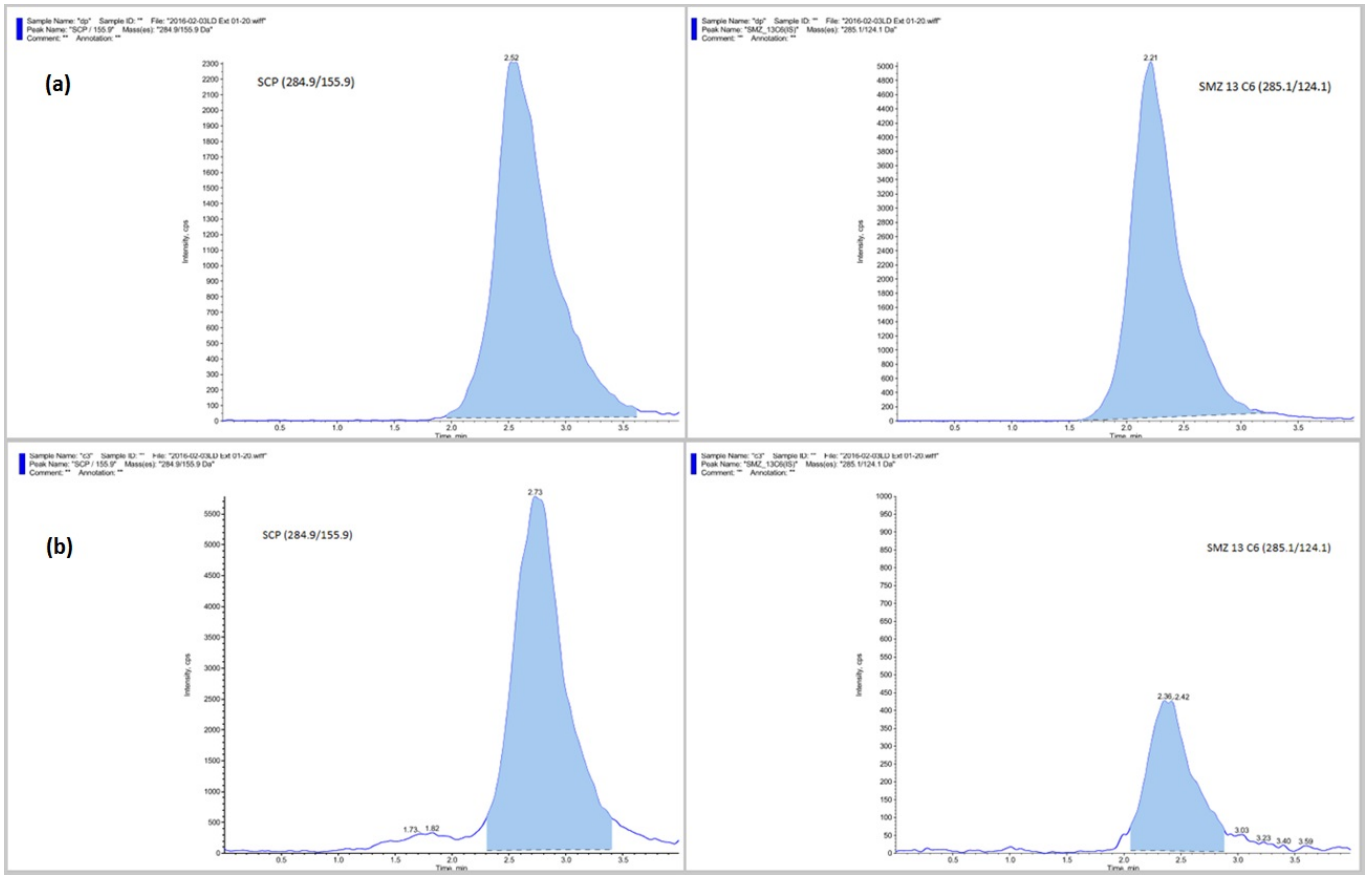

Figure 1. Chromatograms of Sulfachloropyridazine (SCP) standard injection versus control feather sample fortified at LoD level.

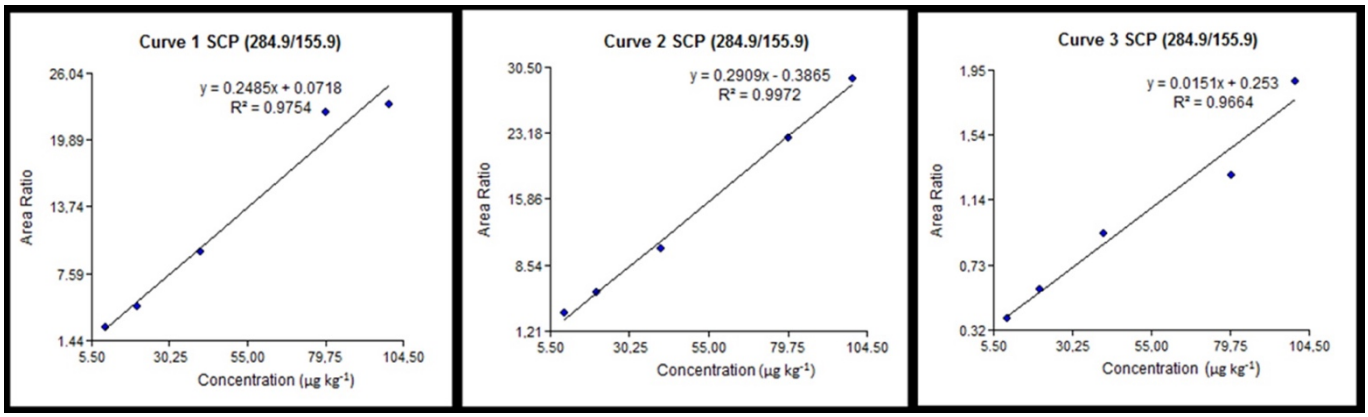

Figure 2. Calibration curves of sulfachloropyridazine (SCP) at five fortification levels (10, 20, 40, 80 and 100 µg kg^{-1}) in feathers.

Discussion and Conclusions

According to the obtained results, the analytical methodology used in feathers detects sulfachloropyridazine showing a good linearity, since R^2 was higher than 0.96. These results meet the acceptance criteria for the validation of analytical methodologies according to the European Community Decision 2002/657/EC. Calibration curves were carried out with 10, 20, 40, 80 and 100 µg kg^{-1} in accordance with the MRL established for muscle (100 µg kg^{-1}; EU Commission Regulation No 37/2010). The implemented method proved to be linear. Considering a signal-to-noise ratio of 3:1, the LoD was established at 10 µg kg^{-1}.

Currently, there are several studies on this antimicrobial family for different poultry products, such as the research recently carried out by Premarathne *et al*. (2015), who implemented a method for sulfonamides detection in muscle and chicken eggs. Nevertheless, until today, there are no methodologies able to detect SCP in broiler feathers. Previous depletion studies in edible tissues and chicken feathers carried out by San Martin *et al.* (2007), Cornejo *et al*. (2011 and 2012) and Berendsen *et al.* (2013) showed that some antimicrobials can accumulate in feathers at higher concentrations than in edible tissues, for longer periods. Because of this, it is important to detect and quantify SCP in feathers of treated birds, in order to assess its accumulation in this matrix.

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DEPLETION STUDY OF OXYTETRACYCLINE (OTC) AND 4-EPI-OXYTETRACYCLINE (4-EPI-OTC) RESIDUES IN FEATHERS OF BROILER CHICKEN BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Abstract

Feathers can be used as an ingredient in feed for other productive animals, having thereby the potential to carry-over antimicrobial residues into the food chain. Thus, it is important to determine whether detectable OTC and 4-epi-OTC remain in feathers at higher concentrations and for longer periods than edible tissues. The levels of these analytes were evaluated in the feathers of 64 broiler chickens slaughtered at 3, 5, 7, 13, 19 and 22 days post-treatment with a therapeutic dosage of OTC 10% during 7 days. Extraction was carried out using EDTA-McIlvain buffer and acetone; solid-phase extraction columns (OASIS HLB®) were used. Instrumental analysis was performed using LC-MS/MS.

Results show high OTC and 4-epi-OTC concentrations at day 7 post-treatment, which is the recommended withdrawal time of the selected pharmaceutical formulation. At day 19 post treatments, mean concentrations of OTC and 4-epi-OTC were 100 µg kg^{-1} and 89 µg kg^{-1}, respectively. Results show that OTC and its metabolite remain for longer periods and at higher concentrations in feathers than in edible tissues.

Introduction

Antimicrobials used in poultry production are administered through water or feed to prevent disease outbreaks during breeding (Stolker and Brinkman, 2005). The use of antibiotics to treat infectious diseases, among other measures, has increased production by lowering the correlated incidence of infectious diseases and mortality. The introduction of these antimicrobial agents in rearing and production of livestock has resulted in the selection, propagation and persistence of antimicrobial resistant bacteria strains capable of causing infections in animals and humans (Agunos *et al*., 2012).

In the case of edible tissues from broiler chickens, such as muscle and liver, several authors (Schneider and Donoghue, 2003; San Martín *et al*., 2009; Cornejo *et al*., 2011; Anadón *et al.,* 2012) have demonstrate that some analytes and metabolites of the antimicrobials drugs may remain after therapy for varying times depending on drug elimination kinetics and pharmaceutical formulations. However, there is scarce information on drug residues behaviour in non-edible tissues, as feathers.

Feathers are a by-product of poultry production not intended for human consumption. It has been calculated that about 37% of the total weight of chicken is not consumed directly by humans (Meeker and Hamilton, 2006). This non-edible fraction consists of heads, bones, guts and feathers. It becomes a source of feeding material that enters the food chain, mainly in the form of feed meal (Swisher, 2009). Love *et al*., 2012 noted the presence of antimicrobial residues in feather meals coming from twelve different states, by quantifying the compounds of different antibiotic families such as fluoroquinolones, macrolides, sulfonamides, streptogramins and tetracyclines, ranging from two to ten different molecules in total per sample.

San Martín *et al.* (2007) and Cornejo *et al.* (2012; 2011) have previously reported the transfer of fluoroquinolones to feathers of chickens treated with therapeutic doses of these drugs. Their concentrations were high compared to those determined in edible tissues such as muscle and liver. These studies suggested that the withdrawal period of the drugs, based on concentrations of the antimicrobial residues in the studied edible tissues, were unsatisfactory to reduce antimicrobial residues in the feathers of treated animals. However, despite the evidence that points it as an unrecognized route for antibiotics re-entering into the food chain (Love *et al.* 2012), there is scarce information about the transfer and bioaccumulation of antimicrobials in this matrix. Also, the results indicate the need for further research in this area by studying the behaviour of other antimicrobials used in poultry production and its presence in the feathers of treated birds.

Tetracyclines are broad-spectrum acting agents exhibiting activity against a wide range of gram-positive and gram-negative bacteria, such as Chlamydia, Mycoplasms, Rickettsiae, and protozoan parasites. The favourable antimicrobial properties of these agents and the absence of major adverse side effects have led to their extensive use in animal production.

Berendsen *et al.* (2013) studied the disposition of oxytetracycline (OTC) in feathers after poultry treatment. These researchers showed that after the withdrawal period, OTC concentrations in feathers were higher than in muscle and liver. Also, based on the analysis of individual segments of feathers from OTC-treated chicken, the authors acknowledged that the administered OTC is built into the feather rachis, concluding that the analysis of feathers is an extremely valuable tool in residue analysis of antibiotics.

However, depletion of OTC and 4-epioxytetracycline in feathers has not yet been reported. In the present study, concentrations of both analytes where quantified and depletion of OTC in feathers of broiler chicken after oral administration was studied.

Materials and Methods

Experimental Animals

In order to define the size of the experimental groups, criteria established by the European Medicines Agency guideline were applied (Approach towards harmonization of withdrawal periods EMA/CVMP7036795, 1997). Eighty male one-day-old broiler chickens (Ross 308 genetic) were housed in individual cages (25 ± 5°C, 50-60% relative humidity) with *ad libitum* access to water and non-medicated feed. Cages had an elevated wire floor in order to avoid faecal contamination of feathers. Animal welfare guidelines in Directive 2010/63/EU for the protection of animals used for scientific purposes were respected.

After 25 days of breeding, chickens were randomly allocated in two experimental groups A and B, with 64 and 16 birds respectively. Group A was treated with 50 mg kg^{-1} bodyweight (bw) of 10% OTC, orally administrated once a day for seven consecutive days, premix powder with a WDT of 7 days. Group B remained as untreated control group.

Eight treated and two non-treated birds were euthanized at 3, 5, 7, 13, 19 and 22 days post-treatment. Samples were collected, individually processed and stored at -20°C in identified plastic bags until extraction and analysis.

Reagents and standards

All reagents were of analytical grade quality, and solvents such as methanol and acetonitrile of HPLC-grade. In addition, water of HPLC-grade (Millipore or similar) was used with OTC and 4-epi-OTC were of certified standard. The internal standard (IS), deuterated tetracycline (TC-d6), was purchased from Toronto Research Chemicals (Toronto, Canada). Primary stock standard solutions of OTC, 4-epi-OTCand TC-d6 were prepared in methanol at the concentration of 500 ng mL^{-1} and 1,000 ng mL^{-1}, respectively. The working solution used for spiking blank samples was obtained by dilution in methanol to produce a 2.5 ng mL^{-1} concentration for OTC and 4-epi-OTC, and 20 ng mL^{-1} for TC-d6. All solutions were stored at -80°C.

Sample preparation

Feathers were processed as described by Berendsen *et al*. (2013). For the analysis 10 ± 0.10 g of sample were weighed in 50-mL polypropylene centrifuge tubes. Blank samples were fortified with an OTC and 4-epi-OTC solution, TC-d6 was added. To extract the analyte from the biological matrix, 20 mL acetone and 20 mL EDTA/McIlvaine were added and mixed in a tube shaker for 30 min. Suspension was sonicated for 5 min and centrifuged for 15 min at 1,800 *g*. Supernatant was then transferred to another 50-mL centrifuge tube, passing through glass wool and a Millipore filter. The filtrate was passed through a solid phase extraction column OASIS HLB®, which was prepared prior to use with 4 mL methanol, 4 mL HPLC-grade water and finally with 4 mL of EDTA/McIlvaine buffer at 1 mL min^{-1}. The column was dried using a vacuum pump for 10 min, and eluted with 3 mL methanol at a flow rate of 1 mL min^{-1}. The solvents were evaporated from the samples under a mild nitrogen flux at 40 to 50° C. The particulate material was reconstituted in 250 µL mobile phase by vortex-mixing for 5 min and then sonication for 5 min. Finally, the sample was centrifuged for 5 min at 1,800 *g*. Next, it was transferred to an Eppendorf tube and centrifuged at 17,000 g for 10 min. The supernatant was transferred to a glass vial for chromatographic analysis.

Table 1. Mobile phase gradient: 0.1% formic acid in water (Phase A) and a 0.1% formic acid in methanol (Phase B).

Time (Minute)	Mobil phase A (%)	Mobil phase B (%)	Flow rate (µL min^{-1})
0.0	85	15	200
5.0	85	15	200
5.1	60	40	200
10.0	60	40	200
10.1	10	90	200
12.0	10	90	200
12.1	95	15	200
25.0	95	15	200

LC-MS/MS Analysis

Chromatographic separation was performed using a mobile phase gradient of 0.1% formic acid in water (Phase A) and 0.1% formic acid in methanol (Phase B) as presented in Table 1. The flow-rate was 0.2 mL min^{-1}, injection volume was 25 µL and column temperature was maintained at 30°C. The MS detector was operated according to the parameters listed in Table 2, and the monitoring of ion masses according to Table 3.

Determination of withdrawal time (WDT)

In order to determine WDT for OTC and 4-epi-OTC in feathers, samples were taken on days 3, 5, 7, 13, 19 and 22 post-treatment. With the concentrations obtained for each sampling point, linear regression analysis was performed. Depletion curve was built on a semi-logarithmic scale, considering a 95% confidence level to determine the WDT for feathers. The LoD was set as cut point.

Table 2. Parameters for operation of the MS/MS detector.

Ionization	Negative ion mode with a Turbo Ion Spray ™
Scan type	MRM
Source temperature	450°C
Nebulizer	40
Turbo ion	40
Curtain gas	12
Collision gas	6
Ion spray voltage	-3,000V
Entrance potential	-10
Dwell time	250.0 ms

Table 3. Monitored mass ions.

Ions	Precursor ion	Fragment ion
OTC / 4-epi-OTC	461.0	426.0
		381.0
TC-d6 (IS)	451.0	416.0

Results

At day 3 post-treatment, high concentrations of OTC and its metabolite 4-epi-OTC were found in the feathers. The average concentration was 2.94 mg kg^{-1} and 2.66 mg kg^{-1}, respectively. At day 5 post-treatment, the analyte concentrations detected in the biological matrix, decreased by 80%, reaching an average concentration of 556 µg kg^{-1} for OTC and 492 µg kg^{-1} for 4-epi-OTC. However, at day 7 post-treatment, corresponding to the withdrawal time established for the formulation used in the experiment (OTC 10%), high average concentrations where found in feathers (451 µg kg^{-1} for OTC and 675 µg kg^{-1} for 4-epi-OTC). Concentrations remained high until day 13 post-treatment at 276 µg kg^{-1} for OTC and 227 µg kg^{-1} for 4-epi-OTC. On day 19 post treatment concentrations of the two analytes were 101 µg kg^{-1} for OTC and 90 µg kg^{-1} for 4-epi-OTC. Table 4 shows the average concentrations, standard deviation (SD) and coefficient of variation (CV)for OTC and 4-epi-OTC in feathers over the post-treatment period.

Withdrawal time for feathers was established on the basis of the statistical method stipulated on EMA Guidelines with a 95% tolerance and 95% confidence. Considering a cut point of 20 µg kg^{-1} (LoD of the analytical method) withdrawal time for OTC was set at 46 (45.76) days and 42 (41.16) days for 4-epi-OTC (Figure 1).

Discussion and Conclusions

During the depletion study, high concentrations of OTC and its metabolite were detected in feathers until 13th day post-treatment. Average levels of OTC and 4-epi-OTC in feathers where high during the whole experiment. An example of this, are the concentrations in feathers observed at day 7 post treatment which were above 450 (OTC) and 650 µg kg^{-1} (4-epi-OTC). At day 19 post-treatment, the concentrations quantified in this matrix were around 100 µg kg^{-1}. Also, depletion in feathers shows that not until day 46 post-treatment, values of the studied analytes were equal or below the LoD of 20 µg kg^{-1}.

High concentrations of OTC and 4-epi-OTC founded in feathers could be attributed to drug distribution throughout the blood stream, reaching these structures and binding to feathers structures. Furthermore, Berendsen *et al*. 2013 proved that OTC is built into the *rachis* during feather growth.

Obtained data agree with results published by other authors in this matrix. San Martín *et al*. (2007) concluded that enrofloxacin and ciprofloxacin remained in feathers at higher concentrations than edible tissues through the whole study (216 h after treatment). Cornejo *et al.* (2011 and 2012) studied depletion of fluoroquinolones in chicken edible tissues as well as their bioaccumulation in feathers, showing that concentrations of these antimicrobials accumulate at higher levels than in edible tissues, and for longer periods after therapy.

Table 4. Average and ranges of concentrations of OTC and 4-epi-OTC in feathers at the indicated post-treatment sampling days.

		OTC		4-epi-OTC	
Post-treatment (day)	Age of animals (days)	Average concentration (µg kg^{-1}) ± SD	CV (%)	Average concentration (µg kg^{-1}) ± SD	CV (%)
3	34	2,947 ± 1,474	49	2,662 ± 1,227	46
5	36	557 ± 142	25	493 ± 121	24
7	38	452 ± 375	83	675 ± 364	53
13	44	276 ± 192	69	228 ± 166	73
19	50	101 ± 72	71	90 ± 70	78
22	53	27 ± 4	14	< LoQ	–-

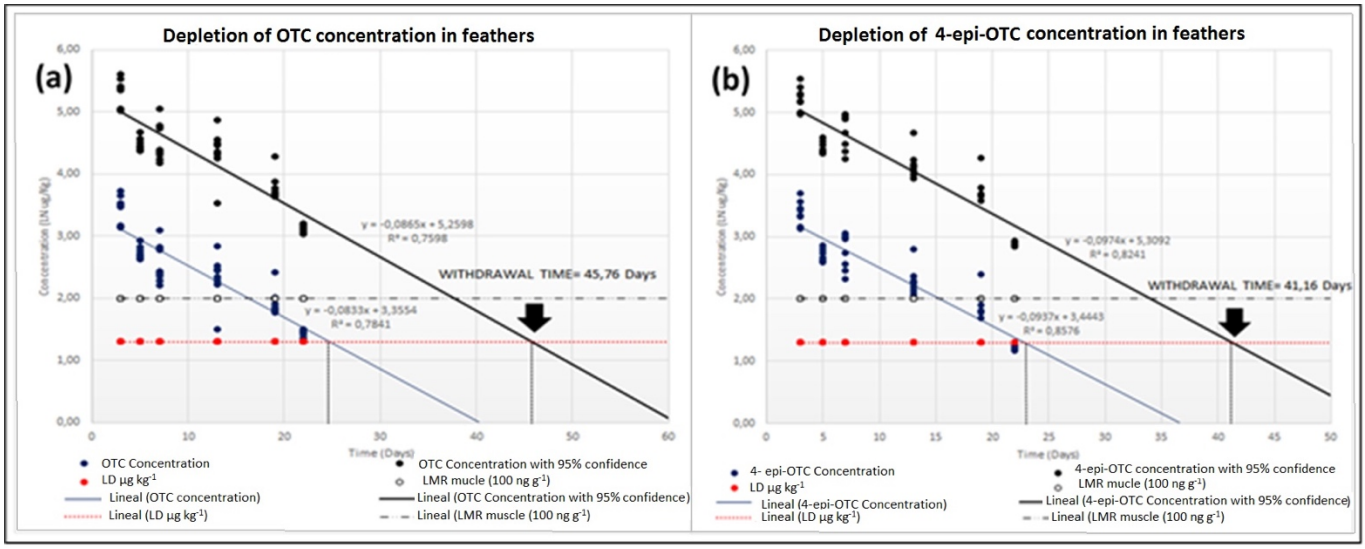

Figure 1. (a) Depletion of OTC concentrations, showing a calculated withdrawal time of 46 days in broiler feathers. (b) Depletion of 4-epi-OTC concentrations, showing a calculated withdrawal time of 42 days in broiler feathers.

Berendsen *et al.* 2013 analysed segments of feathers from chickens treated with OTC, and detected the analyte in the *rachis* of the feathers. The OTC concentrations found in feathers by these authors revealed that the drug concentrations in this non edible tissue remain higher than in edible tissues (muscle and liver). A study carried out by Love *et al.* (2012) in which commercial feather meals coming from different sources were tested, the result showed that antimicrobials were present in all samples. They found 2 to 10 different antimicrobials representing six antimicrobial families in each sample. Tetracycline family was found among the most frequent one.

According to obtained data and the pharmacokinetic properties of these antimicrobials, it can be concluded that OTC and 4-epi-OTC residues are stored and accumulated in feathers at high concentration levels, and may remain in feather meal, becoming a source of antimicrobial residues re-entrance into the food chain. Also, the result obtained enables us to reinforce the idea previously suggested by other authors, about feathers being used as a possible monitoring tissue for the detection of oxytetracycline in poultry.

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IMPLEMENTATION AND IN-HOUSE VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE DETECTION OF FLORFENICOL (FF) AND FLORFENICOL AMINE (FFA) RESIDUES IN FEATHERS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

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Abstract
In order to study the behavior of FF and its metabolite FFA in broilers feathers, validation of a suitable analytical method is needed. Antibiotic-free commercial broiler feathers were fortified with certified standards at five level concentrations (20-50-100-150-200 µg kg^{-1}). Chloramphenicol-d5 was used as internal standard. Extraction of the analytes was carried out using acetone. Dichloromethane was used for the clean-up. By LC- MS/MS analysis, a limit of detection of 20 µg kg^{-1} was stablished. Calculated limit of quantifications was 24.4 µg kg^{-1} and 24.5 µg kg^{-1} for FF and FFA, respectively. Validation parameters such as linearity, recovery and precision were calculated according to the Commission Decision 2002/657/EC. The implemented method met the acceptance criteria for all the parameters. Regarding linearity, all standard curves show a standard coefficient of more than 0.99. Recoveries ranged from 99% to 102% for all studied concentrations. Results show that the analytical method is precise and reliable for detection and quantification of FF and FFA residues in feathers. Therefore, it can be used for detection of these analytes in further research in this matrix.

Introduction
Antibiotics are the main therapeutic tool for the treatment of bacterial infectious diseases in humans, as well as in animals, with the aim of treating bacterial diseases and improving the efficiency of the productive systems. Nevertheless, the use of antibiotics is not free from risks, since the persistence of its residues in products of animal origin. Some of the adverse effects are direct toxic effects, immunologic effects (allergic reactions), mutagenesis, carcinogenicity, and teratogenicity. Residues may contribute to a persistent selection pressure for resistant bacteria (Martínez and Baquero, 2002; Anadón and Martínez-Larrañaga, 2012).

Regarding edible tissues, different studies prove that antimicrobial residues are able to remain for variable periods of time in edible tissues. However, in regards to by-products such as feathers there are no regulations on antimicrobial residues. There is evidence from published studies showing that antimicrobials accumulate in broiler feathers at higher levels and for longer periods than in edible tissue. San Martín *et al.* (2007) found high concentrations of enrofloxacin and its metabolite, ciprofloxacin in feathers from animals treated with these antimicrobials when compared to edible tissues (muscle, liver and kidney). Similarly, Cornejo *et al.* (2011) found higher concentrations of flumequine in feathers when compared to liver and muscle samples. Cornejo *et al.* (2012) show that enrofloxacin and its metabolite ciprofloxacin were transferred to the birds' feathers and that the concentrations of these antimicrobials remained for a longer period and in higher concentrations than in edible tissues.

Considering that they are used in the production of feather meal for feeding other animal species, such as swine, cattle and fish, the carry-over of drug residues in feathers is then a way of re-entry for residues into the food chain (Meeker and Hamilton, 2006; Divakala *et al.,* 2009).

Among the drugs approved for their use in poultry is florfenicol, an antimicrobial used in poultry production due to its effectiveness on several bacteria such as: *Enterobacter cloacae*, *Shigella dysenteriae*, *Salmonella paratyphi*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pasteurella multocida*, *Proteus vulgaris* and *Escherichia coli* (Park *et al.,* 2007). It is considered an effective tool for the treatment of in particular respiratory infectious diseases.

Thus, it is necessary to study the transfer, bioaccumulation and depletion of antibiotics which have not been studied before, as florfenicol. In order to carry out research, a validated analytical method capable of determining florfenicol and florfenicol amine in the feather matrix is needed.

Materials and Methods

Implementation of analytical methodology
For the implementation of an analytical method for the detection of florfenicol and florfenicol amine in feathers, methods published by Hormazabal *et al.* (1993), Li *et al*. (2006), Zhang *et al*. (2008) and the United States Department of Agriculture (USDA) (2010) were used as a reference.

Sample collection and processing
Samples were obtained from feathers from commercial broilers. These were analysed by LC/MS-MS to confirm the absence of florfenicol and its metabolite. During implementation, as well as in validation of the analytical method, samples received a cryogenic treatment with liquid nitrogen, being afterwards treated in a food processor (Robot Coupe R4).

FF and FFA standards
For the analysis and quantification of FF and FFA in feathers, standards of certified purity from Sigma Aldrich were used. As internal standard (IS) chloramphenicol d-5 (CAF-d5, Sigma Aldrich) was used. All solvents used were High-Performance Liquid Chromatography (HPLC) grade.

Analyte extraction process from the biological matrix
For FF and FFA residue extraction from feathers, a 2.00 ± 0.02 g sample was weighed in a 50-mL polypropylene tube. Subsequently, samples were fortified with certified standards of FF, FFA and CAF-d5. As extraction solvents, 20 mL water and acetone were used. Samples were agitated, sonicated and centrifuged. Supernatant was transferred to another polypropylene tube and 15 mL dichloromethane was added. Then, samples were agitated and centrifuged. The inferior phase, was collected and evaporated under a mild nitrogen flow between 40-50°C. Samples were reconstituted in 700 µL methanol: water (7:3).

Instrumental analysis
For instrumental analysis an LC (Agilent, 1290 infinity series) coupled to a triple quadrupole mass spectrometer (API 5500, ABSCIEX) was used. An analytic column Synergi 4u fusion RP 30ª 50 x 2.0 mm was also used. For equipment management Analyst 1.6.3 software was used, and for integration a Multiquant 3.0 software. Chromatographic separation was performed through a mobile phase A: 0.1% of acetic acid in water; and a mobile phase B: 0.1% of acetic acid in water/methanol 1:9; with a gradient flow of 300 µL min^{-1} of 25% phase A and 75% phase B (Table 1). Injection volume was 2 µL and the column oven temperature was set at 37°C.

Table 1. Mobile phase A and B flow gradient

Minute	Flow gradient µL min^{-1}	Mobile phase A	Mobile phase B
0.0	300	25%	75%
2.5	300	25%	75%

Parameters for validation of the analytical methodology
Validation of the analytical method was carried out according to an internal protocol based on the European Union recommendations (Commission Decision 2002/657/EC). The parameters evaluated were: Analyte retention time, Specificity, Linearity of calibration curve (CC), Recovery and Precision (Repeatability and Intralaboratory reproducibility). Limit of Detection (LoD) and Limit of Quantification (LoQ) were calculated according the FDA (Food and Drug Administration) VICH GL49 validation of analytical methods used in residue depletion studies.

Table 2. Parent ion mass, product an average retention times for FF, FFA and CAF-d5

Analyte	Parent ion mass	Product ion mass	Average retention time (min)
FF	356	336	1.46
		185	1.46
FFA	248	230	1.07
		130	1.08
CAF-d5	326	157	1.47

Results
Specific ion masses and retention times for FF and FFA from six pure drug analyses are shown inTable2.

Validation of analytical methodology

Analyte retention time (RT). For determination of RT, CV (%) of the six pure standard injections were analysed. Average RT and coefficient of variation (CV %) are shown in Table 3.

Table 3. Average and CV of retention times from the 6 injections of pure FF and FFA standard.

Analyte	Average retention time	CV (%)
FF (356.0/336.0)	1.46	2.8
FFA (248.0/230.0)	1.07	0.70

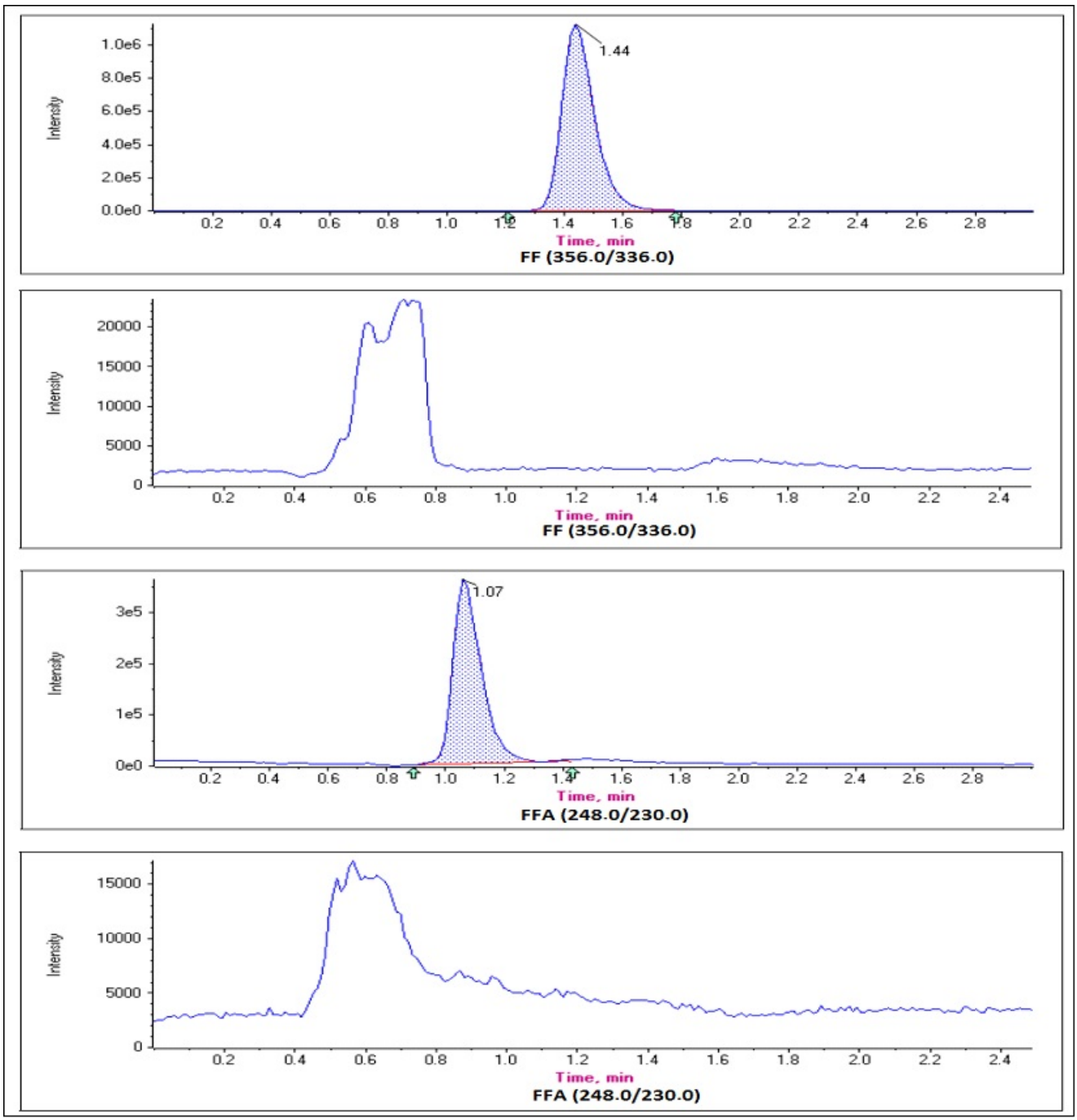

Figure 1. Florfenicol and florfenicol amine standard injection chromatogram versus blank feather sample chromatogram.

Specificity. The analysis of samples showed interferences at analytes RT. Figure 1 shows chromatograms of the injection of pure drug, and of FF and FFA residue-free feathers samples.

Limit of detection (LoD). The LoD was set at 20 µg kg^{-1} at which the signal-to-noise ratio was higher than 3:1. With the aim of validating the parameter, 20 repetitions of matrix fortified at this concentration level were carried out. Average concentrations, standard deviation and CV of the 20 repetitions were determined considering a variation lower than the 25%. Results obtained from the 20 repetitions analysis are summarised in Table 4.

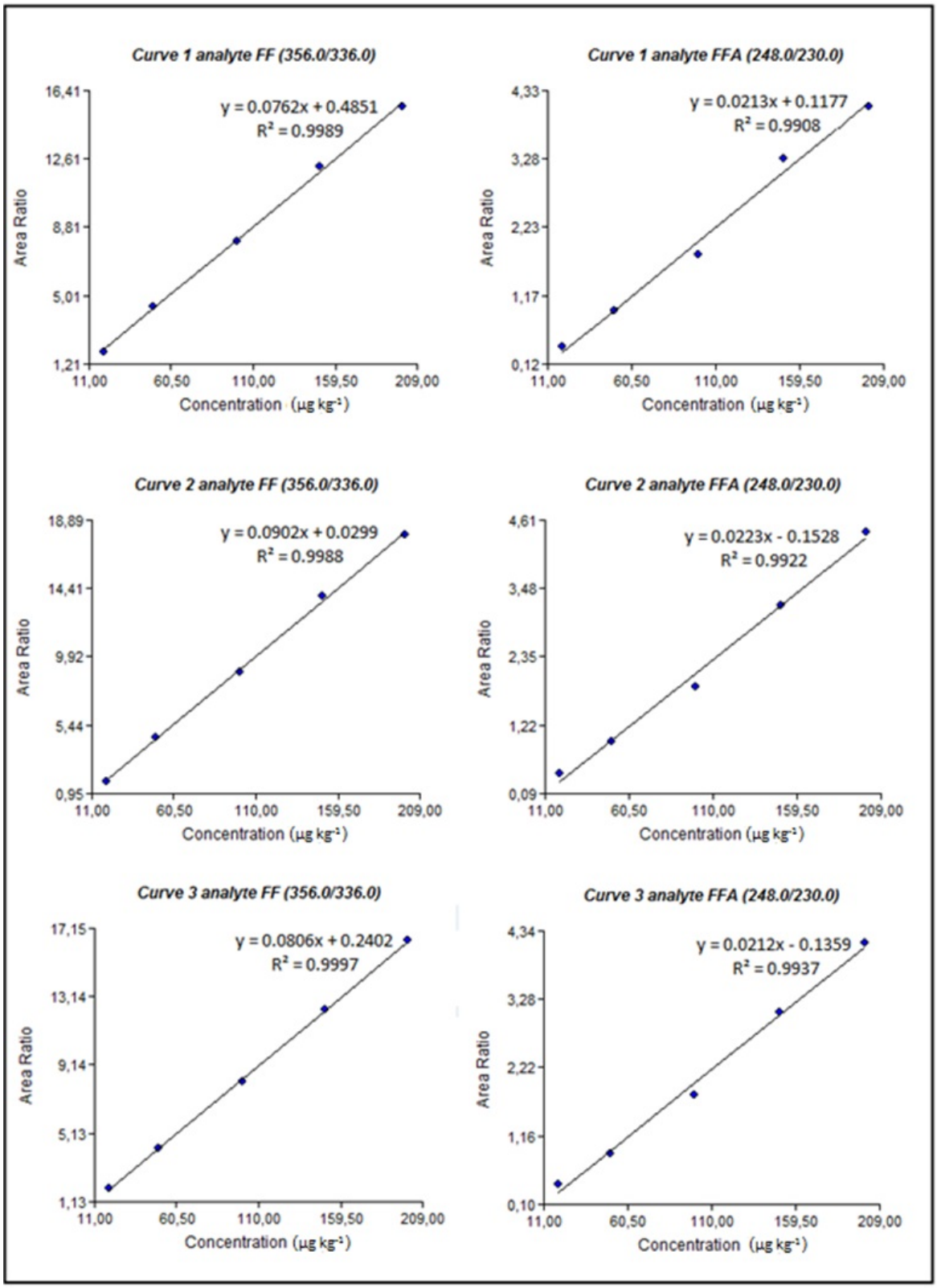

Figure 2. Calibration curves of florfenicol and florfenicol amine at five fortification levels (20, 50, 100, 150 and 200 µg kg^{-1}) in feathers.

Table 4. Average concentration, standard deviation (SD) and CV (%) for FF and FFA residues fortified at 20 µg kg^{-1} (LoD) in 20 repetitive feather samples.

Analyte	Concentration Average	SD	CV (%)
FF (356.0/336.0)	17.6 µg kg^{-1}	2.68	15.2
FFA (248.0/230.0)	19.7 µg kg^{-1}	2.72	13.8

Table 5. Linearity, recovery, repeatability, reproducibility, LoD and LoQ of analytes FF and FFA in feathers.

Parameter	Linearity	Recovery	Reproducibility to LoD	Repeatibility to LoD	LoD	LoQ
Criteria	$R^2 \geq 0.95$ / CV < 25%	80 – 110%	CV < 35%	CV < Reproducibility	-	-
FF (356.0/336.0)	$R^2 > 0.99$ / CV=0.04%	100%	19.4%	10.1%	20 µg kg^{-1}	24.4 µg kg^{-1}
FFA (248.0/230.0)	$R^2 > 0.99$ / CV=0.14%	102%	20.8%	15.6%	20 µg kg^{-1}	24.5 µg kg^{-1}

Table 6. Average recoveries, standard deviations and CVs (%) for FF and FFA residues spiked at working concentrations 20, 100 and 200 µg kg^{-1} in the feather matrix,.

Parameter	Working concentration (µg kg^{-1})	Recovery average (%)	Standard deviation	CV (%)
FF (356.0/336.0)	20	100	0.10	10
	100	100	0.04	4
	200	100	0.01	1
FFA (248.0/230.0)	20	102	0.16	16
	100	99	0.06	6
	200	100	0.01	1

Limit of Quantification (LoQ). The LoQ was calculated by adding 1.64 times the standard deviation of the quantified concentrations in the 20 analysed repetitions of fortified matrix to the LoD. The LoQ was accepted since signal-to-noise ratio was higher than 10:1. An LoQ of 24.4 µg kg^{-1} was determined for FF and 24.5 µg kg^{-1} for FFA in feathers (Table 5).

Linearity of calibration curves (CC). Three calibration curves were fortified at five concentration levels (20, 50, 100, 150 and 200 µg kg^{-1}) with the aim to determine the linearity of the method. Linearity of all calibration curves was accepted when R^2 was higher than 0.96 (Figure 2), meeting the acceptance criteria for R^2. The CV of the calibration curves was lower than 25% of variation (Table 5).

Recovery. The recovery was calculated with blank samples fortified at three levels: 20, 100 and 200 µg kg^{-1}. Analytes FF and FFA showed a recovery of 100% and 102% at 20 µg kg^{-1} with a CV of 10% and 16%, respectively, in feathers. Recoveries, standard deviation and CV (%) for both analytes are listed in Table 6.

Table 7. Average of quantified concentrations, SD, repeatability CV (%) and intralaboratory reproducibility according to fortification concentrations 20, 100 and 200 µg kg^{-1} in feathers.

		Repeatibility			Reproducibility		
Parameter	fortified concentration	Quantified concentration average	SD	CV (%)	Quantified concentration average	SD	CV (%)
FF (356.0/336.0)	20 µg kg^{-1}	20.0 µg kg^{-1}	2.02	10.11	17.5 µg kg^{-1}	3.38	19.36
	100 µg kg^{-1}	100.0 µg kg^{-1}	3.64	3.64	104.6 µg kg^{-1}	6.08	5.82
	200 µg kg^{-1}	200.0 µg kg^{-1}	1.63	0.82	198.0 µg kg^{-1}	2.69	1.36
FFA (248.0/230.0)	20 µg kg^{-1}	20.5 µg kg^{-1}	3.19	15.58	23.3 µg kg^{-1}	4.85	20.84
	100 µg kg^{-1}	99.1 µg kg^{-1}	5.75	5.80	94.1 µg kg^{-1}	8.72	9.27
	200 µg kg^{-1}	200.4 µg kg^{-1}	2.57	1.28	202.6 µg kg^{-1}	3.87	1.91

Precision. Precision was evaluated through repeatability analysis and intralaboratory reproducibility. For repeatability, six curves were performed in one day, by a single analyst, with the same batch of reagents/solvents and analysed during the same day by a liquid chromatographer coupled to a mass detector. Curves were fortified at three concentration levels: 20, 100 y 200 µg kg^{-1}. Average, SD and CV were calculated for each fortification level. Results were compared to the results from the six curves performed for intralaboratory reproducibility (Table 7) at the same fortification levels, but carried out by two analysts on different days with different reagents/solvents batches. Intralaboratory reproducibility CVs were lower than 35% for both analytes. The repeatability CVs were lower than the intralaboratory reproducibility CVs.

Discussion and Conclusions

An in-house protocol for the validation of the analytical method was based on Commission Decision 2002/657/EC with the aim of proving that the method was appropriate for its use in feathers. Moreover, from the tests carried out with fortified matrix, a limit of detection was established at 20 µg kg^{-1} with a signal-to-noise ratio higher than 3:1. The CV of the LoD with 20 repetitions was less than 25%. In this way, data are considered to be statistically homogeneous, and thus, the LoD is accepted, as well as the calculated LoQ.

Results for all parameters meet the acceptance criteria, according to the Commission Decision 2002/657/EC, proving that the method is linear, reproducible and repetitive, and therefore, precise and quantitative.

The importance of this study relies in the fact that through implementation and validation of the analytical methodologies it is possible to detect these antimicrobials in feathers in accurate way. This will allow to carry out further studies on this matrix. Different studies on these drugs were performed in several products of the poultry industry. For example, Zhang *et al.* (2008) determined FF and FFA in broiler muscle, whereas Fizali *et al.* (2014) detected the analytes in eggs. However, there are no studies in non-edible tissues as feathers.

It is calculated that around 37% of the life weight of chickens is not directly consumed by human beings, becoming a source of material for feed (Meeker and Hamilton, 2006; Divakala *et al.,* 2009). Feathers, then, have become an important way of a re-entry in the food chain. It is a risk for public health, mainly for the development of antimicrobial resistance. According to a 2014 WHO report, besides being a serious threat for public health, a cost of at least €1,500 million and 25,000 deaths are estimated each year in the European Union (European Centre for Disease Prevention and Control, 2011).

Considering the published scientific evidence and the importance for public health, further investigation on the subject is needed, regarding the re-entry way of residues of other antimicrobial families into the food chain. Moreover, it is highly important to study bioaccumulation of florfenicol and its main active metabolite in feathers, and its relation with other tissues' concentrations.

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ENROFLOXACIN AND CIPROFLOXACIN RESIDUES IN BROILER CHICKEN FEATHERS AFTER ORAL ADMINISTRATION

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Abstract

Our aim was to study the residue depletion of enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP) in broiler chicken feathers. Broiler chickens were treated through medicated drinking water with 10 mg ENR / kg / day for five consecutive days. Feather samples were taken daily from ten random birds until nine days. Extraction was performed by a liquid/liquid technique. ENR and CIP concentrations were determined in feather samples by a validated liquid chromatography with fluorescence detection.

High levels of ENR and CIP were found in feathers after oral administration and these compounds were detected in feathers for 9 days. Feather meal is a potential source of drug residues that can pass through the food chain when contaminated meal is fed to food-producing animals. This finding cannot be explained by blood distribution to this tissue because feather vasculature reaches only the lower part of the calamus. One possible source for the feather contamination is secretion through the uropygial gland, which may reach the feathers via grooming behaviour.

Introduction

Antibiotics in animal feed are a public health concern. Drug residues could eventually be detected in animal food products intended for human consumption (Sapkota *et al*. 2007). For decades, antibiotics have been added to livestock feeds in low doses to serve as growth-promoters (Love *et al*. 2012). Antibiotics have recently been shown to accumulate in poultry feathers, which is significant because poultry feathers serve as a high protein ingredient in animal feed, such as poultry feed (Love *et al*., 2012).

The continued use of antibiotics as feed additives has inadvertently created antibiotic-resistant micro-organisms, which causes human health concerns. Antimicrobials used in poultry production have the potential to bioaccumulate in poultry feathers but available pharmacokinetics and tissue depletion studies are very scarce (Love *et al*. 2012). Following poultry slaughter, feathers are converted in feather meal (Nachman *et al*. 2012) and sold as fertilizer (Hadas and Kautsky, 1994). Feather meal is often incorporated as a protein source into the diets of other food animals, such as cattle, swine, rainbow trout, shrimp and salmon (Cheng *et al*., 2002; Divakala *et al*., 2009), thereby providing a potential pathway for re-entry of antimicrobials into the human food supply chain.

Considering the pharmacokinetic characteristics of enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP) (Otero *et al*. 2009; Mestorino *et al*. 2009, 2011; Dimitrova *et al*. 2006), drug accumulation in non-edible tissues such as feathers is highly probable. Although feathers after been processed, are introduced in the food chain as a protein source in animal feed, withdrawal periods are not established yet. Hence, the present study has been conducted to evaluate the residue profile of enrofloxacin and its primary metabolite ciprofloxacin in feather of broiler chicken, after ENR (Carval®) administration at 10 mg/kg/day for 5 consecutive days with drinking water.

Materials and Methods

Study Design Treatment and Administration

The experiment was conducted with 90 three week-old chickens. The birds were randomly divided into control (n = 20) and treatment group (n = 70). Treatment group was administered with enrofloxacin at recommended therapeutic dose 10 mg kg^{-1} in drinking water for five consecutive days (0, 1, 2, 3 and 4 days), whereas control group received non-medicated water. The birds were kept in a special space designed for performing experiments on animals. Prior to treatment, chickens were deprived of water. Antibiotic-free food was available *ad libitum*. The chickens treated with enrofloxacin were euthanized 1d, 2d, 3d, 5d, 7d and 9d after final drug administration (ten animals at each time point) and feather samples were collected, washed and stored at -20°C until sample preparation and chromatographic analysis. Control chickens were sacrificed on day 9 post treatment.

The protocol was according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS).

Reagents
Enrofloxacin (ENR) and ciprofloxacin (CIP) standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, trifluoroacetic, triethanolamine and acetone were of HPLC grade and purchased from JT Baker (Phillipsburg, NJ, USA). All other reagents were of analytical reagent grade.

Enrofloxacin and ciprofloxacin analysis
Enrofloxacin and ciprofloxacin feathers analysis was performed by high-performance liquid chromatography (HPLC) with fluorescence detection.

Extraction was performed following a method described by San Martín *et al*. (2007) and modified by us (SOP: ENR/CIP-VMA-04/02). Briefly, feather samples were thinly triturated, placed in centrifuge tubes and 5 mL of acetone was added. Samples were shaken and centrifuged at 2,000 *g* for 10 min at 4°C. The clear supernatant was transferred into drying tubes. The extraction procedure was repeated two times. Supernatants were combined (S1 + S2 + S3) and evaporated to dryness in a vacuum concentrator (AVC 2-25CD Christ, Germany) at 40°C. Residues were dissolved in 75 µL methanol 0.1% tifluoracetic. After 475 µL aqueous 0.1% trifluoroacetic were added. The total solution was vortexed for 2 min and then filtered. Finally, 100 µL of clear solution was injected into the chromatographic system.

Standard curve
Standards were prepared by adding 0.1, 0.25, 0.5, 1.0 and 2.0 µg mL^{-1} of ENR-CIP to test-tubes, evaporating to dryness at 60°C and dissolving as described above.

Apparatus
The chromatographic system consisted of an isocratic pump (Gilson Inc. 307), an automatic injector (Gilson Inc. 234), a FluoroMonitor IM III Detector (excitation 278 nm and emission 446 nm) (Sp Thermo Separation products) and Eppendorf CH-30 Column Heater (set at 30°C). The system is controlled through the Unipoint ® Gilson system. An C_{18} column (Luca, 5 µm, 4.6 mm x 150 mm; Phenomenex, Torrance, CA, USA) was eluted with a mixture of water : acetonitrile : triethanolamine (80:19:1) pH 3, at a flow rate of 1.2 mL min^{-1}. Identification of ENR-CIP in bird feathers was accomplished by comparison with the retention times of the reference standards.

Method validation
The validation procedure was performed following Commission Decision 2002/657/CE of the EU (2002). The following parameters were evaluated for the analysis of ENR and CIP in feathers: linearity (concentrations ranging between 0.1 and 2.0 µg mL^{-1} or µg g^{-1}), precision and accuracy, limit of quantitation (LOQ), limit of detection (LOD) and selectivity. Samples from untreated animals (blank feathers) were analysed to confirm ENR and CIP absence and the specificity of the analytical method. The LOD was estimated through the analysis of ten aliquots of control feathers (free of ENR-CIP). The noise of the base-line was measured; the average and the standard deviation were calculated, the LOD corresponds to three of those SD (sign/noise ≥ 3/1). The LOQ is defined as the level where the reproducibility of the replicate analysis does not exceed a variation coefficient of 20% and the accuracy is from 85-115% after the analysis of twelve replicates of fortified sample matrix with the smallest concentration.

Results
The development and validation were successfully accomplished. This method performed accurately and reproducibly over a range of 0.1 to 2.0 µg mL^{-1} for ENR-CIP.

Precision of the system
One standard solution containing 1 µg mL^{-1} ENR and 1 µg mL^{-1} CIP was prepared and the precision of the system was evaluated after the placement of twenty (20) injections in the chromatographic system. In this manner the efficiency of the column and of the system were evaluated. After twenty injections a coefficient of variation (CV) of 6.8% and 13.0% for ENR and CIP, respectively, were determined.

Assay linearity
This assay exhibited a linear dynamic range between 0.1 and 2 µg mL^{-1}. A linear relationship was obtained with r values ranging from 0.9974 to 0.9984 and 0.9974 to 0.9986 for ENR and CIP, respectively (Figures 1A and 1B).

Specificity
Six different samples from control feathers (free of ENR-CIP) and six feather samples fortified with ENR and CIP were analysed by HPLC and the corresponding chromatograms were compared. No matrix interferences were observed in the chromatograms of the samples with the same retention time as ENR-CIP (Figure 2). The chromatographic analysis time was short; ENR and CIP eluted at 3.4 and 2.8 min, respectively, as sharp and symmetrical peaks without interferences.

Limits of detection (LOD) and quantitation (LOQ)

The LODs were 0.040 and 0.062 µg g^{-1}; and LOQs were 0.050 and 0.080 µg g^{-1} for CIP and ENR, respectively, in chicken feathers.

Intra-day and inter-day accuracy and precision

The method for the analysis of feather samples was thoroughly validated and the results are presented in Table 1. To assess the inter-day (over 3 days) assay accuracy and precision, six sets of feather samples were prepared containing ENR and CIP at 0.1, 0.25 and 2.0 µg g^{-1}. The inter-day variation in accuracy (recovery) and precision were assessed. The mean accuracy (recovery) should be within the range 85-115 % and the variation in precision should be ≤ 20%.

To determine the intra-day accuracy and precision, six replicates of each three concentrations were analysed along with duplicate standard calibration curves prepared from 2 separate stock solutions (Table 1).

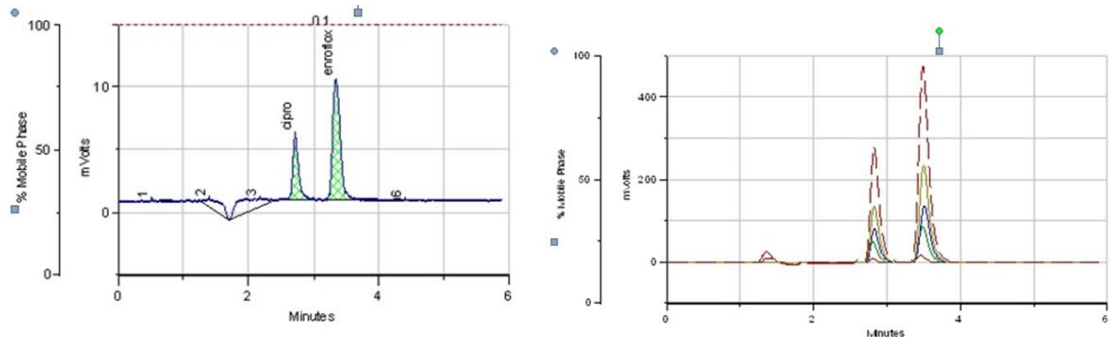

Figure 1. A) HPLC Chromatograms of ENR and CIP 0.1 µg mL^{-1} standard solution (left panel); B: HPLC Chromatograms of ENR and CIP standards calibration curve at 0.1, 0.25, 0.5, 1.0 and 2.0 µg mL^{-1} (right panel).

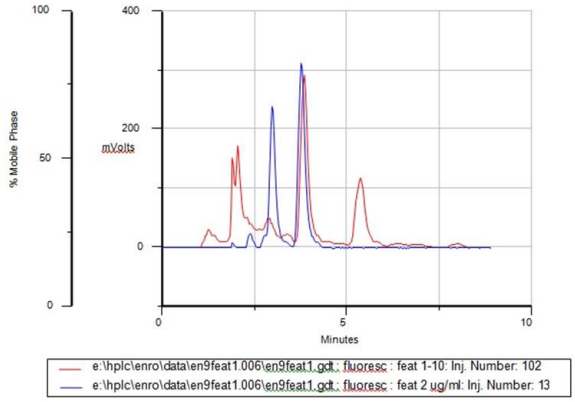

Figure 2. HPLC Chromatograms of feather spiked with ENR and CIP at 2 µg mL^{-1} and problem feather (1-10 = 1 day post treatment / animal N°10).

Table 1. ENR and CIP recovery from feather samples obtained from chickens treated with 10 mg kg^{-1} enrofloxacin in drinking water.

			Intra-day		Inter-day (over 3 days)	
Compound	r	µg g^{-1}	Accuracy (%), n=6	Precision (%), n=6	Accuracy (%)	Precision (%)
ENR	0.9995	0.1	110	4.8	100	10.0
	(0.1-2.0 µg g^{-1})	0.25	96	3.4	95	2.4
		2.0	98	5.6	97	0.59
CIP	0.9986	0.1	110	5.3	100	10.0
	(0.1-2.0 µg g^{-1})	0.25	96	5.5	93	2.5
		2.0	100	3.9	97	3.0

ENR and CIP feather concentrations

High levels of ENR and CIP were found in feathers after ENR oral administration (10 mg kg-1) for consecutive 5 days through medicated drinking water. Both compounds persisted throughout the study period. Mean values of ENR, CIP and ENR + CIP are shown in Figure 3.

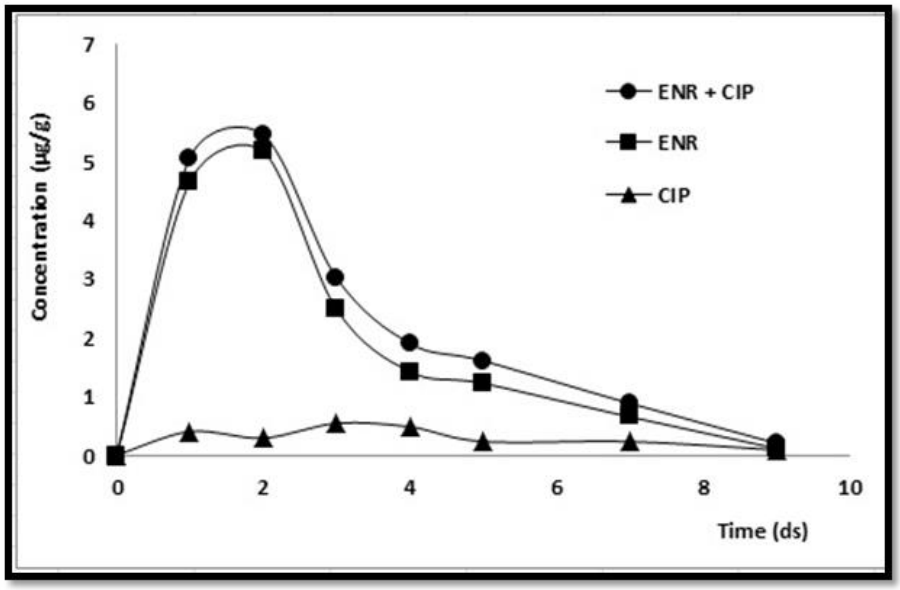

Figure 3. Mean feather concentrations of ENR, CIP and ENR + CIP of chickens after ending the enrofloxacin (Carval®) administration by oral route with drinking water for five consecutive days.

Discussion and conclusions

The analytical method developed to determine ENR and CIP in broiler chicken feathers demonstrated linearity, precision and accuracy under the analytical conditions. The method included acetone extraction and quantitative analysis by liquid-chromatography with a fluorescent detector. This method might have important applications in residues studies of ENR and CIP in feathers.

Feather meal is a potential source of drug residues that can pass to the the food chain when contaminated meal is fed to food-producing animals. In the present study, feathers had high ENR and CIP concentrations, coincident with results from other authors who analysed different compounds in chicken feathers (Malucelli *et al*. 1994). Although feathers are processed and introduced in the food chain as a protein source in animal feed, because poultry feather constitutes the most abundant keratinous material in nature, withdrawal periods are not established yet.

Our results correspond with those reported by San Martín *et al*. (2007) and Love *et al*. (2012). The characteristics of enrofloxacin include good absorption after parenteral and oral applications, large volume of distribution, suggesting wide tissue penetration, including peripheral tissues (feathers) as we observed in our study, and a long terminal half-life (Mestorino *et al*. 2009, 2011; Otero *et al*. 2009).

The high concentrations found in the feathers cannot be explained by blood distribution to this tissue because feather vasculature reaches only the lower portion of the calamus. One possible source of feather contamination is secretion from the uropygial gland, which may reach the feathers via grooming behaviour (San Martín *et al*. 2007, López-Cadenas *et al.* 2013). However, such high concentrations of ENR and CIP found in feathers cannot be attributed only to external contamination of feathers. Feather generation and molting can play an important role in drug disposition kinetics in feathers of treated animals. As the birds grow from chicks to adult birds, they undergo a series of molts, in which four generations of feathers develop and grow from the same follicle. All these follicles are formed during embryo development; once the bird has hatched, the follicle number is fixed. Both the follicle and the emerging feathers are derived from the epidermis of the skin (Kumar *et al*. 2014). The slow elimination of enrofloxacin residues from feathers could be explained by the reabsorption of the vascularized pulp that fills the shaft of the feather throughout the maturation process. This process is discontinued and terminates in a pulp cap, in which the drug can be retained.

Further studies to establish a withdrawal time may be useful to avoid that drug residues could result in adverse health consequences like increasing antibiotic resistance.

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QUECHERS METHOD FOR SIMULTANEOUS DETERMINATION OF VETERINARY DRUGS AND PESTICIDES ANAYLSIS IN MILK BY LC-MS/MS

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Abstract
A rapid and simple multi-class multi-residue method based on liquid-chromatography coupled to tandem mass-spectrometry (LC–MS/MS) was developed to investigate a total of 25 veterinary drug and 187 pesticide residues in milk. Sample preparation was a simple procedure based on liquid–liquid extraction with ethyl acetate containing 0.1% acetic acid, followed by centrifugation and evaporation of the supernatant. The residue was dissolved in ethyl acetate with 0.1% acetic acid and centrifuged prior to LC–MS/MS analysis. Chromatographic separation of analytes was performed on an Inertsil X-Terra C18 column with acetic acid in methanol and water gradient. Repeatability and reproducibility were in the range of 2 to 13% and 6 to 16%, respectively. The average recoveries ranged from 75 to 120%. The developed method was validated according to the criteria set in Commission Decision 2002/657/EC. The validated methodology represents a fast and cheap alternative for the simultaneous analysis of veterinary drugs and pesticide residues and it can easily be extended to other compounds and matrices.

Introduction
Veterinary drugs are used for treatment and prevention of diseases, and for improvement of growth and feed efficiency as well. Incorrect use of these drugs may cause accumulation of such drugs in the animal body and by intake also in humans. Therefore, they should be checked to protect the agricultural environment and food industry. Since there are many veterinary drugs to be monitored, development of methods enabling the analysis of a wide range of drugs representing multiple classes in a single procedure is important.

In this study, milk samples were prepared by a simple and quick procedure using a single liquid–liquid extraction step (Kaufmann 2014; Souza 2016). The reduced amount of chemicals and steps in the sample preparation phase together with the avoidance of further sample clean-up, simplified sample pre-treatment and provided high recoveries of compounds of various polarities. The proposed method reduced total laboratory costs of analysis. Using the method, milk samples were analysed for veterinary drug and pesticide residues simultaneously (Zhang 2015; Mol 2008) using liquid-chromatography tandem mass-spectrometry (LC–MS/MS).

Materials and Methods

Reagents and chemicals
HPLC grade acetonitrile (ACN), methanol, ethyl acetate (Lichrosolve purity ≥99.9) and acetic acid (Emprove, 100%) were purchased from Merck (Darmstadt, Germany). The water used to prepare the solutions was purified in a Milli-Q Plus system (EMD Millipore, Billerica, MA, USA). Magnesium sulphate, sodium chloride, SupelcleanTM Primary secondary amine (PSA), pure tetracylines, sulfonamides, quinolones, macrolides and antibiotics were provided from Sigma-Aldrich (St. Louis, MO, USA) and the pesticides were provided from Dr. Ehrenstrorfer (Ausburg, Germany).

Sample preparation
Upon arrival at our laboratory, milk samples were kept at 10 ± 4°C until analysis. Extraction of analytes was carried out using ethyl acetate extraction without salting procedure. For the preparation, an aliquot of approximately 5 mL milk sample was pipetted in a 50-mL polypropylene centrifuge tube. Then, 200 µL acetic acid was added to 10 mL ethyl acetate. After vortex for 3 min, the mixture was centrifuged at 5,000 rpm for 10 min. The upper phase was transferred into a 15-mL centrifuge tube and dried under a gentle stream of nitrogen. The residue was reconstituted in 1,000 µL of mobile phase A/ Mobile Phase B (80/20). The sample was vortexed vigorously for 10 min. The extract was filtered through a 0.45 µm filter prior to LC-MS/MS analysis.

Results and Discussion

Validation Study

The selectivity of the method was assessed by duplicate analysis of seven blank milk samples. No peaks of interfering compounds were observed within the intervals of the retention time of the analytes in any of these samples. Linearity was evaluated from the calibration curves by triplicate analyses of blank milk samples fortified with the analytes at five concentration levels (0.0, 0.01, 0.025, 0.05, 0.1, 0.2 mg kg^{-1}). Linearity was expressed as the coefficient of linear correlation (r) and from the slope of the calibration curve. The linearity of the analytical response across the studied range was excellent, with correlation coefficients higher than 0.997 for all analytes. Figure 1 shows very satisfactory S/N ratios for all analytes at LOQ level.

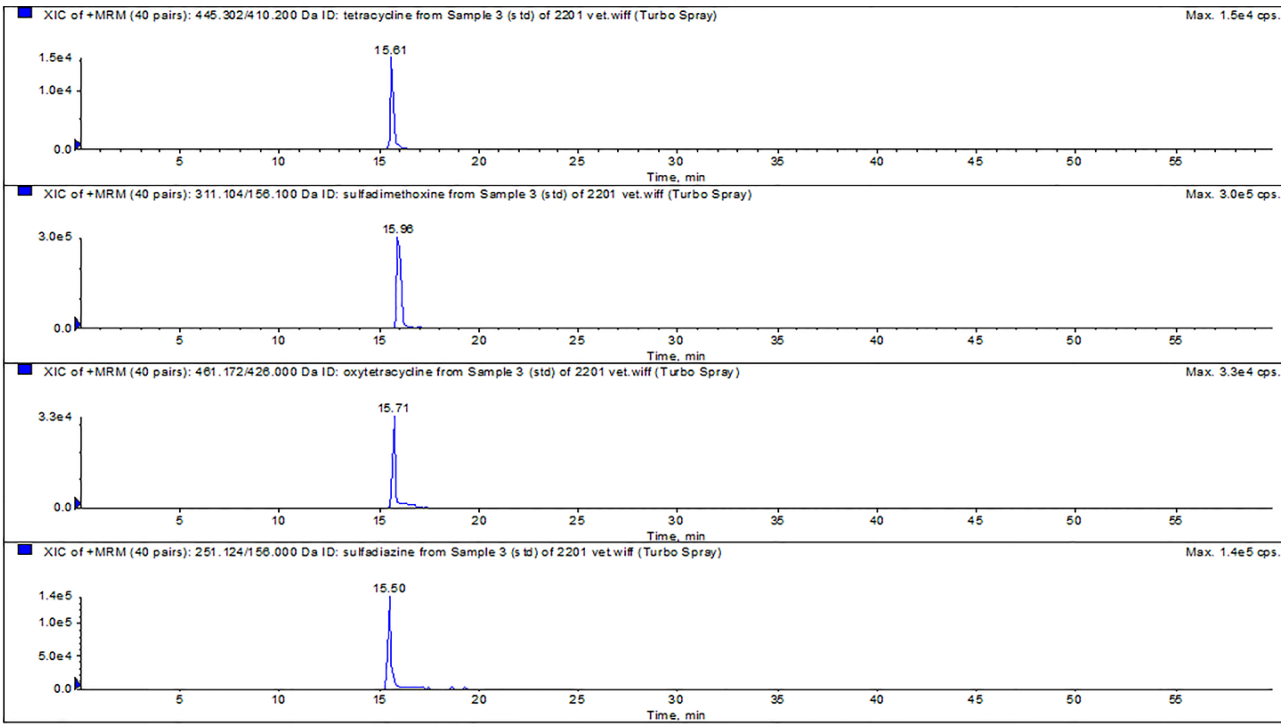

Figure 1. MRM chromatograms of milk samples at the LOQ level of tetracyclines, sulfadimethoxine, oxytetracycline and sulfadiazine (10 µg kg^{-1}).

Conclusions

A multi-class multi-residue procedure with LC-MS/MS detection has been developed and validated to determine and quantify veterinary and pesticide residues in milk. A simple sample preparation method was used and involved liquid-extraction salting-out procedures in an ethyl acetate system, without clean-up steps, and shortening the sample preparation time. Validation of the method was performed according to Commission Decision 2002/657/EC. The method was characterized by good results in terms of recovery, reproducibility and repeatability allowing the detection of veterinary drug and pesticide residues below the recommended analytical level. Based on these results, LC-MS/MS method with ethyl acetate extraction showed the suitability for sensitive quantification of veterinary and pesticide residues in milk samples for food safety applications. The validated method was applied to 220 real commercial samples.

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DYE RESIDUES IN AQUACULTURE PRODUCTS: IMPLEMENTATION OF TARGETED AND NON-TARGETED APPROACHES

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Abstract
Chemotherapy has been applied in aquaculture for the last decades and is of growing concern because of current safety worries: environmental contaminants, emergence of resistance to antibiotics, consumer demand of healthy foods etc. Among these chemicals, pharmacologically-active dyes like the well-known malachite green can be administered because they are cheap and they hold interesting antiseptic and antibacterial activities. But treatment based on malachite green and other related dyes is now prohibited due to toxicity concerns. Then appropriate analytical methods are necessary to control their absence in aquaculture products. The aim of the study was to initiate an exhaustive strategy of control by implementing both targeted and non-targeted approaches. A LC-MS/MS method was developed and validated to target the analysis of fourteen residues belonging to different families of dyes. An oxidative step was integrated in order to recover the parent forms for dyes which are supposed to metabolize in reduced forms after administration to fish. The objective of the non-targeted approach was to investigate the potential presence of biomarkers after treatment of farmed fish and selecting two specific dyes namely malachite green and victoria pure blue BO. The comparison of the metabolic fingerprints of these structurally similar dyes could allow finding appropriate biomarker(s) fit for tracking new illegal practices in aquaculture.

Introduction
Chemical contaminations are regulatory monitored on the French national territory, in particular for residues of veterinary drugs in food of animal origin in the context of the European Union Food Law. Since the last fifty years, the strong global demand for fish for human consumption led to significant growth in aquaculture production. According to the FAO (2012), a consequence of the ever growing world population, will be an increased and intensive food production to meet world market needs. The food-producing animal farming is thus likely to use more chemicals such as antibiotics, antiparasitics, antiseptics, to sustain appropriate sanitation and master the livestock disease. Dyes, especially triarylmethanes, are among the therapeutic substances to be controlled because they were declared harmful to human health and therefore banned for use in aquaculture. They may however still be used fraudulently due to their antiseptic and antifungal properties. These chemicals are even largely used in several industries (textiles, inks) and are thus readily available and cheap. Some triarylmethane dyes known for their potential usage in aquaculture have been included in the French official control method, *i.e*. the malachite green and the crystal violet with their metabolites, and the brilliant green. Malachite green and crystal violet when absorbed by fish are metabolized to form lipophilic reduced leucomalachite green and leucocrystal violet. Hurtaud-Pessel *et al*. (2011, 2013) have shown that brilliant green is also reduced in its leuco form. These dyes have been extensively used as biocides in aquaculture in the past and are still used in some fish farming practices around the world. Tao *et al*. (2011) reported positive samples of malachite green and crystal violet in salmon. Extraction from the RASFF (Rapid Alert Reports for Food and Feed in EU) for alerts due to malachite green and its metabolite residues in fish shows that they were regularly found in farmed trout between 2002 and 2013 (Fallah *et al*., 2014). Moreover, it was assumed that it would be possible that other dyes might be selected to replace the malachite green. Other compounds derived from the family of triarylmethanes as the Victoria blue sub-family, or other dyes including phenothiazines, and phenyl azoic derivatives are often suggested but with no evidence yet. Regarding the Victoria pure blue BO, the RASFF notified in 2010 a first evidence of contamination of farmed fish imported from Vietnam (2010.1372 notification). More interest has recently focused on the possible use of similar compounds to malachite green that would not be sought in aquaculture products (Tarbin *et al*., 2008; Reyns *et al*., 2014.). However, the dyes in their entirety are not allowed to treat fish intended for human consumption due to their carcinogenic, mutagenic and teratogenic properties. It is therefore necessary to start investigating the occurrence of residues of other families of dyes that were previously not forecast for their use in aquaculture. Our project aims at investigating the need to strengthen the current monitoring system either by increasing the number of dyes to be targeted, or by implementing a new strategy based on non-targeted control. On the one hand, a multi-family method based on liquid chromatography and tandem mass spectrometry will be developed. Targeted compounds will include the main triarylmethanes (malachite green, crystal violet, brilliant green, ethylviolet, pararosaniline), and other triarylmethanes derivatives (victoria blue B, victoria blue R, victoria pure blue BO), xanthenes (rhodamine 6G), phenothiazines (methylene blue, azure B, new methylene blue), and phenoxazines (nile blue

A). On the other hand, the second objective is to determine any marker(s) of dye treatment through an untargeted approach which will be based on the analysis by liquid chromatography coupled to high resolution mass spectrometry of samples after farmed fish experimentations. The untargeted research strategy is intended to provide significant information relative to a targeted strategy, necessary to assess and strengthen controls. The experimental farmed fish study will permit to compare the general metabolic fingerprinting obtained from fish treated with a reference triarylmethane, the malachite green, with the one of fish treated with a derivative of triarylmethane, the victoria pure blue BO. Similarities between the fingerprints could allow to determine a strategy to track the presence of other dyes and therefore allowing to limit fraud in aquaculture and ensuring the consumer food safety.

Materials and Methods

Chemicals, standards and solutions

The dyes standard substances were purchased from Sigma-Aldrich (France). Stock solutions of the individual substances with a concentration of 100 µg mL^{-1} were prepared with methanol. Further working standard solutions of the substances and their mixtures with appropriate concentrations were also produced by dilution with acetonitrile. The dyes were divided into three groups during the validation according to their sensitivity. The solution of internal standards MG-d_5 and CV-d_6 was prepared at 100 µg L^{-1} in acetonitrile. The solution of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) was prepared at 5 mM in acetonitrile. The final solution of reconstitution was prepared by mixing 80% of acetonitrile and 20% of a solution of acid ascorbic at 1 g L^{-1}. The mobile phase A consisted of a mixture of ammonium acetate 10 mmol L^{-1} and formic acid 0.1% in deionized water. The mobile phase B was 100% acetonitrile.

Extraction procedure

Two grams of mixed muscle (fish or prawn) tissue was weighed into a centrifuge tube. The sample was spiked with an adequate volume of the working solution. Then 50 µL of the internal standard solution and appropriate volumes of acetonitrile were added. The sample was vortex-mixed and let to stand for 10 min in a dark place. Six mL of acetonitrile was added and then 0.5 g of $MgSO_4$ and the sample was again vortex-mixed to homogenize the material with the solvent. The sample was further placed on a mechanical rotary shaker for 10 min at 100 rpm and then centrifuged for 5 min at 4,000 g refrigerated at 4°C. The supernatant was transferred to a polypropylene tube and 2 mL of the DDQ solution was added. The sample was again placed on a mechanical rotary shaker for 30 min at 100 rpm. The sample was evaporated to dryness under gentle nitrogen flow at 50°C. The residue was dissolved in the mixture solution for reconstitution (80% acetonitrile and 20% ascorbic acid), vortex-mixed and then centrifuged for 5 min at 20,000 g refrigerated at +4°C. The sample was transferred into the LC vial by filtration of the residue through a 0.45 µm syringe PVDF filter.

Animal experiment

The objective of the animal experimentation was based on two experimental procedures. During the pilot phase, the toxicity of malachite green (MG) and victoria pure blue BO (VB) was evaluated. In the literature a dose administrated for malachite green can be found but no data suggested for the administration of victoria pure blue BO in aquaculture. The objective of the second phase was to determine metabolic fingerprints following administration of the two dyes on separate groups of trout. The fingerprints were compared in order to identify a possible metabolic correspondence.

The experiment involved a total of 81 individuals in three groups: a control group, a group treated with malachite green and a group treated with victoria pure blue BO. The administration dose was 0.1 mg L^{-1} in a water bath during 2 days for MG and 0.05 mg L^{-1} in a water bath during 2 days for VB.

At each date (two days of treatment, 48 h withdrawal after end of treatment, 120 h withdrawal after end of treatment), nine trout were taken from each group simultaneously. The different tissues taken were: muscle with skin, plasma, liver.

LC-MS/MS targeted analysis

Chromatography was performed on a Shimadzu LC-20AD-XR system (Kyoto, Japan) fitted with a Phenomenex Kinetex C18 column (100 mm · 2.1 mm, 2.6 µm) and protected with a C18 security guard system from Phenomenex. A gradient was applied and started with 30 % B during 1 min. It was then raised to 80% B over 5.5 min, then set to 95% B for 0.1 min and hold for 3.4 min, and again set to 100% B for 0.1 min and hold for 4.4 min. The initial composition was then recovered over 1-min delay. The flow rate was 0.40 mL min^{-1}. The MS equipment consisted of a Sciex API5500 mass spectrometer (San Jose, CA, USA) controlled by the Analyst software (Vers. 1.6.2) operating in positive ion electrospray mode for the detection (1 MRM transition) and confirmation (2 MRM transitions) of the dye analytes. The positioning of the source was adjusted; cone voltage and collision energy were tuned to optimize the transition of the precursor ion to the most abundant product ions monitored in the MRM mode. The following MS-MS parameters were used: source temperature, 600°C; turbo-ion spray voltage, 2,000 V; sheath gas pressure (air), 50 psi; auxiliary gas pressure (air), 55 psi; curtain gas, 30 psi. A chromatogram of some individual transitions is displayed in Fig 1.

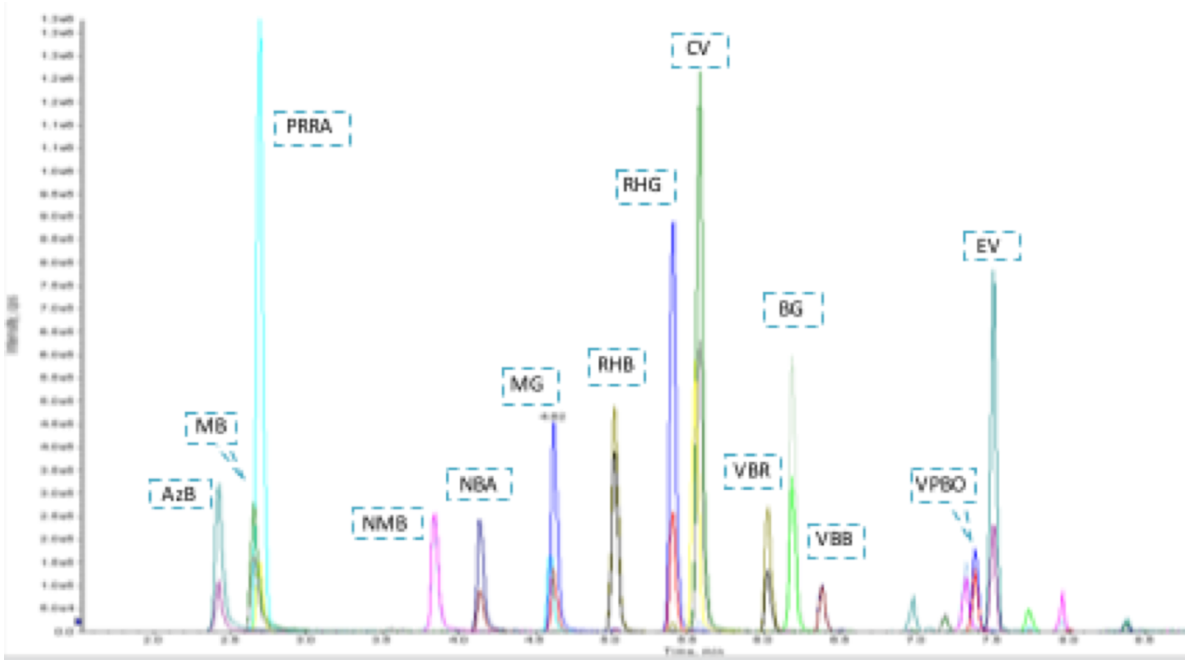

Figure 1. Chromatogram of a spiked muscle of trout at level 1.

LC-HRMS untargeted analysis

Chromatography was performed on a ThermoFischer U-HPLC Accela system (Bremen, Germany) fitted with a Waters Xbridge BEH C18 column (100 mm · 2.1 mm, 2.5 µm) and protected with a C18 security guard system from Phenomenex. A gradient was applied and started with 2% B. It was then raised to 98% B over 13 min and hold for 4 min. The initial composition was then recovered over 1-min delay. The flow rate was 0.30 mL min^{-1}. The MS equipment consisted of a LTQ Orbitrap XL MS mass spectrometer (ThermoFisher, Bremen, Germany) controlled by the Xcalibur software (Vers. 1.6.0) operating in positive ion electrospray mode of the analytes. The positioning of the source was adjusted to obtain the optimal signals. The following parameters were used: capillary temperature, 275°C; turbo-ion spray voltage, 5,000 V; sheath gas pressure (air), 30; auxiliary gas pressure (air), 10; sweep gas, 2 psi. The instrument was calibrated to reach mass accuracies in the 1-3 ppm range. It was operated in full-scan FTMS over *m/z* range of 100-1000 Da at a resolving power of 60,000 (full width at half maximum). For each matrix, LC-HRMS samples acquisitions were randomized and quality-control (QC) samples for each batch, prepared by combining 5 µL of each sample for each batch were injected regularly in the sequences.

Data were converted by MSconvert (Kessner *et al*., 2008) and processed under R (R core Team, 2015 version 3.2.0) with the xcms package (version 1.44.0) and further analysed with the workflow4metabolomics (Giacomoni *et al*., 2014) to set up descriptive and predictive models and highlight candidate biomarkers.

Results and Discussion

Targeted approach

According to Annex I of Directive (EC) No 96/23 for the substances to be controlled in foodstuffs of animal origin, the pharmacologically active dyes belong to the Group B3e. Currently, only the malachite green has been set a MRPL of 2 µg kg^{-1} for the sum of malachite green and its metabolite (*Commission Decision (EC) No 2004/25*).

In France, the current official method for controlling residues of dyes of interest monitors the malachite green, the crystal violet and the brilliant green. The occurrence of the malachite green seems to decrease since the 15 past years when other dyes are now disclosed in the literature as potentially used. During our project, a targeted and very sensitive method was developed and validated to extend the scope of investigation of these compounds. The method aims at confirming and quantifying by LC-MS/MS the residues of fourteen dyes in aquaculture products. Some of these dyes are already known to be rapidly metabolized in their reduced leuco form and most of the methods described in the literature target also the known leuco-bases (leuco malachite green, leuco cristal violet). However, potentially existing leuco forms of other dyes may have to be monitored as well and all the relevant standards are not necessarily commercially available. This is the case for instance for the leuco brilliant green already described by Hurtaud-Pessel *et al*. (2011). The method developed in the project, as well as some others in the literature, aims at oxidizing the leuco metabolites to finally dosing the parent molecules instead. During the development of our standard operating procedure, it was noticed that some compounds undergo a loss caused by oxidation. So it was proposed to validate the method in two parts, one with oxidation and one without oxidation, and to col-

lect and compare the data. For the validation without oxidation, a small fraction of 300 µL was removed just before the oxidation step and sent to LC-MS/SM directly after filtration. The validation was performed in accordance with Commission Decision No (EC) 2002/657. Matrix-fortified calibration standards (SC) and matrix-fortified validation samples (SV) were selected to estimate the required qualitative and quantitative performance parameters to be validated. The adequate levels for fortifying the SC and SV samples were chosen following the sensitivity of the compounds and then separated in two groups of levels. The validation levels ranged between 0.1 and 0.4 µg kg^{-1} for eleven compounds and between 0.5 and 2.0 µg kg^{-1} for three compounds (see Table 1). All validation parameters, *e.g.* the decision limit CCα, the detection capability CCβ, the precision, and the recovery were evaluated (*data partially submitted here*). The results fulfilled the requirements when applying the method with an oxidation step for eleven compounds with CCα ranging from 0.023 µg kg^{-1} for Victoria blue R and 0.336 µg kg^{-1} for Methylene blue. For three compounds, new methylene blue, pararosalinine, and victoria pure blue BO, results were satisfactory in terms of accuracy without the oxidation step.

Table 1. Partial performance parameters.

Analyte	***Method*** ***Ox. : with oxidation*** ***NOx. : no oxidation***	***Calibration range (µg kg^{-1})***	***Mean validation CCα (µg kg^{-1})***
Crystal violet	***Ox.***	0.1-0.4	0.024
Victoria blue B	***Ox.***	0.1-0.4	0.028
Azure B	***Ox.***	0.5-2.0	0.266
Victoria blue R	***Ox.***	0.1-0.4	0.023
Methylene blue	***Ox.***	0.5-2.0	0.336
Brilliant green	***Ox.***	0.1-0.4	0.051
Rhodamine B	***Ox.***	0.1-0.4	0.038
Malachite green	***Ox.***	0.1-0.4	0.034
Ethyl violet	***Ox.***	0.1-0.4	0.145
Nile blue A	***Ox.***	0.1-0.4	0.037
Rhodamine 6G	***Ox.***	0.1-0.4	0.061
New methylene blue	***NOx.***	0.5-2.0	0.141
Pararosalinine	***NOx.***	0.1-0.4	0.042
Victoria pure blue BO	***NOx.***	0.1-0.4	0.067

Non-targeted approach

The targeted method allows analysing an exhaustive list of dye residues (fourteen analytes) and to take into account their leuco-metabolites already known (three analytes). The aim of the untargeted approach was totally different with starting to compare fingerprints between batches of trout treated with malachite green, and trout treated with victoria pure blue BO. These two dyes are relatively close in their chemical structure both derived from the triarylmethanes. Then, it was assumed that a similarity in the fingerprints may arise between the two treatments. Highlighting common biomarkers could permit to evidence new farming practices with use of new dyes in aquaculture. The animal experiment involved homogeneous animal population in terms of age, breed and nutrition. The biological matrices (liver, muscle, and plasma) were stored immediately at -80°C. The data acquired by LC-HRMS in Full Scan for the treated trout from each dye group: the MG group and the VB group were compared together to the group of non-treated trout or control group, by operating statistical data treatments on the generated datasets. The repeatability of data acquisition was checked by injection of quality-control (QC) samples for each batch. Deviations observed between QCs were corrected by data normalization following a correcting algorithm as mentioned by Van der Kloet (2009). Then Log10 transformation and Pareto scaling were applied before multivariate statistics. The data were processed for the three sets of matrices but are presented here only for muscle. After a first pre-processing, the retention time alignment of all samples was not satisfactory due to a dense chromatographic region between 600 and 900 s. RT alignment was improved by removing the masses higher than 750 Da. Principal-components analysis (PCA) was first used to detect intrinsic clusters or outliers. For muscle, the analysis revealed six outliers, three for malachite green and three for victoria pure blue BO. Partial least squares discriminant analysis (PLS-DA) was then used to model the relationship between exposure conditions and LC-HRMS data. A preliminary PLS-DA analysis was able to discriminate the group Control from the group Treated with a validated model on two components. The model revealed 19 features significantly different among the three groups, with a VIP value > 2.0 (variable influence on projection (VIP) is an established parameter that summarizes the importance of the X-variables in a PLS model with many components (Galinto-Prieto *et al.*, 2014), but some ions were still correlated. So the model was reprocessed with removing these ions. The resulting model revealed two components explaining 66% of the variation (R^2X) and with a predictive capacity (Q^2Y) of 0.7. The model was validated by using a

permutation test (see Fig. 2). Briefly, these three parameters: R2(X), corresponding to the proportion of the total variance of the dependant variables that is explained by the model, R2(Y), defining the proportion of the total variance of the response variable (*i.e*. the class of the samples) explained by the model, and Q2(Y), which is similar to R2(Y) excepted that it is computed by cross-validation. The score-plot obtained, presented in Fig. 2, enables separation of the control group from the treated group either for malachite green or for victoria pure blue BO. Seven ions (*data not shown*) were revealed to be significantly different among the groups, with a VIP value > 2.0 and a Kruskal-Wallis test p-value < 0.05. At this time, no common significant ion has been found yet between the trout treated by malachite green and trout treated with victoria pure blue BO, but there are already several signals that seem of particular interest in each of the two treated groups in regard to those in the control group.

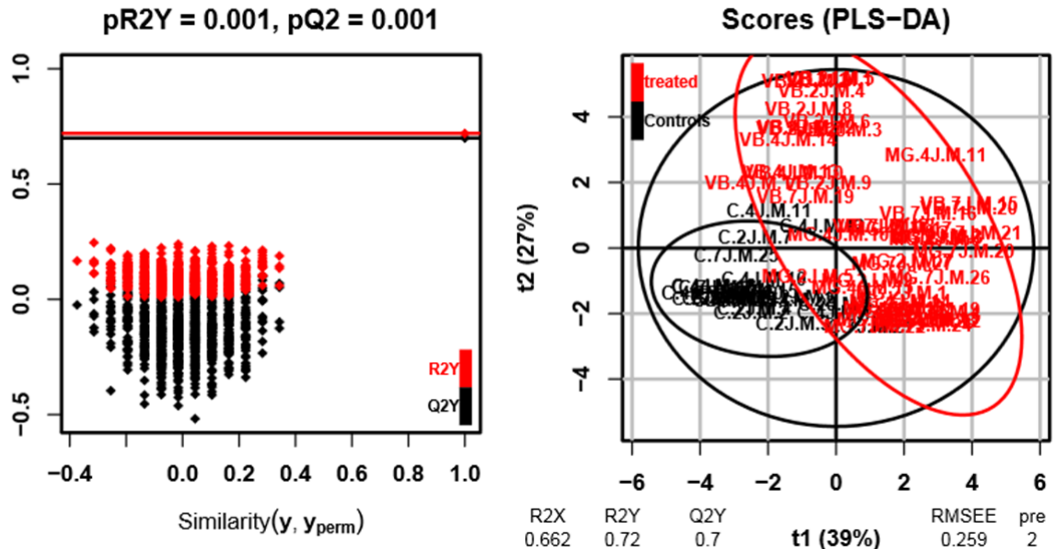

Figure 2. Two-dimensional PLS-DA score plot and permutation test graph of trout muscle samples for the control group and treated group.

Conclusion

The need to strengthen the control in particular for dye residues in aquaculture leads to the development of ever enlarged targeted methods and/or to the implementation of new non-targeted approaches. The project permitted in a first part to develop a multi-residue method able to confirm fourteen dyes in aquaculture products with an oxidation step running for eleven out of the fourteen analytes. The method may allow controlling the aquaculture deviant practices in most of the potential usage of prohibited dyes. To combine with this targeted method, a metabolomic strategy is proposed to highlight possible common biomarkers between trout treated with different triarylmethane dyes: malachite green and victoria pure blue BO. Data analysis is still in progress but promising results were already found in particular in muscle tissue and also in the fish liver. At the moment, no common biomarker for triarylmethanes seems to be highlighted but biomarkers specific for each of the two treatments. The data analysis will be completed up to the annotation of the generated ions of interest.

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MULTIPLEX BEAD BASED ASSAY FOR THE SIMULTANEOUS ON-SITE DETECTION ANTIMICROBIALS IN DRINKING WATER AND FEATHERS.

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Abstract

Because of the increasing threat of antimicrobial resistance, restrictive measures apply to the prescription and use of antibiotics. Monitoring is increased and rapid on-site screening methods to identify abuse of antimicrobials can support (governmental) inspections. For the on-site detection of at least forty-two frequently used antimicrobials, a multiplex microsphere (bead) based assay was developed. This assay uses paramagnetic carboxylated polystyrene beads (MagPlex^{TM}) which are internally dyed with different ratios of two fluorophores and of which fifty different bead sets are available. Each bead set can be chemically coupled with different biomolecules and ligands or antigens (*e.g*. antibodies, receptors, drugs, drug-protein conjugates and even DNA). In the present test for the detection of forty-two antimicrobials, only seven bead sets are used of which three with specific antibodies, three with "generic" antibodies and one with a receptor.

The newly developed on-site multiplex bead based assay detects at least forty-two antimicrobials that belong to the aminoglycoside, sulfonamide, tetracycline, (fluoro)quinolone and β-lactam groups in drinking water and feathers. The sensitivity for all these antimicrobials in drinking water is well below 100 ng mL^{-1}, and in most cases even below 10 ng mL^{-1}.

Introduction

For many years, antimicrobial agents have been used as feed additives and for prophylactic treatments to improve the performance of farm animals. They were added at sub-therapeutic concentrations to animal feed. The serious emerging problems caused by the dissemination of antimicrobial resistance were the reason for an EU-wide ban on the use of antimicrobials as growth-promoters in animal feed in 2006 (EC Regulation, 2003). Any use of antimicrobials must be authorized by a veterinarian and documented at the production site. At the moment, antimicrobials are only allowed for therapeutic use and are often administered by adding them to the drinking water, as Dutch feed mixing companies (NEVEDI) stopped the production of medicated feed in January 2012. The restricted carry-over level from medicated feed into normal feed by the Dutch Ministry of economic affairs was the main reason for this production stop.

To stimulate prudent use and proper registration of antibiotic use, monitoring is increased and rapid on-site screening methods to identify abuse of antimicrobials can support inspections to reach the regulatory goals. For this purpose, samples through the whole food chain should be monitored. However, monitoring at the farm has several advantages, as concentrations are expected to be relatively high and specific measures to prevent undesirable residues of entering the food chain can still be undertaken.

A reliable and rapid screening method for the on-site detection of a broad spectrum of antimicrobials is of great value but are still very rare. Most screening assays that are suited for on-site applications detect a specific compound or an antimicrobial group at the target level (*e.g*. lateral flow test). Tube tests have a broader range of detection and can be used on-site, but are not sensitive enough for all antimicrobial groups and take more time.

The microspheres (referred to as beads) based technology has been selected for the development of an assay for a broad, rapid and sensitive detection of antimicrobials in animal drinking water and feather extracts. This technology is based on colour-encoded beads and when making use of antibodies, the assays are comparable with other well-known immunoassays. Applying these assays on bead sets provides the opportunity of combining the favourable properties of immunoassays with the opportunity of multiplexing (combining assays developed on different bead sets). Moreover, instead of antibodies, the specific coloured bead sets can also be used with aptamers, receptors or even DNA and together with planar imaging detection, on-site screening becomes possible too.

In the present test comprising the detection of at least forty-two antimicrobials, only seven bead sets are used: six with antibodies and one with a receptor. All seven assays are developed in the competition format. There is a competition in the first incubation step between the bound antimicrobial competitor on the bead and the free antimicrobial in the sample for the binding site on the antibody or receptor. The bound antibody on the bead can be measured after the second incubation step with a fluorescent PE protein label (R-phycoerythrin). The measured mean fluorescent intensity (MFI) on the bead corresponds with the amount of bound antibody and is thus inversely related to the analyte concentration in the sample (Figure 1).

The newly developed multiplex assay thus detects the most commonly used antimicrobials in animal husbandry that belong to the groups of sulfonamides, quinolones, aminoglycosides, tetracyclines and β-lactams. The opportunity of on-site screening has been investigated with water samples and aqueous extracts of feathers for the detection of antimicrobials.

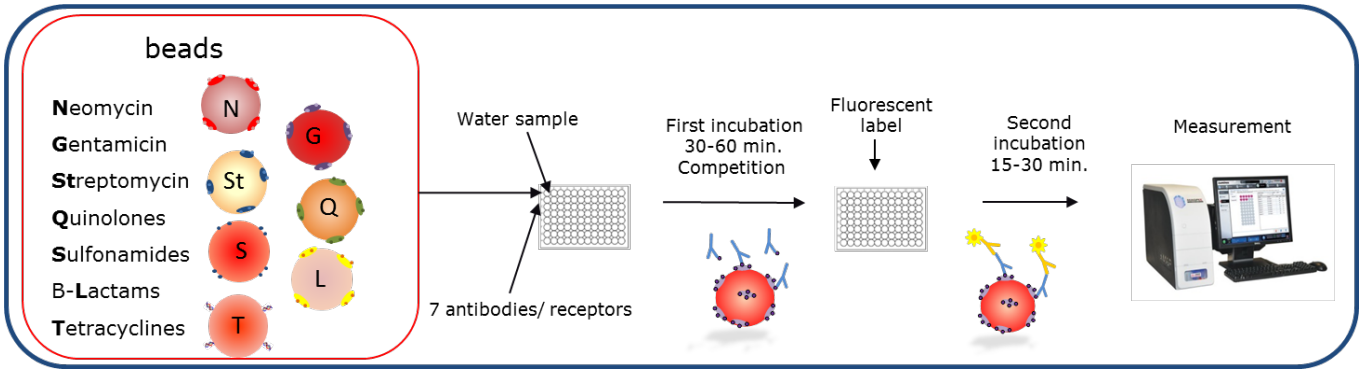

Figure 1. The general principle of the bead-based multiplex assay.

Materials and Methods

Materials

The carboxylated (paramagnetic) beads (MagPlex®) and the planar imaging detector (MAGPIX®) were obtained from Luminex (Austin, Texas, USA). The reference standards were obtained from Sigma-Aldrich.

Gentamicin- and neomycin-*bovine* serum albumin (BSA) conjugates were delivered by Tubu-bio (Heerhugowaard, The Netherlands). The norfloxacin-HRP conjugate was supplied by EuroProxima (Arnhem, The Netherlands). The norfloxacin-NH_2 derivative was obtained from CER (Marloie, Belgium). Gentamicin-, streptomycin- and neomycin-ovalbumin (OVA) conjugates were prepared according to Haasnoot *et al.* (2002) and ampicillin- horseradish peroxidase (HRP) conjugate was prepared according to Peng *et al*. (2013). The sulfonamide derivative (TS) was prepared according to Haasnoot *et al.* (2000).

The monoclonal antibodies (mAbs) directed against gentamicin and neomycin were delivered by Tubu-bio. The anti-streptomycin mAb F62 was a gift from Wageningen UR Food & Biobased Research (Wageningen, The Netherlands). The polyclonal antibodies directed against (fluroro)quinolones were applied from EuroProxima. The polyclonal ampicillin was delivered by Randox (Antrim, UK). A mutant recombinant antibody (rAb) M.3.4. directed against sulfonamides was obtained from the University of Turku (Turku, Finland) and the tetracycline reagents were gifts form Unisensor (Liege, Belgium). Goat-anti-rabbit-R-phycoerythrin conjugate (GAR-PE), rabbit-anti-mouse-R-phycoerythrin conjugate (RAM-PE) and streptavidine-R-phycoerythrin conjugate (SAPE) were from Molecular Probes (Leiden, The Netherlands), donkey-anti-sheep-R-phycoerythrin conjugate (DAS-PE) from Bioconnect (Huissen, The Netherlands).

On-site water and feather samples were supplied by the NVWA. Drink water samples were acquired from an animal facility.

Immobilisation of antimicrobial-protein conjugates or antimicrobial derivatives on the beads

All conjugates were coupled to a unique paramagnetic bead set according to a standard Luminex protocol for protein coupling. The derivatives were prepared using a previously described procedure (Haasnoot *et al.,* 2002).

Sample preparation

For preparing the water extract of feathers, about 2 mL tap water was added to a few feathers. This was shaken for one min. The feather water extracts and drink water samples were treated similarly. Both were mixed 9:1 (v/v%) with a 10 times concentrated phosphate-buffered saline solution (PBS) containing 1% *bovine* serum albumin and thereafter 100 µL was analysed.

Bead based immunoassay protocol

Serial dilutions of the individual antimicrobials (neomycin, gentamicin, streptomycin, ampicillin, sulfamethoxazole, tetracycline and norfloxacin) and a mixture of antimicrobials were prepared in sample buffer (PBS containing 0.1% BSA). Bead suspensions and antibody dilutions (individual or as mixture) were made in 0.1% BSA blocking buffer (Luminex, 2012). Subsequently, 10 µL of this bead suspension, 100 µL sample (or 100 µL of serial dilutions of the standards and mixtures to prepare calibration curves) and 10 µL of antibody solution were added to a well of a flat-bottom 96-well plate and the plate was then incubated on a microplate shaker at RT in the dark for 1 h. After three manual washings with wash buffer (PBS containing 0.05% Tween-20 and 0.004% antifoam), 100 µL of diluted SAPE, GAR-PE, DAS-PE and/or RAM-PE were added. After 30 min of incubation, on the microplate shaker at RT in the dark, the beads were washed and ready to be analysed in the Luminex analyser. The antibody or receptor binding was quantified by the response (MFI) obtained from the amount of fluorescent reporter molecule (PE). The responses of the samples were compared with responses of positive and negative controls and used to classify the samples as compliant or suspect positive.

Results and discussion

For each antimicrobial group, except for the three aminoglycosides, a (multicomponent) assay was developed on a single bead set using "generic" antibodies or receptor. In the present study, seven single assays were developed; four of such "generic" single assays for tetracyclines, sulfonamides, (fluor)quinolones and β-lactams and three with specific immunoassays for aminoglycosides (gentamicin, neomycin and (dihydro)streptomycin). The assays were developed on paramagnetic beads to be measured with the MAGPIX. Within the aminoglycoside antimicrobial group the structural relations are very limited, except for streptomycin and dihydrostreptomycin. As a result, a specific antibody was needed for almost every aminoglycoside, for now resulting in three aminoglycoside assays.

After investigating the performance and optimising the conditions of the singleplex assays, the assays were combined in the multiplex format in order to investigate possible interferences. Therefore, the cross interaction of the antibodies/receptors with non-corresponding beads sets was tested. Combining six assays, *i.e*. the three aminoglycoside, the sulfonamide, the β-lactam and the quinolone assay, could be done without any problems. Only low cross-interactions of the antibodies with the other beads were observed and comparable calibration curves and maximum responses (MFI) were obtained in the singleplex and multiplex format. The tetracycline assay was used in the same 96 well plate but in a different well because a very high cross interaction of a tetracycline assay component was observed with the sulfonamide, quinolone and ampicillin bead. The MFI value can be corrected for daily inter-plate fluctuations by calculating the percentage of relative binding (%B/B0) from the maximum response (B0), which is obtained from a blank sample.

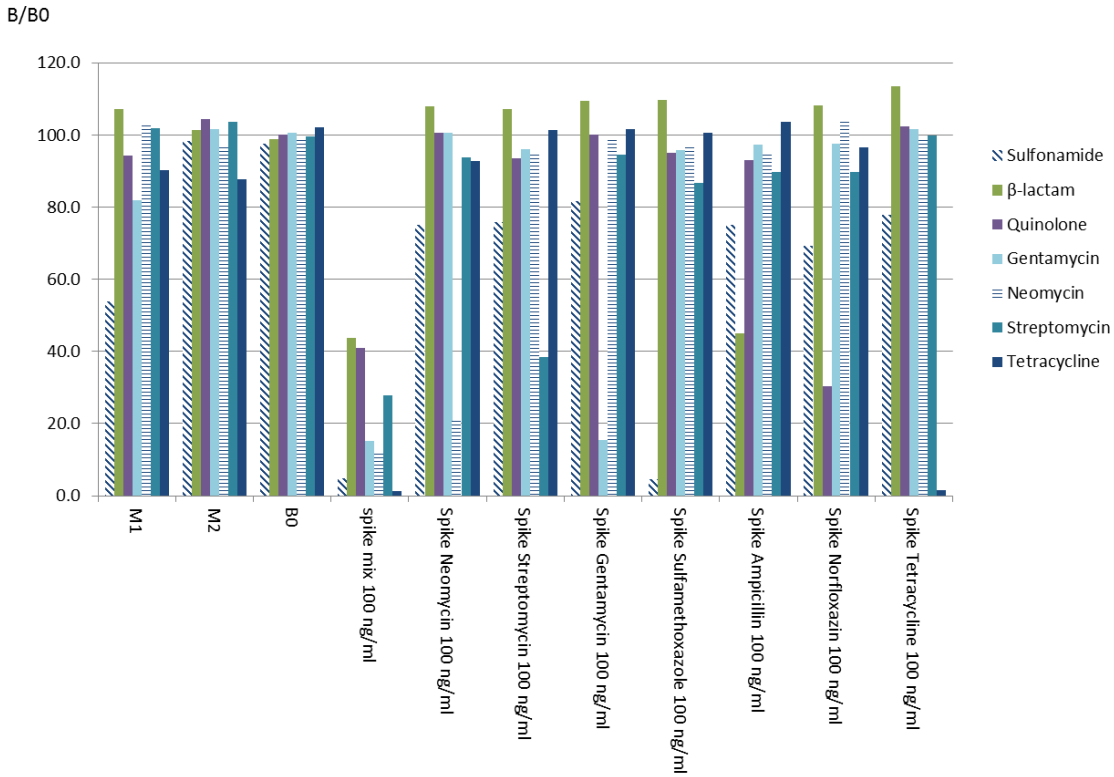

Figure 2. Relative responses obtained with blank drinking water samples and one spiked drinking water samples (with a single antimicrobial and with a mixture of seven antimicrobials at 100 ng mL^{-1} level).

Oral administration through the drinking water is often applied for antimicrobial treatment of farm animals. Therefore, the assay was applied on drinking water samples, which is also convenient for direct on-site screening, as sampling water is not invasive for the animal and very convenient and easy for the inspectors as well. For the validation of the test, blank drink water samples were collected. These samples were also spiked with single standards antimicrobials (neomycin, gentamicin, streptomycin, ampicillin, sulfamethoxazole, tetracycline and norfloxacin) or the mixture of these antimicrobials (all at 100 ng mL^{-1} for each antimicrobial) and analysed (Figure 2). Blank samples had a B/B0 around 100%. Both samples showed a compliant result for all antimicrobials, except sample M1 showing a clear response in the sulfonamide assay, showing an inhibition of about 50%, which corresponds to a concentration < 1 ng mL^{-1}. Unfortunately, this could not be confirmed with LC-MS. The spike samples resulted in inhibitions > 58% (B/B0 < 42%). As expected, when a single antimicrobial was added only the target assay showed inhibition, while all assays showed inhibition when a mixture of seven antimicrobials was added. Also as expected, similar inhibition results were obtained, comparing samples with a single antimicrobial added or a mixture

of antimicrobials, as the concentration both in the single standard and the mixture was 100 ng mL^{-1}. From this it can be concluded that the different antimicrobials have only influence on the assay of their target antimicrobial group, and do not interfere with the other (non-target) assays.

Sensitivity and specificity of the screening assay are important as the assay should at least detect the antimicrobials important for legislation/enforcement. Besides this, the newly developed antibody and receptor-based multiplex screening assay can potentially detect structurally related compounds as well. For these reasons, it is important to have information about the detection spectrum. The sensitivity and specificity of the multiplex test was therefore mapped using fifty different standard solutions of the different antimicrobials groups at 100 ng mL^{-1} water (a level well below that of medicated water).

An inhibition of >30% of the maximum signal was regarded as significant and means that this antimicrobial will be detected in the sample. Using this criterion, forth-two of the fifty antimicrobials could be detected by one of the assays (Table 1). As expected no cross-reactivity was observed for trimethoprim (TMP), tylosin (a macrolide) and chloramphenicol (CAP) in all assays. These antimicrobials have no structural relation with any antimicrobial from the other groups. The limited structural relation between the different aminoglycosides was also the reason why four aminoglycosides could not be detected with the antibodies of the three aminoglycoside assay. As a result, specific antibodies are needed for almost every aminoglycoside. The same phenomenon can be observed for the β-lactams. All β-lactams belonging to the penicillin group showed inhibition at the 100 ng mL^{-1}.

Table 1. Antimicrobials tested in water at 100 ng mL^{-1} level

Antimicrobial group	Antimicrobial	Detection at 100 ng mL^{-1}	Antimicrobial	Detection at 100 ng mL^{-1}
Aminoglycoside	Streptomycin	√	Kanamycin	-
	Dihydrostreptomycin	√	Apramycin	-
	Neomycin	√	Paromomycin	-
	Gentamicin	√	Spectinomycin	-
Quinolonen	Enrofloxacin	√	Sarafloxacin	√
	Flumequine	√	Marbofloxacin	√
	Ciprofloxacin	√	Darofloxacin	√
	Norfloxacin	√	Oxolinic Acid	√
	Difloxacin	√		
Tetracyclines	Tetracycline	√	4-Epi-tetracycline	√
	Doxycycline	√	4-Epi-chlortetracycline	√
	Oxytetracycline	√	4-Epi-oxytetracycline	√
	Chlortetracycline	√		
Sulfonamides	Sulfadimidine	√	Sulfamethiazole	√
	Sulfamethoxazole	√	Sulfadimethoxine	√
	Sulfathiazole	√	Sulfamethoxypyridazine	√
	Sulfaoxazole	√	Sulfapyridine	√
	Sulfatroxazole	√	Sulfamerazine	√
	Sulfachloropyridazine	√	Sulfamonomethoxine	√
	Sulfaquinoxaline	√	Sulfadoxine	√
β-Lactam	Ampicillin	√	Cloxacillin	√
	Amoxicillin	√	Oxacillin	√
	Penicillin G	√	Nafcilline	√
	Penicillin V	√	Cefquinome	-
	Dicloxacillin	√		
Others	Trimethoprim	-		
	Tylosin	-		
	Chloramphenicol	-		

Cefquinome showed a low inhibition because the cephalosporins are structurally less related to the penicillin group. The sensitivity and specificity of the sulfonamide assay in milk samples has been described before (De Keizer *et al*., 2008) and showed that sulfadiazine and sulfamethoxydiazine can also be detected at this level. However, with this bead based assay it is relatively easy to extent the spectrum by adding beads and cognate antibodies for other compounds, thereby expanding the multiplex.

Subsequently, the applicability with real on-site samples has been tested in a small pilot experiment. One farm was selected because, according to the registration, one stable of chickens was treated with amoxicillin and the other one stayed untreated. From both stables feather and water samples were taken. All water samples and feathers of untreated chickens were compliant. The feathers of the treated chickens were suspect positive for the presence of β-lactam and sulfonamides (Figure 3). The water samples from this stable were found compliant. Amoxicillin is a β-lactam, therefore the suspect positive screening of the feather in the β-lactam assay is in accordance with farmer's administration. The suspect positive outcome of the sulfonamide screening could not be confirmed by the administration, and was further analysed by LC-MS, but could not be confirmed. It therefore remains uncertain whether the result is a false or true positive, however, the high sensitivity of the multiplex screening assay was later confirmed by a study using bath water of ornamental fish, which showed that the multiplex was able to detect most antimicrobials well below 10 ng mL^{-1} (data not shown).

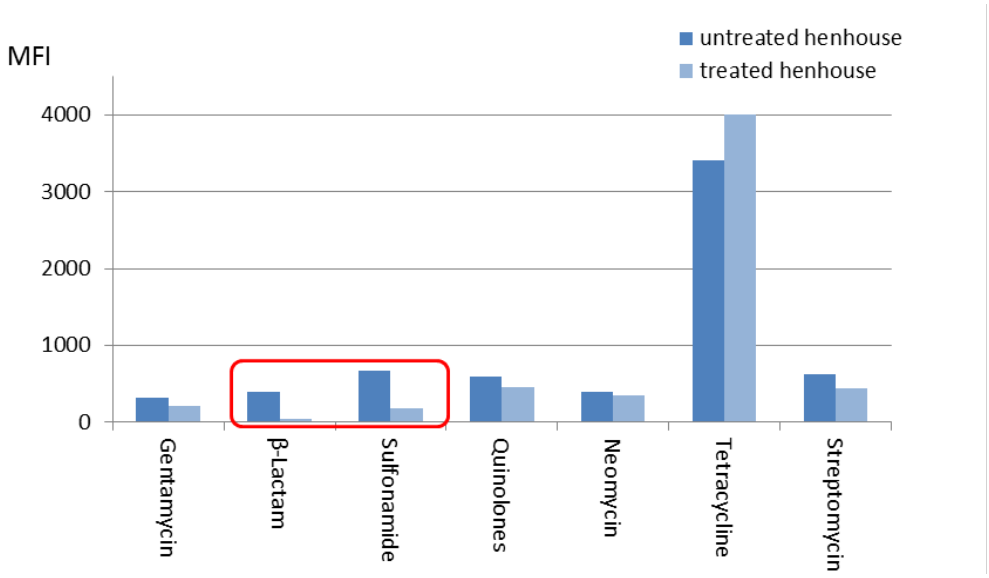

Figure 3. On-site assay performance in feathers of treated and untreated chickens.

Conclusions

A multiplex bead based assay has been developed for the detection of antimicrobials and an initial validation with water samples has been performed. Finally, the method has to be fully validated to be used as a screening assay for enforcement or monitoring purposes. In the future, the antimicrobial detection can be extended with other assays, *e.g*. for the detection of macrolides, extra assays for the aminoglycosides that are not yet included and assays for other types of growth promoters, *e.g*. β-agonists.

This assay has to compete with other on-site tests, like lateral flow devices (dip sticks). The strength of the bead based assay is its sensitivity, multiplex capability (and thus broad screening), and its low costs. The more the method is extended with other assays, the lower the relative costs for each analyte will become, ultimately potentially outcompeting dip sticks for broad on-site screening.

Acknowledgements

Unisensor for supplying us with all assay components for the tetracycline assay and the NVWA for supplying us with on-site sample materials.

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SCREENING AND VERIFICATION OF STEROID ESTERS IN *PORCINE* HAIR USING LC-QTOF-MS

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Abstract

The use of growth-promoting steroids and steroid esters in meat-producing animals is prohibited in the EU. The detection of the abuse of steroids and steroid esters is hampered by the rapid metabolism of these into endogenous compounds. However, a small fraction of steroid esters administered to the animals is reported to be deposited in hair. The detection of steroid esters in hair provides unequivocal evidence for illegal use as these esters are not formed endogenously.
An analytical method for screening of 16 steroid esters of testosterone, nortestosteron, boldenone and estradiol was developed. *Porcine* hair was washed in lukewarm water, dried and extracted with methanol using ultrasonification. The extract was purified by dispersive SPE, a fraction was evaporated to dryness, re-dissolved and analysed by HPLC connected to a QTOF mass spectrometer via an electrospray ion source. Identification of target compounds was based on retention time (±0.1 min), accurate mass (±3 mDa) and isotopic pattern ($m\sum < 50$). Positive findings were verified using alternating full scan MS and MRM comparing ion ratios with those of authentic standards.

Introduction

The use of growth promoting agents such as steroid hormones in meat producing animals is prohibited within the EU. Steroid hormones undergo rapid metabolism in the animals to substances which structurally are similar to endogenous hormones and this makes illegal use of hormones difficult to detect (Aqai *et al*. 2009). Due to this rapid metabolism, steroids are usually administered as steroid esters. The ester function facilitates uptake and transport to target organs in the body, where the esters are hydrolysed so the parent compound can exert its biological activity (Duffy *et al*. 2009). Steroid esters do not occur naturally in animals and their presence is accordingly evidence for illegal use (Groot *et al*. 2012).

Due to the rapid metabolism of steroids and their esters evidence for illegal use of the substances is hard to obtain using conventional samples such as urine and blood. However, several studies have shown that steroid esters as well as other drugs are incorporated into the growing hairs in *bovines*, horses, pigs, guinea pigs and humans and that the esters can be persistent for several months (Regal *et al*. 2010; Gray *et al*. 2013; Groot *et al*. 2012; Shen *et al*. 2009; Nielen *et al*. 2007; Duffy *et al*. 2009).

Analytical methods for extraction and detection of steroid esters in hair from various species have been developed and are mainly based on detection with triple quadrupole mass spectrometers (Stolker *et al*. 2009; Regal *et al*. 2010; Groot *et al*. 2012). Here, we report the measurement of steroid esters in *porcine* hair using UPLC connected to high resolution accurate mass spectrometry which is an attractive detection system giving good sensitivity and specificity and allows easy incorporation of additional esters in the analytical method.

Materials and Methods

Standards and solvents

Boldenone undecylenate, estradiol 3-benzoate, 1-testosterone acetate, 1-testosterone tetrahydropyranyl ether, testosterone acetate, testosterone benzoate, testosterone cypionate, testosterone decanoate, testosterone isocaproate, testosterone phenylpropionate, testosterone propionate, testosterone undecanoate, nortestosterone decanoate, nortestosterone phenylpropionate and testosterone enanthate were obtained from Steraloids (Newport, RI, USA). 4-chlorotestosterone acetate, HPLC-MS grade ethylacetate, acetonitrile, methanol, formic acid and ammonia were obtained from Sigma-Aldrich (Brøndby, Denmark). All aqueous solutions were prepared using ultrapure water obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). Isolute C-18 (EC) bulk sorbent was obtained from Biotage (Sweden). Stock solutions of steroid esters at a concentration of 1 mg mL^{-1} in methanol were stored at -20˚C. Working solutions at a concentration of 20 to 100 ng mL^{-1} in methanol were prepared daily.

Preparation of porcine *hair samples*

Porcine hair samples were collected by the National Food Administrations control laboratory during routine inspections of local farms. Hair samples of not less than 100 mg were suspended in water for several hours and then washed in lukewarm water until visually clean. The hair samples were transferred to a 100 mL beaker glass and cut into 2 -3 mm length and dried overnight at 40 ˚C. Hair samples of 70 ± 1.5 mg were weighed into a 15-mL Sarstedt vial and 7 mL methanol was added. The mixture was treated with ultra-sonication for 30 min followed by centrifugation at 1,500 x g for 5 min. The supernatant was subjected twice to dispersive SPE with 2 x 200 mg of Isolute C-18 for 4 min each. Five mL of the resulting supernatant was

evaporated to dryness, dissolved in 0.125 mL methanol and analysed by UPLC-QTOF-MS. Optimisation of the extraction conditions and extraction time was performed on samples of incurred *bovine* hair kindly provided by dr. Marco Blokland (RIKILT, Wageningen, NL).

Liquid chromatography- time of flight mass spectrometry

The UHPLC-QTOF-MS consisted of a Dionex Ultimate 3000 RS liquid chromatograph (Thermo Scientific, Sunnyvale, CA, USA) connected to a Bruker maXis time-of-flight mass spectrometer equipped with an electrospray ion source operated in positive mode (Bruker Daltonics, Bremen, Germany). The analytes were separated on a Poroshell 120 SB-C18 column with dimensions of 100 x 2.1 mm and 2.7 µm particle size (Agilent Technologies, Santa Clara, CA, USA) The column was held at 50˚C and the sampler at 20˚C. Mobile phases were: A: 0.2% formic acid with 5 mM ammonium hydroxide and B: acetonitrile with 0.1% formic acid. The analytes were eluted using the following gradient: isocratic 30% B for 0.5 min followed by a linear gradient to 100% B at 15 min, isocratic 100% B to 25 min followed by return to initial conditions. The flow rate was 0.4 mL min^{-1} from 0 to 0.5 min followed by a linear gradient to 0.5 mL min^{-1} to 15 min which was held until 25 min followed by return to initial conditions. The injection volume was 5 µL. The ion source settings were: nitrogen nebulizer pressure 2 bar, nitrogen drying gas 10 L min^{-1}, 200˚C, capillary voltage 4,500 V. Mass spectrometry data were collected from 80 – 700 Da at a frequency of 2 Hz in either full scan mode (screening analyses) or in alternating full scan/MRM mode (verification). In the MRM mode the chromatogram was divided into 6 segments containing between 1 to 4 precursor selections each. The collision energy in MRM mode was 30 eV with nitrogen as collision gas. In a segment from 0.2 to 0.4 min sodium formate was introduced into the ion source and the cluster ions generated were used for mass axes calibration of the data files, additional lock mass calibration was performed using hexakis(1H, 1H, 2H-perfluoroethoxy)phosphazene which was continuously present in the ion source (Apollo Scientific, Manchester, UK).

Data analysis

Full scan MS data were processed with the TargetAnalysis software package (Bruker Daltonics, Bremen Germany). This software package uses a csv file containing retention times and elemental composition of all the steroid esters included in the analysis for construction of extracted ion chromatograms ±3 mDa of all the esters. The software automatically compares measured retention times with expected, measured exact mass and measured isotopic pattern with the theoretical values and reports according to predefined scoring parameters.

Verification of positive samples was done using alternating full scan/MRM mode of acquisition. Data were processed with the software package QuantAnalysis (Bruker Daltonics, Bremen Germany). This software package is used to construct extracted ion chromatograms ± 3 mDa of both precursor and fragment ions and following integration ion ratios can immediately be compared to values obtained from analysis of authentic standards.

Validation

Twenty blank samples and 20 samples spiked at CCβ level were analysed to assure that no interferences with the analytes were present in the blank samples. Construction of calibration curves was not attempted. The use of steroid esters in livestock production is not permitted and accordingly the presence of one of these esters is proof of illegal use.

Results and Discussion

Sixteen steroid esters were included in this screening method for steroid esters in *porcine* hair. Figure 1 shows an overlay of extracted ion chromatograms of the 16 esters after analysis of a standard sample using the exact mass of each of the esters ±3 mDa. A good separation was achieved using a linear gradient from 30% to 100% acetonitrile in 15 min. The chromatogram shows the presence of 18 peaks because 1-testosterone tetrahydropyranyl ether occurs as two diastereomers resulting in two peaks with different retention times and estradiol 3-benzoate is detected both as proton and ammonium adducts with the same retention time.

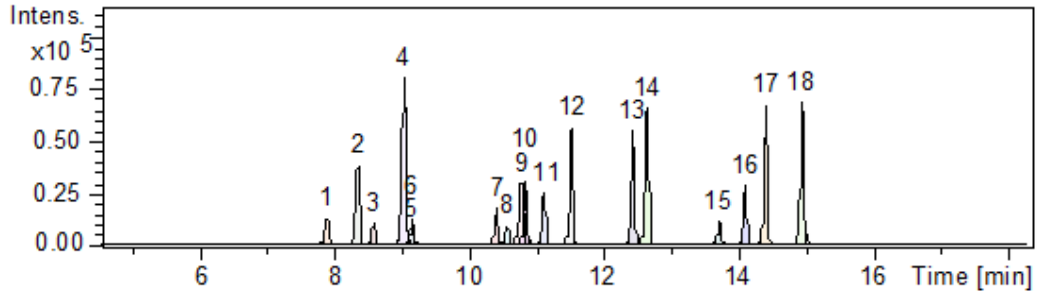

Figure 1. Overlaid extracted ion chromatograms using exact mass ± 3 mDa of the 16 steroid esters in a standard sample.

Data were acquired in full scan mode in this method and narrow extracted ion chromatograms were constructed later in the data analysis, this means that additional steroid esters can easily be included in the method. A determination of retention time and addition of the elemental composition of new analytes to the csv file used in TargetAnalysis is the only information needed. Construction of extracted ion chromatograms with a narrow width of ± 3 mDa resulted in a good signal-to-noise ratio.

Porcine hair obtained from live farm animals are rather dirty and extensive washing with lukewarm water was needed prior to extraction of the analytes. Initial attempts to wash incurred *bovine* hair samples with water containing the detergent SDS resulted in loss of analytes and was therefore not pursued. Steroid esters were extracted from the hair using methanol as solvent combined with ultrasonication.

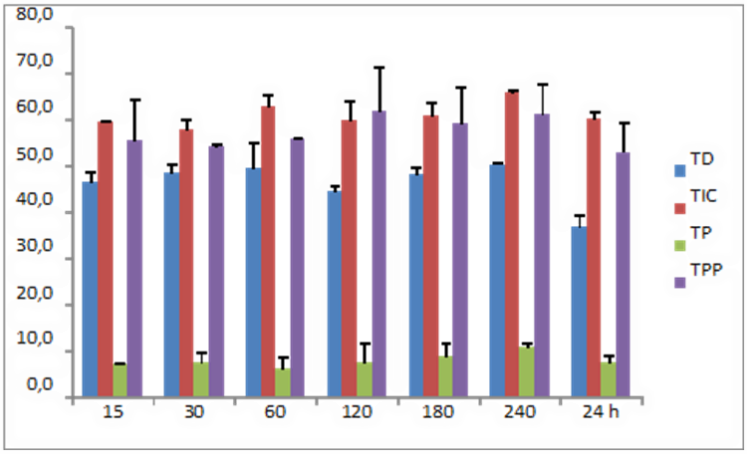

Figure 2. Concentrations of steroid esters measured in extracts after 15, 30, 60, 120, 180, 240 min and 24 hours. Incurred bovine hair samples were suspended in methanol and treated with ultrasonication. TD: testosterone decanoate; TIC: testosterone isocaprionate; TP: testosterone propionate and TPP: testosterone phenylpropionate.

Figure 2 shows that the 4 steroid esters present in incurred *bovine* hair are essentially extracted after 15 min of ultrasonication in methanol, longer extraction times did not result in additional release of esters from the hair. On the contrary, long extraction times resulted in release of more matrix components from the hair. An extraction time of 30 min was chosen for the further work. Addition of reducing agent such as TCEP during extraction did not improve the yield of steroid esters in the extract compared to ultrasonication alone (results not shown) (Stolker *et al*. 2009).

Figure 3 shows a chromatogram of an extract of blank hair spiked with a standard mixture at CCβ level. The light grey line shows the base peak chromatogram with a scaling reduced from 2 x 106 to 2 x 105 in order to be able to visualize also the presence of the steroid esters in the sample. The extract was subjected twice by dispersive SPE in an attempt to remove some of the most lipophilic compounds in the extract, however, a large number of compounds at high concentrations were still present in the extract. Searches in the human metabolome database using the exact mass of the matrix compounds indicated that these included ceramides, fatty acids, monoglycerides and diglycerides containing fatty acids of different chain length and unsaturation (http://www.hmdb.ca/).

Despite the large number of compounds in the matrix, it was still possible to detect the steroid esters in the extract using extraction of chromatograms with a narrow width around the exact mass of the different esters.

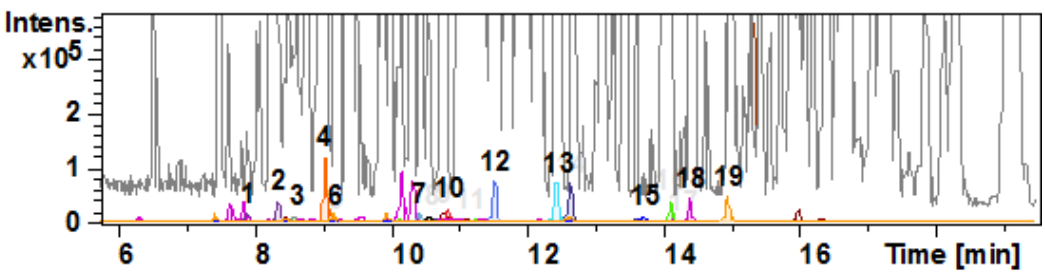

Figure 3. Overlaid base peak (grey line) and extracted ion chromatograms of the steroid esters (coloured lines).

The specificity of the analysis was evaluated by analyses of 20 blank samples and 20 blank samples spiked at CCβ level. The average concentration measured in the spiked samples was all higher than in the blank samples. The lowest difference was observed for the two TPH ethers giving 17.3 and 7.3 ng mL^{-1} in the spikes samples versus 9.9 and 5.4 ng mL^{-1} in the blanks. For *e.g*. nortestosteron decanoate the corresponding concentration were 9.7 versus 0.5 ng mL^{-1}. The extraction yields of the esters were between 60 and 120% estimated by comparison with a standard dissolved in solvent alone.

Verification of positive finding of steroid esters in samples was performed by alternating full scan and MRM analyses of the samples using precursor ion selection. In the MRM mode the chromatogram was divided into 6 segments containing between 1 to 4 precursor selections each. Ion ratios varied between 0.04 and 0.7 dependent on the type of ester. Analyses of 20 samples spiked at CCβ level gave ion ratio tolerances within 20% for ratios above 0.5, 25% for ratios above 0.2, 30% for ratios above 0.1 and 50% for ratios lower than 0.1.

Conclusions

In order to be able to detect illegal use of steroid esters in pig meat production, we developed a method for analyses of steroid esters in pig hair. The method included extraction of hair samples, dispersive SPE to remove the most lipophilic contaminants in the extracts and analyses by UPLC connected to high resolution accurate mass spectrometry. Using extraction of ion chromatograms with a narrow width around the exact mass of the esters resulted in a method with good sensitivity and specificity. Verification of positive findings in samples was accomplished by using alternating full scan and MRM and comparison of ion ratios between fragment and parent ions with ratios obtained from analyses of standards.

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PROFICIENCY TEST FOR RESORCYLIC ACID LACTONES IN *BOVINE* URINE

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Abstract
Proficiency testing is conducted to provide laboratories with a powerful tool to evaluate and demonstrate the reliability of the data that are produced. Next to validation and accreditation, proficiency testing is an important requirement of the EU Additional Measures Directive 93/99/EEC [1] and is demanded by ISO 17025:2005 [2].
The aim of this proficiency test was to give laboratories the possibility to evaluate or demonstrate their competence for the analysis of resorcylic acid lactones (RALs), group A4 of Commission Decision 96/23, in *bovine* urine. Also, the model designed by Launay *et al.* [11] was applied to determine whether findings of RALs are due to abuse of zeranol or feed contamination with *Fusarium* toxins.
This proficiency test was conducted in accordance with guideline ISO/IEC 17043 [3]. The preparation of the materials, including the suitability testing of the materials and the evaluation of the quantitative results were carried out under accreditation by RIKILT Wageningen UR. Five samples were sent to 43 participants. The results were evaluated on the basis of the participant's scope which included one to six analytes.

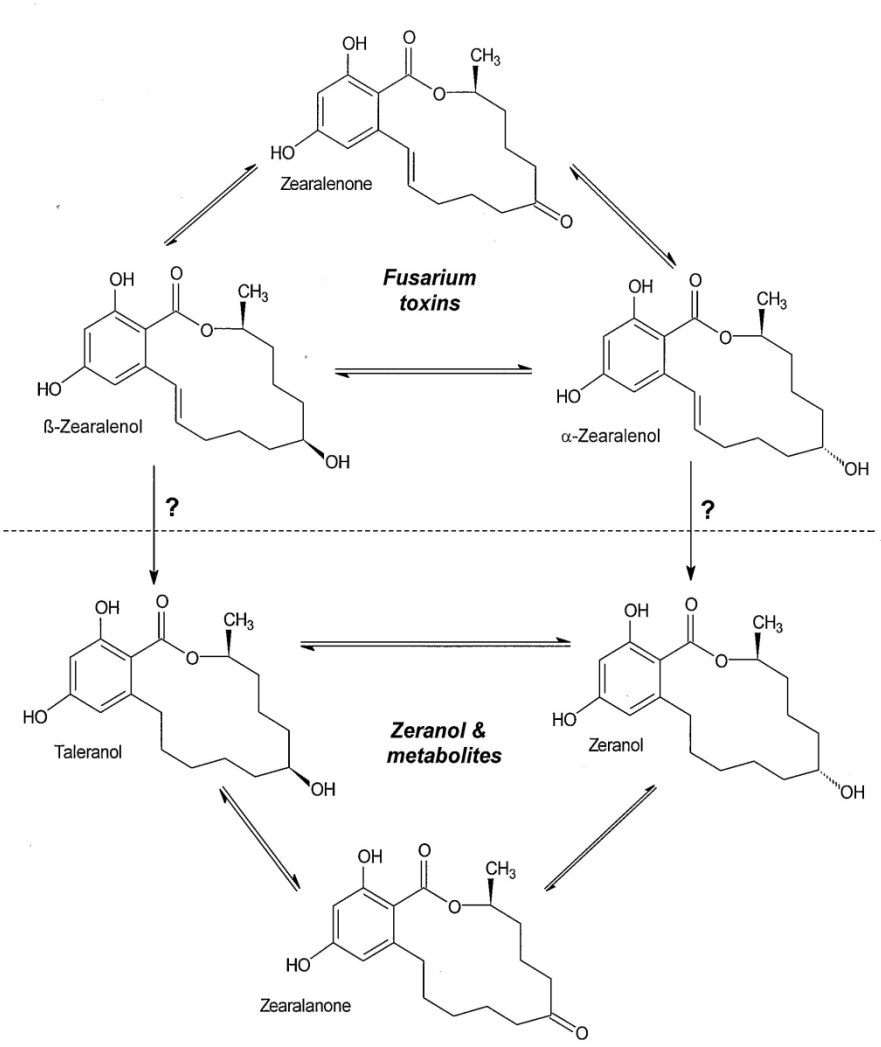

Figure 1. The structures and possible metabolic interconversions of zeranol and its metabolites (zeranol, taleranol and zearalanone) and the Fusarium *spp. toxins (zearalenone, a-zearalenol and ß-zearalenol). Chemical species above the dotted line are* Fusarium *spp. toxins and those below the dotted line are derived from zeranol [12].*

Introduction

The use of zeranol is banned in the European Union for use in food-producing animals. When administered to animals, zeranol (α-zeralanol, α-ZAL) metabolizes to taleranol (ß-zeralanol, ß-ZAL) and zearalanon (ZAN). Next to the abuse of zeranol, RALs can also be present in animal tissues due to the metabolism of the *Fusarium* spp. toxin zearalenone (ZEN) resulting in α-zearalenol (α-ZEL) and ß-zearelenol (ß-ZEL) (Figure 1). For this proficiency test five samples containing RALs originating from treatment with zeranol ranging from 0.5-10 µg L^{-1} were prepared. Additionally, two samples were enriched with ZEN and its metabolites.

Materials and Methods

Preparation of the materials

One blank material (A) and four incurred/spiked materials (B, C, D and E) were prepared. For materials B and C a urine sample obtained from a heifer treated with zeranol was mixed with blank urine to obtain target concentrations. Material D was prepared by adding a methanol solution of α-ZEL, ß-ZEL and ZEN to material A. Material E was prepared by mixing the sample obtained from the treated heifer with the same methanol solution of α-ZEL, ß-ZEL and ZEN to material B. Each of the materials was homogenized according to in-house standard operating procedures [13] and lyophilized. Samples were stored at <-18°C.

Homogeneity

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [4] and ISO 13528 [5], taking into account the insights discussed by Thompson [6] regarding the Horwitz equation. With this procedure the between-sample standard deviation (s_s) and the within-sample standard deviation (s_w) are compared with the target standard deviation for proficiency assessment derived from the Horwitz equation (σ_P). The method applied for homogeneity testing is considered suitable if $s_w < 0.5*\sigma_P$ and a material is considered adequately homogeneous if $s_s < 0.3*\sigma_P$.

Table 1. Concentrations of RALs in bovine urine in µg L^{-1} based on homogeneity test.

	Material A	Material B	Material C	Material D	Material E
α-ZAL, zeranol	-	0.52	1.2	-	0.36
ß-ZAL, taleranol	-	2.5	6.3	-	2.3
α-ZEL, α-zearalenol	-	-	-	1.9	2.1
ß-ZEL, ß-zearalenol	-	-	-	2.1	2.4
ZAN, zearalanon	-	-	1.1	-	-
ZEN, zearalenon	-	-	-	8.9	9.9

Ten containers of materials B, C, D and E were analysed in duplicate for all compounds to determine the homogeneity of the materials [9]. All materials demonstrated to be sufficiently homogeneous for use in the proficiency test. The results are included in Table 1. No extensive homogeneity study was carried out for material A (blank sample).

Stability

In a previous proficiency test for zeranol, taleranol and other metabolites in *bovine* urine (RIKILT report 2011.502) the stability in lyophilised urine was established: no consequential significant difference was observed between samples stored at <-70°C and samples stored at room temperature. Therefore, the samples in this test are considered sufficiently stable.

Evaluation of the performance

The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [6], elaborated by ISO, IUPAC and AOAC and ISO 13528 [5] in combination with the insights published by the Analytical Methods Committee [7,8] regarding robust statistics.

For the evaluation of the quantitative results, the assigned value, the uncertainty of the assigned value, a target standard deviation and z_a-scores were calculated according to ISO 13528. If the calculated uncertainty of the assigned value was significant ($u>0.3\sigma_P$) it influenced the evaluation. Therefore, the uncertainty was taken into account when calculating the z_a-scores.

Applied methods

Six laboratories applied GC-MS and the remaining applied LC-MS/MS for the confirmation and quantification. One lab applied RIA. The most frequently applied combination of purification and detection technique is the use of two SPE purifications (C_{18}

and NH_2) combined with LC-MS/MS (21 participants). Three laboratories applied a combination of immuno affinity purification with LC-MS/MS, two a combination of C_{18} and NH_2 SPE and GC-MS(/MS) detection and two a combination of immuno affinity purification and GC-MS(/MS). The remaining participants applied other clean-up procedures like centrifugation, filtration, liquid-liquid extraction or the use of a polymer SPE purification. Fifteen laboratories applied a hydrolysis step, the remaining labs did not report deconjugation steps. Several different internal standards were used.

Results

Forty-three participants subscribed for the proficiency test and 38 reported results. All labs included α-ZAL and ß-ZAL in their method, 31 included α-ZEL and ß-ZEL, 26 included ZAN and 31 included ZEN.

The results of this test are presented in Table 2 and Figure 2. In some cases (ß-ZEL in materials B and C, ZAN in B and E and ZEN in B and C) the variation in the reported results was too high and statistical evaluation was not possible, since this would result in unjustified conclusions.

Table 2. Results of RALs in materials B-E.

Material		Range µg L^{-1}	# Compliant/non-compliant	Consensus value µg L^{-1}	# results	Correct results (%)
A	-	-	27/3**	-	-	-
B	α-ZAL	0.25-0.94	5/25	0.48	26	69
	ß-ZAL	0.4-5.36		1.9	35	69
	ß-ZEL	0.05-2.31			9	*
	ZAN	0.2-3.82			8	*
	ZEN	0.09-4.1			8	*
C	α-ZAL	0.4-2.28	6/25	0.98	34	76
	ß-ZAL	0.8-12.8		4.5	37	62
	ß-ZEL	0.5-2.88			8	*
	ZAN	0.5-1.77		0.98	15	80
	ZEN	0.2-5.39			10	*
D	α-ZEL	0.81-3.8	25/7	1.8	33	67
	ß-ZEL	0.15-8.2		2.1	32	56
	ZEN	3.45-66.8		9.0	33	67
E	α-ZAL	0.25-1.06	19/11	0.45	26	69
	ß-ZAL	0.4-6.21		1.8	37	70
	α-ZEL	0.62-3.82		1.8	33	67
	ß-ZEL	0.1-11.4		2.1	32	59
	ZAN	0.25-1.077			7	*
	ZEN	2.83-70.5		9.1	33	73

** no statistical evaluation possible, due to high uncertainty of the consensus value*
***These non-compliant results are likely due to misinterpretation of the protocol*

No false positive results were reported in material A, which was the blank sample. Samples B and C were correctly identified as originating from a treated animal due to the presence of zeranol (α-ZAL) and its metabolite taleranol (β-ZAL). Laboratories that did not detect these compounds scored a false negative (FN) result (Table 3). The presence of α-ZEL (> 1 µg L^{-1}) is considered a false positive result. Material D contains ZEN and its metabolites. Failing to detect these compounds results in a false negative result, detecting zeranol or taleranol a false positive result. Material E contained all of the five RALs included in this PT and failing to detect them is considered a false negative result.

Table 3. False positive (FP) and false negative (FN) results.

	Material A	Material B	Material C	Material D	Material E
α-ZAL, zeranol		3 FN	4 FN	1 FP	3 FN
ß-ZAL, taleranol		2 FN	5 FN	3 FP	4 FN
α-ZEL, α-zearalenol		1 FP	2 FP	5 FN	4 FN
ß-ZEL, ß-zearalenol				5 FN	6 FN
ZAN, zearalanon					
ZEN, zearalenon				2 FN	1 FN

In this test seven false positive and 44 false negative results were reported. Concentrations of these false positive results ranged from 1.77 µg L^{-1} α-ZEL to 7.08 µg L^{-1} α-ZEL in material C. A result was assigned a false negative result if a compound was not detected, taken into account the reported scope of the participant, the consensus value and the reported CCα.

Figure 2 presents the results per analyte in terms of the absolute z_a-scores; either exceeding 2 or between 0 and 2. Additional, the percentage false negative and qualitative results are shown.

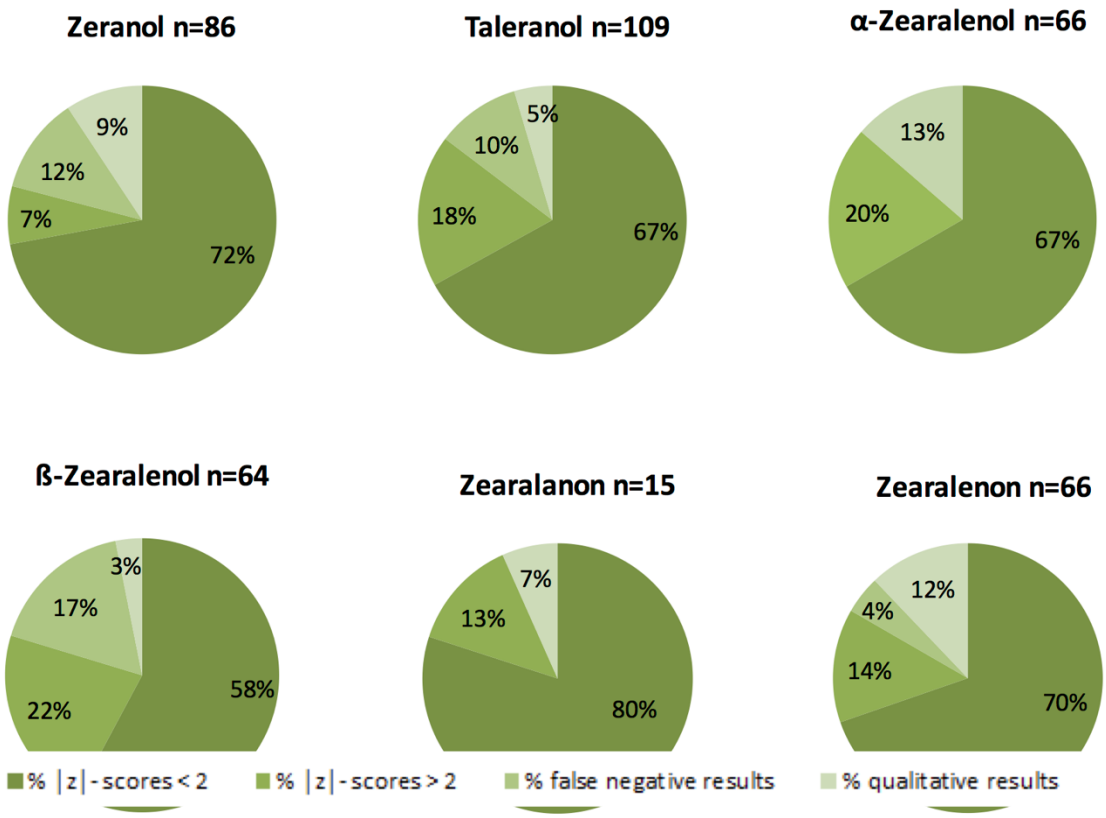

Figure 2. Overview of |z|-scores <2 and >2, false negative results and qualitative results.

From a control point of view zeranol and taleranol are the most important compounds since they are directly related to abuse of zeranol. For these analytes the percentage false negative results is approximately 10. Since there is in this respect no difference between samples B (lower level of both compounds) and C (higher level of both compounds), it seems to be an intrinsic problem with the methods used, and not the CCα. Of the seven laboratories that reported these false negative results, there is no analogy in the applied methods and/or in the deconjugation step. For the other RALs false negative rates varied from 4 to 17%.

In a similar proficiency test for RALs in *bovine* urine in 2010 86% of all results for zeranol was correct, 78% for taleranol, 42% for ß-zearalenol and 52% for zearalanone. Due to a limited number of reported results in 2010, α-zearalenol and zearalenone were not statistically evaluated.

Abuse or contamination, compliant or non-compliant?

Based on the research of Launay *et al.* [10] as also summarized in the EURL Reflection paper [11], material A, the blank sample, should be considered compliant in all cases. Materials B and C, in which zeranol and taleranol are the major compounds present, should be considered as samples for which there was proof of zeranol abuse. The majority of participants indeed classified these samples as non-compliant. Materials D should be evaluated as compliant in which the detected RALs as a consequence of feed contamination with *Fusarium* spp. toxins. Most of the laboratories classified this sample correctly. Sample evaluation of the results for E is the most challenging. Most of the laboratories classified this sample as non-compliant. However, due to the presence of *Fusarium* spp. toxins, a significant number of laboratories classified this sample as compliant.

Based on the consensus values as well as the initial values, the protocol of Launay *et al.* would result in a classification "compliant". Next to the equation of Launay *et al.*, a quick decision about abuse or contamination can be made by dividing the

sum of α-ZAL and ß-ZAL (abuse) by the sum of α-ZEL, ß-ZEL and ZEN (contamination). A value of around 1 is the transition point; <<1 means the samples are contaminated and ≫1 means the samples are proof of abuse.

These results show that masking abuse of zeranol with *Fusarium* spp. toxins is possible. However, it must be realized that the samples used were artificially spiked and are not representative for most of the real samples analysed since feed contamination with *Fusarium* spp. does not represent a large scale problem.

Conclusions

Forty-three laboratories, of which 26 National Reference Laboratories within Europe, subscribed for the proficiency test for resorcylic acid lactones in *bovine* urine. Out of these labs, 8 labs (7 NRLs) showed optimal performance by correct quantification of the samples within the participants' scope and the absence of false positive and false negative results. Twenty-eight labs reported FN, FP, questionable or unsatisfactory z-scores for one or more individual results and two labs showed suboptimal performance (qualitative results, no information about compliancy of samples). Four labs were unable to report results and one lab reported no results due to high CCα-values. A total of seven false positive and 44 false negative results was reported.

The optimal performing labs applied a variety of purification methods, GC- or LC-MS and several different internal standards. Therefore, there is no specific method providing "the best" results.

Correct quantification of low levels of RALs remains a challenging task for laboratories. A small number of laboratories should invest in improving the method used.

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EFFECT OF STORAGE, THERMAL PROCESSING AND PH ON THE STABILITY OF PENICILLINS G AND V IN COW MILK

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Abstract
The effect of various storage conditions, thermal processing methods and pH on the stability of penicillin G and penicillin V in cow milk was investigated. The results showed that penicillin G, contrary to penicillin V, is very sensitive at acidic conditions, but both penicillins are stable for at least one hour at a pH range of 6-10. Penicillins G and V are stable for approximately 24 h at 25°C in water whereas further storage results in a gradual degradation, which is completed in 30 days. The presence or absence of light did not significantly affect the stability of both penicillins. The aqueous solutions of penicillin G and penicillin V can be stored for 6 and 15 days, respectively, at 4°C, whereas they can be stored with spiked and incurred cow milk samples for at least 3 months at -28°C and -80°C. Finally, the pasteurisation of aqueous solutions and of spiked and incurred cow milk samples does not affect the stability of these penicillins, but boiling and, especially, sterilization significantly reduce their concentration.

Introduction
Penicillins, especially penicillin G, are the drugs of choice for treating infections in livestock. However, their administration in food-producing animals could imply the possible presence of their residues in food products. The presence of such residues is an important issue due to the potential health hazards arising from ingestion of such products by the consumer. Milk is the foodstuff of greatest interest, in terms of public health, because of its daily consumption, especially by children. Information on the effect of cold storage, thermal processing and pH on the stability of penicillins G and V residues in milk could be useful for assessments of the actual risk to the consumers' health.

For residue analysis, milk samples should be transported to a testing laboratory and stored at low temperature (frozen or refrigerated) pending analysis. Therefore, the evaluation of the stability of penicillin residues under different storage conditions and the knowledge of the optimal storage and processing conditions of the milk samples are key issues for successful laboratory analysis (Botsoglou and Fletouris, 2001). A literature survey shows that the data regarding the effect of storage conditions on the stability of penicillin G in milk are limited (Shahani *et al*., 1956; Riediker *et al*., 2004; Różańska and Osek, 2013), whereas there are no studies concerning penicillin V.

Information about the effect of thermal processing on the stability of residues of penicillins G and V in milk is also important from a toxicological point of view. Although most milk produced is consumed after some type of thermal processing, relatively few studies (Shahani *et al*., 1956; Zorraquino *et al*., 2008; Roca, 2011) have reported the effect of heating on penicillin G residues, and therefore on the actual dietary exposure of this antibiotic and any breakdown products to the consumer. On the contrary, most information on penicillins G and V residues in milk and regulatory considerations are based on raw milk.

Most of the above mentioned studies (Shahani *et al*., 1956; Zorraquino *et al*., 2008; Różańska and Osek, 2013) used microbiological and/or receptor assays but these assays are limited owing to their lack of specificity, accuracy and precision. Moreover, some discrepancy between the results of these studies was observed and they had been conducted by fortifying antibiotic-free milk with penicillin G.

The aim of this study was to use an accurate and precise liquid-chromatographic method to produce quantitative data about the change in concentration of penicillin G and penicillin V residues in fortified and incurred cow milk, and in aqueous solutions during cold storage and common heating processing methods.

Materials and Methods

Chemicals and equipment
LC-grade acetonitrile and reagent-grade dichloromethane, chloroform, diethyl ether, sodium hydroxide, phosphoric acid, sulphuric acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, ammonium sulphate, and tetrabutyl-ammonium hydrogen sulphate were obtained from Merck (Darmstadt, Germany). Penicillin G sodium and penicillin V potassium were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Liquid-chromatography was carried out using a model UFLC Shimadzu system (Shimadzu Corp., Kyoto, Japan) equipped with an LC-20AD pump, a DGU-20A5 on-line degassing unit, a SIL-20AD automatic sample injector, a CTO-20A column oven and an SPD-M20A photo-diode array detector. LC solution software (Shimadzu) was utilized to control the system. Analyses were

performed on a reversed-phase Nucleosil 100-5 C_{18}, 5 µm material in Macherey-Nagel (Düren, Germany) analytical (250 x 4.6 mm i.d.) and guard (10 x 4.6 mm i.d.) columns.

An FT74P miniature-scale UHT/HTST processing system (Armfield, Ringwood, United Kingdom), an HA-300MII sterilization system (Hirayama, Tokyo, Japan) and a waterbath (Memmert, Schwabach, Germany) were used for thermal processing of milk, a MDF-U7386S deep freezer (Sanyo Electric Co., Osaka, Japan), a freezer, a refrigerator and a controlled temperature incubator (J.P. Selecta S.A., Barcelona, Spain) were used for sample storage, a Milli-Q purification system (Millipore, Bedford, MA, USA) was used for ultrapurification of tap water, and a rotary evaporator (Büchi, Flawil, Switzerland), a G-560E vortex mixer (Scientific Industries, Bohemia, NY, USA), a Centra-MP4 centrifuge (IEC, Needman Heights, MA, USA), and an evaporation unit (ReactiTherm, Pierce Chem., Rockford, IL, USA) were used for sample preparation.

Animals, drug administration and sampling

Five (5) healthy Holstein Friesian cows of 4-5 years of age were used in this study. Each cow was administered three successive intramammary infusions with a penicillin G formulation at the dose of 300,000 IU 24 h^{-1}. The trial was carried out in compliance with Directive 2010/63/EU (European Commission, 2010) on the protection of animals used for scientific purposes. The animals were milked twice daily (12 h intervals) and the milk of the second and third milking, after medication, of all five cows was mixed and stored frozen at -20°C pending processing. Control milk was collected two days prior to the time the cows were administered the intramammary infusions.

Stability of penicillins in aqueous solutions

Effect of pH. Standard solutions containing 1.98 µg mL^{-1} penicillin G and 2.08 µg mL^{-1} penicillin V were prepared in 0.02 M phosphate buffer solution at pH 2, 3, 4, 5, 6, 7, 8, 9 or 10. These solutions were stored in capped glass centrifuge tubes at 25°C for 60 min and the concentration of the analytes in each tube was measured after 10, 20, 30, 40, 50 and 60 min of storage.

Effect of storage temperature. Standard solutions containing 1.98 µg mL^{-1} penicillin G and 2.08 µg mL^{-1} penicillin V were prepared in water. These solutions were stored for 90 days in capped polypropylene centrifuge tubes at 25°C in the dark and in the light, and at 4°C, -28°C and -80°C in the dark. The concentration of the analytes in each tube was measured after 3, 6, 9, 12, 15, 20, 30, 45, 60 and 90 days of storage. Moreover, the effect of thawing and freezing the standard solutions (three cycles) on the stability of penicillins at -28°C and -80°C was also studied.

Effect of thermal processing. Standard solutions containing 495 ng mL^{-1} penicillin G and 520 ng mL^{-1} penicillin V were prepared in water. These solutions were dispensed into capped glass centrifuge tubes, which were subjected to pasteurization (65°C and 72°C for 30, 45 and 60 min, and for 15 s, 2 and 10 min, respectively), boiling (100°C for 2, 5 and 10 min) and autoclaving (120°C for 10, 15 and 20 min).

Six replicates were carried out for all the above mentioned treatments concerning the stability of penicillins in aqueous solutions.

Stability of penicillins residues in milk samples

Effect of freezing temperature. Fortified (penicillin G and penicillin V) and incurred (penicillin G) milk samples were frozen in capped polypropylene centrifuge tubes at -28°C and -80°C for 90 days. The concentration of the analytes in each tube was measured after 15, 30, 45, 60 and 90 days of storage.

Effect of thermal processing. Fortified (penicillin G and penicillin V) and incurred (penicillin G) milk samples were dispensed into capped glass centrifuge tubes, which were subjected to pasteurization (65°C and 72°C for 30, 45 and 60 min, and for 15 s, 2 and 10 min, respectively), boiling (100°C for 2, 5 and 10 min) and autoclaving (120°C for 10, 15 and 20 min).

Six replicates were performed for all the above mentioned treatments concerning the stability of penicillins in milk samples.

Sample preparation and LC analysis

Analysis of the milk samples was performed according to the method of Fletouris *et al*. (1992). In brief, milk is acidified at pH 3 and extracted with dichloromethane. The extracted penicillins were partitioned into a phosphate buffer, pH 7, and following addition of ammonium sulphate, the extracts were purified by treatment with diethyl ether and repartitioned into acetonitrile. The acetonitrile extracts were concentrated into phosphate buffer, pH 7, and after addition of tetrabutyl-ammonium hydrogen sulphate the formed penicillin ion pairs are extracted into chloroform. The chloroform is evaporated to dryness and the residue is dissolved in mobile-phase to be further injected (100 µL) into the LC system and analysed using a mobile phase consisting of acetonitrile and 0.02 M phosphate buffer (38:62 v/v), containing 5 mM tetrabutylammonium hydrogen sulphate and adjusted at pH 6.

Method validation

Validation was performed according to the Commission of the European Communities guidelines (European Commission, 2002) using control cow milk. The specificity, linearity, sensitivity, accuracy, precision, applicability, and stability of the analytes were the criteria used to evaluate the developed method.

Statistical analysis

All data were submitted to analysis of variance using the general linear model of the SPSS 17.0 statistical package (SPSS Ltd., Woking, Surrey, UK). Before statistical analysis, the Levene's test was applied to test the homogeneity of the variances. A probability level of $P \leq 0.05$ was used in testing the statistical significance of all data.

Results and discussion

Stability of penicillins in aqueous solutions

Effect of pH. Both neutral and alkaline pH did not affect the stability of penicillin G and penicillin V in aqueous solutions at 25°C. The data collected in this study show that both penicillins were stable for 60 min at pH values from 7 to 10. These findings are not consistent with those of Kheirolomoom *et al*. (1999) and Lu *et al*. (2008) who reported degradation of penicillin G at a pH above 8. This inconsistency should be attributed to the fact that their experiments lasted much longer (100 and 240 h, respectively).

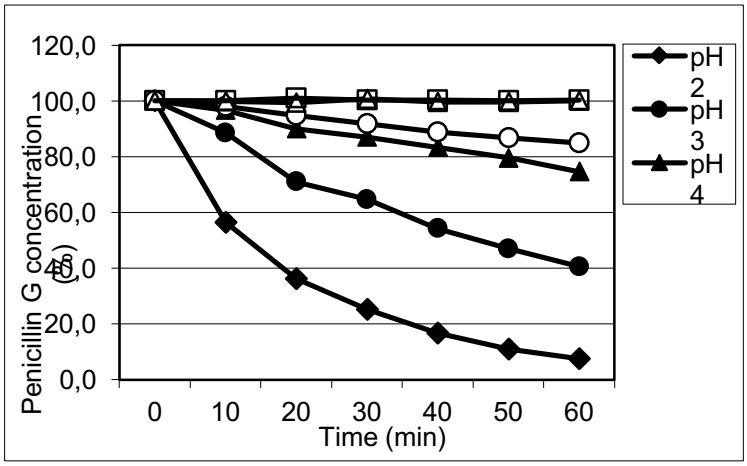

Figure 1. Effect of acidic pH on the stability of penicillin G (1.98 µg mL^{-1}) in aqueous solutions at 25°C.

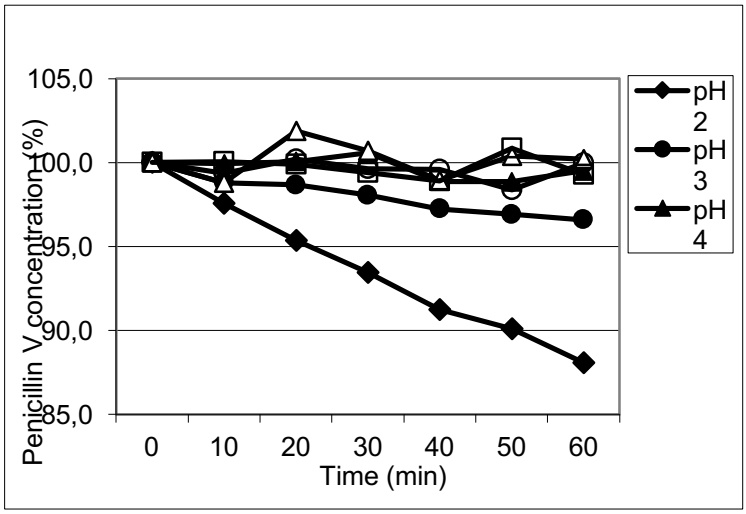

Figure 2. Effect of acidic pH on the stability of penicillin V (2.08 µg mL^{-1}) in aqueous solutions at 25 °C.

Contrary to neutral and alkaline pH, acidic pH significantly affects the stability of penicillin G (Figure 1) and penicillin V (Figure 2) in aqueous solutions at 25°C. Figure 1 shows clearly the high sensitivity of penicillin G under acidic conditions. The re-

duction of the concentration begins at pH 5 and becomes more pronounced with decreasing pH. When the pH of the aqueous solution of penicillin G was adjusted at 2, only 10 and 60 min were required for a 40 and 90% reduction of its concentration, respectively. These findings are consistent with those of Kheirolomoom *et al*. (1999), Lu *et al*. (2008), and Ren *et al*. (2010) who reported stability of penicillin G at neutral pH and degradation at pH values lower than 5. Moreover, our results do not differ from those of McKellar and Horspool (1995) who indicate an 84.7%-reduction of the concentration of penicillin G at pH 1.9 after one hour.

With regard to penicillin V, the experiments performed in this study confirmed the acid resistant nature of this compound (Figure 2). Aqueous solutions of Penicillin V exhibit remarkable stability for 1 h at pH 3 to 6, while at pH 2 a 12% reduction of its concentration was observed after storing the solution for 1 h at 25°C.

Effect of storage temperature. Storage temperature significantly affects the stability of both penicillins (Table 1). In particular, at 25°C, degradation of these penicillins is rapid, whereas penicillin V seems to be relatively more stable than penicillin G. A storage time of 30 days is required for a 100% reduction of the concentration of both penicillins. Regarding the effect of light on the stability of the aqueous solutions of penicillins G and V at 25°C, compared with that of darkness, the results show that there is no significant difference (P >0.05) on the reduction of their concentration.

Contrary to 25°C, the stability of penicillins G and V in aqueous solutions at 4°C significantly increases. More specifically, Table 1 shows that penicillin G remains stable for 6 days and penicillin V for 15 days at 4°C. Under these conditions more than 60 days are required for the complete degradation of both penicillins.

Table 1. Stability of penicillin G (1.98 µg mL^{-1}) and penicillin V (2.08 µg mL^{-1}) in aqueous solutions during storage at 25^{o}C (light and dark) and 4^{o}C (dark).

Time (days)	Concentration change (%) ± SD*					
	Light (25°C)		Dark (25°C)		Refrigerator (4°C)	
	Penicillin G	Penicillin V	Penicillin G	Penicillin V	Penicillin G	Penicillin V
0	0.0	0.0	0.0	0.0	0.0	0.0
3	-14.8 ± 2.0	-9.2 ± 0.6	-13.6 ± 2.7	-8.3 ± 1.4	-0.2 ± 1.6	-2.9 ± 1.9
6	-69.5 ± 4.9	-41.4 ± 15.4	-64.4 ± 6.5	-36.2 ± 6.1	0.1 ± 2.4	-0.4 ± 2.6
9	-87.4 ± 1.9	-62.6 ± 15.4	-81.4 ± 3.5	-57.6 ± 8.9	-5.4 ± 5.5	1.7 ± 0.7
12	-97.9 ± 1.1	-87.0 ± 10.4	-96.0 ± 2.7	-80.9 ± 15.0	-8.8 ± 3.1	-0.1 ± 2.1
15	-98.1 ± 2.1	-89.6 ± 9.0	-96.6 ± 3.1	-83.9 ± 10.7	-13.7 ± 5.3	-1.0 ± 3.8
20	-99.5 ± 0.8	-98.9 ± 1.3	-97.7 ± 2.2	-98.3 ± 2.7	-19.9 ± 6.1	-9.7 ± 5.1
30	-100.0	-100.0	-100.0	-100.0	-30.0 ± 6.6	-19.5 ± 9.2
45	-100.0	-100.0	-100.0	-100.0	-57.6 ± 16.4	-43.7 ± 16.8
60	-100.0	-100.0	-100.0	-100.0	-98.2 ± 2.0	-92.9 ± 6.9
90	-100.0	-100.0	-100.0	-100.0	-100.0	-100.0

** Six replicates.*

As far as the stability of penicillins G and V to freezing is concerned, the results showed that 3 months of storage at -28 and -80 °C, did not produce any change on the concentration of both analytes in aqueous solutions. Furthermore, the effect of thawing and freezing the aqueous solutions (three cycles) at -28 °C and -80 °C did not significantly affect (P >0.05) the concentration of these penicillins.

The results of our study are not consistent with those of Okerman *et al*. (2007), who reported a 21% reduction of the concentration of penicillin G in aqueous solutions after three months of storage at -20 °C. This inconsistency might be attributed to the higher storage temperature used in this study.

Effect of thermal processing. The results concerning the stability of penicillin G and Penicillin V in aqueous solutions during common heating processing methods are presented in Table 2. The results show that both penicillins are resistant to heating treatments analogous to milk low temperature long time (LTLT) and high temperature short time (HTST) pasteurization, even when these treatments last longer.

Contrary to pasteurization, boiling significantly (P ≤0.01) affects the concentration of penicillin G and penicillin V in aqueous solutions (Table 2). Our findings are consistent with those of Rose *et al*. (1997) who reported 50% reduction of penicillin G concentration in aqueous solution after boiling for 60 min.

A dramatic reduction (P ≤0.0001) in the concentration of penicillins G and V was observed when the aqueous solutions were subjected to sterilization (Table 2). When the temperature-time combination was proportional to milk sterilization, the reduction of the concentration of both penicillins was greater than 81%.

It is worth noting that there are no bibliographic data relating to the effect of storage temperature and common heating processing methods on the stability of penicillin V in aqueous solutions.

Stability of penicillins residues in milk samples

Effect of freezing temperature. As far as the stability of penicillin G and penicillin V residues to freezing is concerned, the results showed that 90 days storage at -28°C and -80°C did not produce any change in the concentration of the analytes in fortified (penicillin G and penicillin V) and incurred (penicillin G) milk samples. Furthermore, three thawing-freezing cycles of both fortified and incurred milk samples at -28 and -80°C did not significantly affect (P >0.05) the concentration of these penicillins. The finding concerning storage stability of penicillin G residues at -80°C supports a previous finding reported by Riediker *et al*. (2004), but our results are in direct contrast to those reported by the same authors who observed significant reduction of the concentration of penicillin G residues during three days of storage at -20°C. This inconsistency might be attributed to the higher storage temperature used in this study.

Table 2. Effect of heating treatments on the stability of penicillin G (495 ng mL^{-1}) and penicillin V (520 ng mL^{-1}) in aqueous solutions and cow milk.

Heating treatment	Temperature/ time	Concentration change (%) ± SD*			
		Penicillin G		Penicillin V	
		Aqueous solution	Cow milk	Aqueous solution	Cow milk
Pasteurization	65°C/30 min	0.06 ± 0.45^{aA}	-2.84 ± 5.69^{aAB}	-1.17 ± 0.69^{aB}	-1.44 ± 5.37^{aAB}
	65°C/45 min	-1.72 ± 1.86^{aA}	-1.19 ± 5.25^{aA}	-2.18 ± 1.85^{aA}	-1.97 ± 8.02^{aA}
	65°C/60 min	0.22 ± 0.87^{aA}	0.97 ± 6.84^{aAB}	-0.56 ± 1.17^{aB}	-0.87 ± 5.92^{aAB}
	72°C/15 sec	0.47 ± 0.48^{aA}	-0.98 ± 6.59^{aA}	0.37 ± 0.44^{aA}	0.05 ± 5.58^{aA}
	72°C/2 min	-0.28 ± 0.43^{aAC}	2.90 ± 8.98^{aAD}	-0.50 ± 0.38^{aBD}	-2.69 ± 6.43^{aBC}
	72°C/10 min	-2.02 ± 1.74^{aA}	-1.38 ± 7.07^{aA}	-1.08 ± 2.01^{aA}	-2.47 ± 5.61^{aA}
Boiling	100°C/2 min	-12.09 ± 4.94^{bA}	-10.03 ± 9.55^{abA}	-8.91 ± 2.95^{bA}	-8.98 ± 8.32^{abA}
	100°C/5 min	-21.10 ± 7.71^{bA}	-18.03 ± 9.34^{bA}	-14.67 ± 3.55^{bcA}	-12.14 ± 8.96^{abA}
	100°C/10 min	-34.57 ± 3.18^{cA}	-30.04 ± 11.24^{bAB}	-23.85 ± 7.63^{cAB}	-18.21 ± 9.73^{bB}
Sterilization	120°C/10 min	-80.68 ± 4.92^{dA}	-81.26 ± 10.77^{cA}	-77.02 ± 7.26^{dA}	-70.80 ± 10.04^{cA}
	120°C/15 min	-84.86 ± 3.64^{eA}	-88.08 ± 10.22^{cdA}	-81.67 ± 5.11^{eA}	-78.35 ± 9.32^{cdA}
	120°C/20 min	-93.01 ± 5.67^{eA}	-94.18 ± 6.07^{dA}	-87.24 ± 7.75^{eA}	-85.34 ± 8.54^{dA}

** Six replicates; ^{a,b,c,d,e} Values in the same column with a superscript in common do not differ significantly (P >0.05); ^{A,B,C,D} Values in the same line with a superscript in common do not differ significantly (P >0.05).*

Effect of thermal processing. The results concerning the stability of penicillins G and V residues in fortified cow milk samples during common heating processing methods are presented in Table 2. The results show that both penicillins are resistant to common pasteurization treatments of milk (LTLT and HTST), even when these treatments last longer. Moreover, heating of incurred (penicillin G) cow milk samples under the same conditions do not affect the concentration of penicillin G residues. Our findings concerning penicillin G residues are in accordance with those in the study of Riediker *et al*. (2004), but are inconsistent with the results of Shahani *et al*. (1956) and Zorraquino *et al*. (2008).

In contrast to pasteurization, boiling significantly (P ≤0.01) affects the concentration of penicillin G and penicillin V residues in fortified milk samples (Table 2). The reduction of the concentration of penicillins G and V was more pronounced when the fortified milk samples were subjected to sterilization (Table 2). Analogous reduction of the concentration of penicillin G residues in incurred cow milk samples was observed when these samples were subjected to boiling and sterilization conditions. The results of our study are consistent with those of Zorraquino *et al*. (2008) who reported more than 65% inactivation of penicillin G residues during milk sterilization.

As regards to penicillin V, this paper reports for the first time findings concerning the stability of residues of this antibiotic in fortified cow milk samples during cold storage and common heating processing methods.

Conclusions

The results of the present study clearly indicate that penicillin G, contrary to penicillin V, is very sensitive at acidic conditions, but both penicillins are stable for at least one hour at a pH range of 6-10. Penicillins G and V are stable for approximately 24 h at 25°C in water, regardless of the presence or absence of light. The aqueous solutions of both penicillins can be stored for at least six days at 4°C, whereas they can be stored with spiked and incurred cow milk samples for at least 3 months at -28°C

and -80°C. Moreover, the pasteurization of aqueous solutions and spiked and incurred cow milk samples does not affect penicillins concentration, but boiling and sterilization significantly reduce their concentration. The data presented in this study could help to improve laboratory analysis of penicillin G and penicillin V, and to protect public health.

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LONG TERM DETECTABLILITY OF RECOMBINANT *BOVINE* SOMATOTROPIN IN SERUM AND MILK

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Abstract
The recombinant-DNA protein hormone *bovine* somatotropin (rbST) is known to enhance effectively milk production in cattle. For this, a syringe containing 500 mg of rbST in a slow release formula needs to be administered subcutaneously once every two weeks to ensure continuous rbST bioactivity between subsequent administrations. Knowledge about how equally rbST is released in the period between the subsequent administrations, its detectability in blood and differences between individual cows is lacking. This information is of great added value for control purposes, as rbST use is forbidden in the European Union. To obtain the required knowledge, ten dairy cows were treated with rbST via slow release formulae and blood samples were taken daily during a 14-day period to obtain a detailed release profile from a comprehensive sample set. Presence of rbST in serum was monitored by a quantitative detection method based on immuno-enrichment followed by liquid-chromatography tandem mass-spectrometry. Release profiles in serum will be presented and additionally combined with preliminary results of rbST concentrations in associated milk samples, detected with a newly developed method.

Introduction
Milk production in dairy cattle can be enhanced 10 to 25% by administration of recombinant *bovine* somatotropin (rbST) (1). For this, the rbST which is stored in slow release formulae, needs to be injected subcutaneously once every two weeks. This frequency is low due to the slow release formulae, which enables gradual discharge of rbST into the bloodstream, via which it is expected to transfer to the milk. Presence of rbST in both milk and blood products in dairy cattle treated according to the manufacturers manual (2) is expected and useful for control purposes. Control is required, as the use of rbST is forbidden in Europe according to Council Decision 1999/879/EC (3), but control can also be desirable in case of consumers concern like for instance is seen in the USA.

To pinpoint rbST treatment, methods need to be able to discriminate endogenous bST from administered exogenous rbST. This is a major challenge due to i) low rbST concentrations in blood and milk, ii) the sequence similarity; only one amino acid out of in total 191 amino-acids differs from endogenous bST. Despite these challenges antibodies with high specificity for exogenous rbST were developed and used in an ELISA, but unfortunately the required sensitivity to enable detection of rbST in serum or milk samples was not reached (4). Two liquid-chromatography tandem mass-spectrometry (LC-MS/MS) methods able to detect rbST in serum at relevant concentrations of below 10 ng mL^{-1} were described in literature (5, 6).

Preliminary results showed that the method described by Smits *et al.* (6) was able to detect rbST during the 14-day period between two subsequent administrations. But for milk, until now, rbST was only detected in spiked blank milk samples (7) and never in incurred samples. Although the method for rbST detection in serum is suitable, a method to detect rbST in milk is preferred. This preference is due to the sample taking, as taking milk samples from individual cows or milk collection tanks, is much easier and faster than drawing blood from individual cows. Another important consideration in favour is that milk sampling is non-invasive, in contrast to blood drawing.

In this study we will extend the data for serum rbST concentrations. From nine rbST-treated cows, results were obtained from daily taken serum samples between two subsequent rbST administrations. Furthermore, preliminary results of spiked milk samples and an incurred milk sample are presented.

Materials and Methods

Serum and milk samples
Serum and milk samples from two different controlled animal experiments were used for analysis. Both controlled animal treatment studies used the same administration schedule. Before treatment, first an adaptation period of 2 weeks was taken into account, then cows were treated every second week for four times with subcutaneous injections of 500 mg rbST from Posilac® in a slow-release formula according to manufacturers' guidelines. In the first study, eight cows (a-h) and in the second study two cows (i,j) were treated with this rbST formulae. The cows of both studies were all lactating not pregnant Holstein Friesian, which varied in age (from 2 to 7 years) and were in different stages of lactation. Moreover, in the first study

two lactating Holstein Friesians cows followed the same treatment schedule, however with vehicles containing the slow-release formulae only. During the treatment period, blood and milk samples were collected shortly before injection, and at a day and a week after injection. Additionally, after the fourth subcutaneous injection, for both the rbST treatment and the vehicle treatment group, serum and milk were collected daily (day 1 – 14, with rbST administration on day 1 after sample collection). After blood collection, the blood samples were placed at room temperature for 4 h to coagulate. After coagulation, the samples were centrifuged for 10 min at 3,000 *g*, and serum was collected and stored at -20°C until further use. After milk collection, the milk samples were stored at -20°C directly until further use. For the first study, the experimental procedure was authorized by the ethical committee of ID-DLO in Lelystad, the Netherlands. For the second study the experimental procedure was authorized by the ethical committee of Wageningen University, the Netherlands.

Sample treatment and rbST measurement
To extract and concentrate rbST from serum samples, followed by digestion to obtain the specific N-terminal peptide and LC-MS/MS measurements different steps has to be followed. All these steps are described by Smits *et al*. (6). To concentrate rbST from milk samples roughly the same steps were used with the exception that after thawing, the milk was centrifuged for 10 min at 4°C, then 1 mL from the aqueous layer was used and diluted with 1 mL 20 mM phosphate buffer followed by extraction of the rbST from the milk samples according to the same method as described for serum. A calibration curve was made in milk by spiking raw milk samples with rbST to concentrations of 0.5, 1 and 1.5 ng mL^{-1}. This calibration curve was repeated 5 times on the same day.

Results and Discussion

Serum rbST concentrations after rbST treatment
RbST concentrations were determined in serum samples taken daily over a period of 14 days from nine rbST-treated cows and two untreated cows. No rbST was detected in any sample of the untreated cows, as expected. For the rbST-treated cows, rbST was detected over the full period of time between subsequent rbST injections. Although cows are all treated similar, with 500 mg rbST in a slow release formula, the concentrations found in the serum differ a lot, with extremes of 10-fold differences. Daily averages of serum rbST concentrations from nine rbST-treated cows were calculated and results are shown in Figure 1. For all cows an elevation in serum rbST concentration is seen the first days after rbST administration. The third day after administration this elevation is reduced.

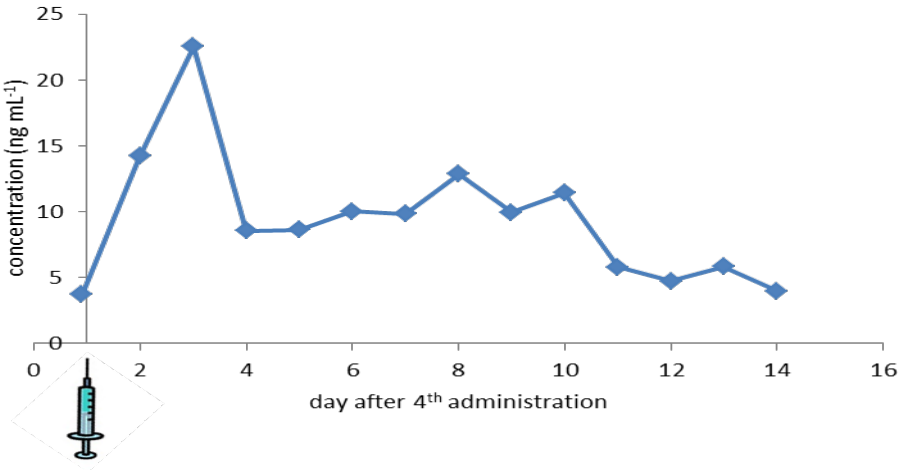

Figure 1. Daily average (n=9) serum rbST concentrations after rbST administration.

This data shows that the detection method described by Smits *et al.* (6) is sensitive enough to measure rbST in serum over the full length of treatment when administration takes place according to the protocol of the manufacturer. This enables control of rbST abuse, however taking blood samples is considered invasive for cows and therefore not preferable. In practise collecting milk samples is much easier. Milk samples can be collected per cow, but also a mix sample containing milk from all the lactating cows from the farm can be taken. Therefore, only a sample from the collection tank where milk of all cows is combined needs to be taken.

Milk rbST concentrations after rbST treatment
It is expected that blood circulating rbST ends up in the milk, however rbST concentrations are predicted to be about 10 times lower in milk than in blood. Therefore, an even more sensitive method is needed and the method also needs to deal

with another type of complex matrix, the fatty matrix milk. This study will show preliminary results of the adjusted serum method applied to defatted raw milk samples. To demonstrate the potential of this method, a small calibration curve in five-fold was made of blank milk samples, and milk samples spiked with three different concentrations of rbST, respectively 0.5, 1 and 1.5 ng mL^{-1}. The variation within similar concentrations showed to be large, and too large to obtain a proper calibration curve. Although the variation coefficient is high, the lowest point of the calibration curve, 0.5 ng mL^{-1}, could be measured in 4 out of the 5 samples. So accordingly, sensitivity appropriate for detection of rbST in milk can be reached. As a proof of principle a milk sample (cow a) is measured and the chromatogram is shown in Figure 2 together with a chromatogram obtained from a milk sample spiked with 1.5 ng mL^{-1} rbST. These chromatograms show that not only rbST can be detected in spiked milk samples, but also rbST presence in incurred milk can be detected.

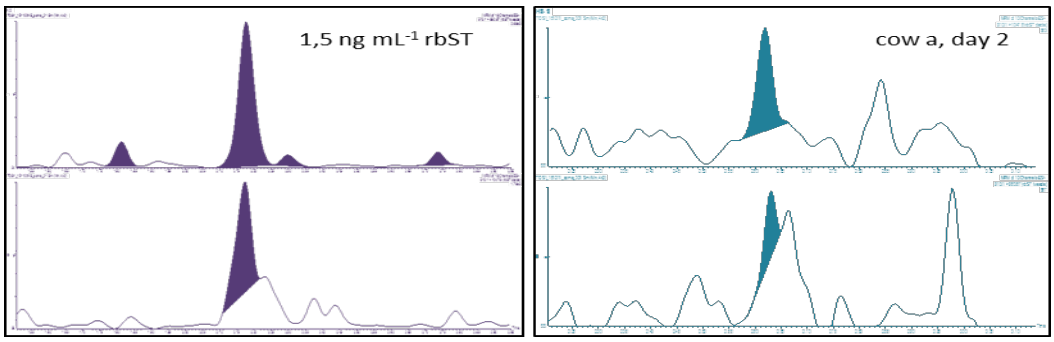

Figure 2. Chromatograms of two different transitions, 913>1047 and 913>960 specific for the N-terminal peptide of rbST of a 1.5 ng mL^{-1} rbST spiked milk sample (left panel) and an incurred milk sample at day 2 of cow a (right panlel).

Conclusions

In this study, it is shown that administration with rbST in slow release formulae ensures continuous rbST activity in the cow. It can be concluded that it is possible to detect rbST, by rbST-enrichment on monolith micro-columns, for the whole period of time in serum between two subsequent administrations in nine lactating cows. Results show that the highest concentration of rbST in serum of cows can be detected the first days after administration and then the rbST concentration decreases in time, but in a fluctuating fashion. Furthermore, preliminary results are shown for rbST detection in milk. The used method still has a large variation in determination of the concentration, however, for the first time, to the best of our knowledge, rbST is detected in a milk sample of a cow treated with rbST under commonly used treatment conditions. In the near future, this should enable to pinpoint rbST abuse using easily obtained milk samples.

Acknowledgements

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TREATMENT OF LAYING HENS WITH NITROIMIDAZOLES – DETECTABILITY OF RESIDUES IN EGGS, FEATHERS AND PREEN OIL

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Abstract
5-Nitroimidazoles are banned from use in food-producing animals in many countries. In order to give recommendations for an efficient residue control of these drugs, an animal study with laying hens was performed. Laying hens were treated via drinking water with different nitroimidazoles for several days. Before, during and after treatment egg samples were taken, feather samples were collected additionally. Preen oil samples from the preen gland were taken as well. Residues in eggs were detected directly after the commencement of the treatment and were detectable up to at least two weeks after the withdrawal for the drug. In preen oil swap samples residues could be detected for a similar period of time. In contrast to egg samples, hydroxy-metabolites of the nitroimidazoles were not detected in the oil. Residues in feathers could be detected for several weeks after treatment. The ratio of drug and hydroxy-metabolite is significantly lower compared to that in egg suggesting that external contamination of the plumage *e.g*. via preen oil may be the source for the presence of the analytes rather than uptake during the growing process of the feathers. With respect to an efficient residue control, it can be concluded that residues of nitroimidazoles in eggs may be present for more than two weeks after the end of treatment and feather samples proved to be a very efficient matrix to screen for nitroimidazoles even several weeks after treatment.

Introduction
There are only few studies dealing with the suitability of the matrix feathers for residue control. It was reported that β-agonists showed a remarkable accumulation in feathers of broilers treated with clenbuterol, salbutamol, and terbutaline. Furthermore, a longer lasting presence of several antibiotics in feathers of medicated broilers was observed. Actually, feathers (of wild birds) are a well-known matrix to screen for the presence of environmental contaminants [1,2,3].

Since 5-nitroimidazoles are banned compounds and tend to degrade rapidly in animal matrices, it was decided to investigate the suitability of feathers as analytical matrix for assessment of misuse of this substance group.

In an earlier study with metronidazole-medicated turkeys, a remarkable accumulation of metronidazole in feathers was observed [4]. Residues in feathers were detected over six months after treatment. The basic findings were that metronidazole (MNZ) could be found in feathers directly after treatment (within one day), and that “external“ sources were the most probable mechanism of uptake of this compound, since only very low levels of the hydroxy-metabolite MNZOH (1-2% of MNZ) were detectable. Possible sources could be the grooming of the plumage with preen gland oil or a contamination via medicated drinking water.

In the present study, these findings were checked for another species and preen oil as a potential source for the presence of residues in plumage was investigated. Laying hens were treated with nitroimidazoles in order to produce residues in eggs. The duration of the detectability of nitroimidazoles in this matrix and the ratio of parent drug and corresponding hydroxy metabolite from start of treatment until after the withdrawal were investigated. In addition, their detectability period in the feather and preen oil matrices was examined. Conclusions for residue control of these antibiotics are drawn.

Materials and Methods

Treatment of the animals
Ten brown laying hens (20 weeks old, average body-weight 1.5 kg) were kept for five months to conduct this study. They were kept in two groups of five animals each in floor husbandry on wood shavings. Once a week, the litter was exchanged completely to give the stable a thorough cleaning. Compound feed and water were accessible *ad libitum*.

The animals were treated via drinking water at 120 mg L^{-1} for all treatments corresponding to 21.5 to 23.6 mg kg^{-1} body-weight. Two groups of animals were treated with one nitroimidazole each. After the first treatment, another treatment with two additional nitroimidazoles for four weeks each was done. The following results refer to the treatment of the hens with metronidazole as an example.

Sampling

Eggs were sampled daily and analysed individually. All moulted feathers were collected from the litter and freed from wood shavings. They were combined to daily samples during the first week of treatment and to weekly samples during the following weeks. Preen gland swap samples were taken daily during the first week of treatment and weekly to monthly during the following weeks. The feathers and swaps were vacuum-packed in coated aluminium bags. All sample material was stored at -25°C until analysis.

Analysis of samples

Feather samples

- Washing of unground feathers with water, phosphate buffer pH 3, water, 0.05 M HCl in ethanol and pure ethanol;
- Keeping the washing solutions for analysis;
- drying of the feathers at 70°C;
- Milling of the dry feathers in a ball mill, mixing in an overhead tumbler;
- Spiking of the samples with internal standard;
- Proteolysis with protease (pH 3);
- Extraction with buffer pH 3, using ultrasonic treatment.

Washing solutions

- Addition of internal standards;
- Ethanolic solutions: evaporation to 0.5 mL, reconstitution with phosphate buffer pH 3;
- Aqueous solutions: addition of phosphate buffer pH 3, adjustment to pH 3.

Egg samples

- Homogenisation of a whole egg;
- 1 – 2 g aliquot of egg (or reconstituted egg if applicable);
- Addition of internal standards, extraction with acetonitrile;
- Evaporation to 0.5 ml, reconstitution with phosphate buffer pH 3.

Preen oil samples (cotton swaps)

- Addition of internal standards, extraction with acetonitrile;
- Evaporation to 0.5 mL, reconstitution with phosphate buffer pH 3.

Common clean-up

- Defattening with 5mL of *n*-hexane;
- Adjusting to pH 6 and clean-up on XTR cartridges;
- Evaporation to dryness;
- Reconstitution in eluent A and measurement with LC-MS/MS.

Chromatography, analytes and mass spectrometry parameters

Gradient UPLC using a BEH C18, 100 x 1.0 mm, 1.7 µm column eluted with solvent A (formic acid /ACN / water: 1+29+970, v/v/v) and B (ACN/ formic acid: 999 + 1, v/v).

Table 1. Analytes, internal standards and ions/ion transitions for LC-MSMS measurement.

Analyte	base ion	1st product ion	2nd product ion	internal standard
Metronidazole (MNZ)	172	128	82	$^{13}C_2{}^{15}N_2$ MNZ
MNZOH	188	144	68	D_2-MNZOH
Dimetridazole (DMZ)	142	96	54	D_3-DMZ
Ronidazole (RNZ)	201	140	55	D_3-RNZ
HMMNI ("DMZOH")	158	140	94	D_3-HMMNI
Ternidazole (TNZ)	186	128	82	$^{13}C_2{}^{15}N_2$ MNZ
Secnidazole (SNZ)	186	128	82	$^{13}C_2{}^{15}N_2$ MNZ
Tinidazole (TIZ)	248	128	82	D_3-IPZOH
Ornidazole (ONZ)	220	128	82	D_3-IPZOH
Ipronidazole (IPZ)	170	109	124	D_3-IPZ
IPZOH	186	168	122	D_3-IPZOH

Decision limits CC_α for the detection of the analytes listed in Table 1 in eggs ranged from 0.07 ng g^{-1} to 0.3 ng g^{-1}. The method has been fully validated in accordance with Commission Decision 657/2002/EC applying the in-house validation procedure with the use of the software package InterVal.

Results and discussion

In Figure 1 the results for the metronidazole-treated hens are exemplarily represented. Residues in eggs are present after the first day of treatment. The ratio of MNZ and MNZOH varies between 1.5 and 3 during the treatment and drops down to 0.01 after approximately one week after the withdrawal. After two weeks of withdrawal the ratio is close to one, but here concentrations decreased already to levels below 1 ng g^{-1}. In the swap samples taken around the preen gland of the hens, residues could be detected after the first day of treatment as well. The amount of secrete taken with the swap sample was around 1 mg. In the swap extract, only MNZ could be detected, MNZOH was not present. The concentration drops relatively quickly after withdrawal of the drug to those found in eggs. Anyhow, even after one month of withdrawal, residues were detected in this matrix.

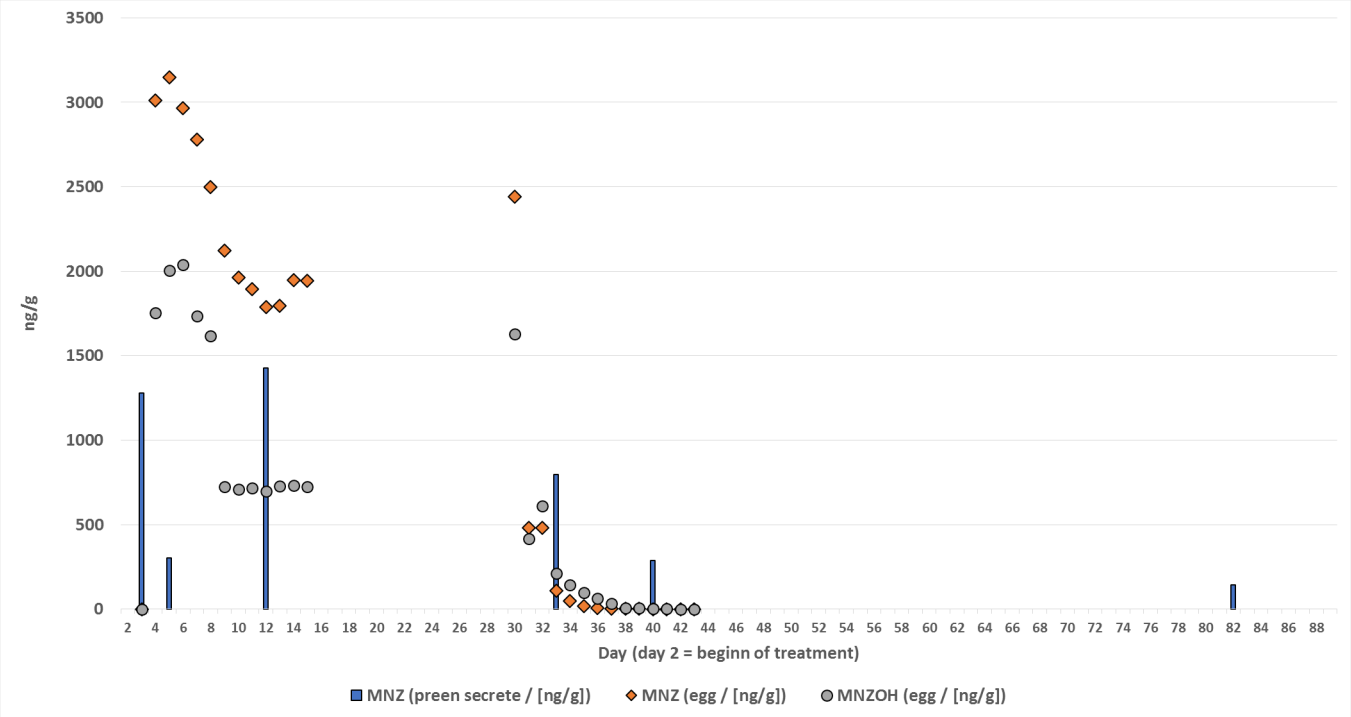

Figure 1. Nitroimidazole residues (MNZ and MNZOH) in different matrices [ng g^{-1}] in the course of the experiment [days]; treatment from day 2 to day 30 via drinking water.

During the experiment, the laying hens lost very few feathers naturally. For animal welfare reasons, cutting of feathers was consciously avoided accepting that only low amounts of feather samples would be available. In order to be able to analyse the feather samples adequately, samples were partly combined to acquire an amount of at least 0.5 g of feathers. The feathers were washed consecutively with water and ethanolic solutions. The washing solutions as well as the remaining feather pieces were analysed separately.

Table 2. Results for MNZ and MNZOH in feather samples, washing solutions (calculated as residue per g feather) and dried (washed) feather material.

Analyte	Withdrawal time [d]	Water [µg kg^{-1}]	buffer pH3 [µg kg^{-1}]	water [µg kg^{-1}]	Ethanol [µg kg^{-1}]	Ethanol [µg kg^{-1}]	sum [µg kg^{-1}]	In washed feathers [µg kg^{-1}]
MNZ	14	5200	2500	950	370	120	9140	75
MNZOH	14	310	100	30	10	4	454	9
MNZ	80	3200	1800	990	520	220	6730	160
MNZOH	80	75	35	16	10	4	140	15

The residue levels in feathers are very high and decrease only slowly even after several weeks of withdrawal of the medication. The highest shares of residues were present in the aqueous extracts. MNZOH could be detected in feather samples too, but the ratio of MNZ and MNZOH is with values between 20 and 50 significantly higher than for egg samples.

For MNZ, the residue concentrations in washed feathers are slightly lower than calculated on the basis of the last extraction step. In contrast, MNZOH is present at significantly higher concentrations compared to values resulting from the last ethanol extraction step.

The high MNZ to MNZOH ratio indicates that an external uptake of nitroimidazoles by the feathers is likely. This may result from the preen oil (which contained mainly MNZ) but also from the medicated water (*e.g*. via the beak of the birds). Anyhow the comparison of the MNZOH concentration in the washed feather sample with the concentration in the last extract may hint that incorporation of the drug during the growing process can play also a (minor) role.

Conclusions

Residues of nitroimidazoles in eggs may be detected for at least two weeks after withdrawal of the drug. The ratio of MNZ to MNZOH changes after the withdrawal of the drug and may be an indicator of the withdrawal time.

The results with respect to the presence of residues of nitroimidazoles in feathers of laying hens were comparable to an earlier study with turkeys. Residues were detected several months after withdrawal of the drug, the parent drug being by far the mayor residue. In swap samples of the preen gland, residues of the parent drugs could be detected too and hence could be one of the possible sources for the residue levels in feathers. Anyhow, a contamination of the plumage via medicated drinking water cannot be excluded. In order to clarify the origin of the nitroimidazole residues, additional animal studies with different ways of medication of the animals (via feed and via drinking water) is required.

Nevertheless, the suitability of the matrix feathers is demonstrated as a simple and efficient way to screen for the misuse of nitroimidazoles in poultry, even long time after the withdrawal of the medication.

Acknowledgements

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GEL-PERMEATION CHROMATOGRAPHY CLEAN-UP FOR THE DETERMINATION OF GROWTH PROMOTERS IN KIDNEY FAT BY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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Abstract
A rapid multi-analyte method for the determination of 21 growth-promoters (β-estradiol, ethynylestradiol, diethylstilbestrol, hexestrol, dienestrol, α/β-trenbolone, methyltestosterone, α/β-zearalanol, dexamethasone, flumethasone, triamcinolone acetonide, α/β-boldenone, α/β-nortestosterone, chlormadinone acetate, megestrol acetate, melengestrol acetate and medroxyprogesterone acetate) in animal kidney fat was developed. Fat is considered as a complex matrix and extensive clean-up procedures are needed focusing mostly on the determination of corticosteroids and gestagens. This procedure consists of an automated clean-up procedure with gel-permeation chromatography (GPC), evaporation and LC-MS/MS measurement. The decision limits CCα ranged from 0.10 to 0.30 ng g^{-1}, while the detection capabilities CCβ ranged from 0.17 to 0.48 ng g^{-1}. The recoveries of growth promoters spiked in kidney fat ranged between 81% and 121% with relative standard deviations (RSD) less than 12%. This reliable method can be used to efficiently separate and quantify residues of growth-promoters in kidney fat with advantages of an automated clean-up procedure, short retention times and more sensitivity.

Introduction
For years, the use of steroid hormones for food-producing animals has been prohibited in the European Union, because of their possible toxic effect. Effective residue control plans have been implemented for monitoring the illegal administration of growth-promoters by analysis of different type of matrices at various stages in the food chain. At farm level, urine, faeces and hair are monitored and at slaughterhouse level, injection sites, meat or fat tissue samples and organs (liver and kidney). Consumable parts of the animal like liver, kidney and muscle tissue, are target matrices for residue analysis. Many steroid hormones are lipophilic compounds and can accumulate in fat. Kidney fat is considered to be the tissue of choice for detection at slaughterhouse level mainly for gestagens and corticosteroids. Therefore, the European Union (EU) has established minimum required performance limit (MRPL) for medroxyprogesterone set to 1 ng g^{-1} in kidney fat. Concerning other gestagens a recommended concentration of 5 ng g^{-1} is set based on the Community Reference Laboratory (CRL) Guidance Paper of 2007. EU has also established maximum residue limits (MRL) for corticosteroids in different matrices and MRPLs for dexamethasone and flumethansone in kidney fat at 1 ng g^{-1}. The perinephric kidney fat is composed of adipose tissue, which is a collection of fat cells bound together by connective tissue and supplied with blood vessels. So, except of gestagens and corticosteroids, other steroid hormones can also travel in the bloodstream bound to transport proteins and diffuse through the membrane of target cells.

Several analytical methods have been developed for the determination of corticosteroids (Tölgyesi *et al*. 2011) and gestagens (Tao *et al*. 2015; Rejtharová *et al*. 2013) in kidney fat by applying mostly liquid-chromatography tandem mass-spectrometry (LC-MS) and less gas-chromatography mass-spectrometry (GC-MS), as the last technique requires additional steps to hydrolyse and derivatise samples prior analysis. Commonly, sample preparation methods are based upon extensive extraction of fat with organic solvents followed by procedures for defatting of the extracts obtained. The extract is cleaned with solid phase extraction (SPE), liquid/liquid extraction (LLE), high performance liquid chromatography and novel approaches for the extraction using accelerated solvent extraction (ASE) or supercritical fluid extraction (SFE) have been developed.

The goal of the present study was to develop a highly sensitive and automated multi-analyte method for the analysis of growth-promoters in kidney fat by combining gel-permeation chromatography (GPC) as clean-up step and analysis by LC-MS/MS.

Materials and Methods

Chemicals and Reagents
All standards were purchased from Cerilliant (Promochem, Wesel, Germany), NARL (Pymble, NSW, 132Australia) and (Sigma–Aldrich, Steinhem, Germany). The internal standards were obtained from the EURL for growth-promoters (RIKILT, Wageningen, The Netherlands). Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany), *n*-hexane, and dichloromethane were from Sigma (Sigma–Aldrich, Steinhem, Germany) and ultrapure water was produced with a Pure Lab system (Sation9000, Spain).

An appropriate amount in mg of each compound was weighed accurately and dissolved in methanol in order to produce the stock standard solutions. The intermediate working solutions were prepared by dilutions of the stock solution in methanol. All standard solutions were stored at -20^{o}C in the dark.

Samples
Kidney fat samples collected from untreated *bovine* animals at slaughterhouses were used as blank and, after fortification with the growth promoters, as quality control samples. Kidney fat samples from *bovine* animals were collected as part of the national program for residue control in Greece and were stored at -20°C until analysis.

Instrumentation
LC–MS/MS analysis was performed on a ThermoElectron TSQ Quantum AM mass-spectrometer equipped with a Finnigan Surveyor MS pump Plus, a Finnigan Surveyor Autosampler plus and a Dell computer system with Xcalibur data acquisition software (ThermoElectron, San Jose, CA, USA). The clean-up was performed on a GPC system equipped with a marathon III pump (RigasLabs, Thessaloniki, Greece), an automatic sampler of large volume Midas (SparkHolland, Emmen, The Netherlands), a Glass column Omni fit (1000 mm), a UV scanning detector Fasma 506 (RigasLabs), two Rheodyne valves (Cotati, CA), a fraction collector CHF 122SB (Toyo Seisakusho Kaisha, Japan) and a Clarity Data chromatography software (DataApex, Prague, Czech Republic).

GPC clean-up
For GPC, a glass column was used 1000 mm × 25mm ID, filled by Bio Beads (S-X3) 200–400 mesh (Bio-Rad Laboratories, Richmond, CA, USA). The flow rate was kept at 5 mL min^{-1} with dichloromethane used as the mobile phase. Injection volume was 5 mL throughout the study. The wavelength of the UV scanning detector (used to monitor the GPC column effluent in order to collect the fractions) was set at 254 nm. The samples run time was 50 min and the time frame for the collection of the gestagens was from 36.5 to 50 min (13.5 min).

LC–MS/MS detection
The chromatography was performed on a Prevail C18 analytical column (150 mm × 4.6 mm, 3 µm – Alltech, Deerfield, IL, USA) applying a gradient elution with the mobile phase consisted of water as solvent A and methanol as solvent B. Gradient elution started with 60% solvent A (v/v), decreased to 20% over 6 min, which was kept over a period of 3 min and then changed to 60% solvent A (v/v) to 13 min for re-equilibration of the analytical column. The total analysis time was 13 min with the injection volume was 15 µL. The flow rate was 700 µL min^{-1} and the temperature of the column was set at 20°C.
Atmospheric pressure chemical Ionization (APCI) was selected for the ionization of the compounds and was operated in the positive and negative mode. Nitrogen was used as sheath and auxiliary gas, at flow rates of 40 and 5 arbitrary units, respectively. Capillary and vaporizer temperatures were set at 300 and 450°C, respectively. The discharge current was optimized at 7 µA. Multiple Reaction Monitoring (MRM) mode was applied acquiring two products ions per precursor ion.

Sample preparation
An amount of 2 g of fat, formed as oil by heating, was weighed in a 10-mL volumetric flask, diluted with 1 mL *n*-hexane and filled up to 10 mL with dichloromethane. The sample was transferred in a 10 mL vial and injected on the GPC system. The fraction of the gestagens (eluted between 36.5 and 50 min) was collected according to the program of the GPC system. The fraction was evaporated to dryness in a water bath at 40°C under a stream of nitrogen. The residue was dissolved in 0.6 mL methanol, transferred to an injection vial, evaporated under a stream of nitrogen at 55°C to dryness, dissolved in 80 µL methanol and analysed on the LC-MS/MS.

Results and Discussion

Optimization of sample preparation
Sample preparation methods in the literature have aimed at the exhaustive extraction of fat using organic solvents. The extracts were cleaned with SPE and/or subsequent LLE extraction. Our aim was to develop a simple, rapid and automated method for the clean-up of fat samples. GPC has been used in our laboratory for the purification of gestagens from fat samples prior to LC–MS analysis (Kaklamanos *et al*. 2009). Hence, we optimize such an approach in order to include more growth-promoters. Different flow rates were tested. A flow rate at 5 mL min^{-1} provided a balance between the operating pressure on the system and the resolution between the two extracts of fat and steroid hormones in order to collect a clear final extract.

Optimization of liquid chromatography-mass spectrometry

In order to evaluate the optimum separation of the analytes, three different analytical columns were tested Hypersil ODS column 150 mm × 4.6 mm, 5µm (ThermoElectron, San Jose, USA,), an Alltima C18 218 column 150 mm × 4.6 mm, 3µm (Alltech, Deerfield, IL, USA) and a Prevail C18 column 150 mm × 4.6 mm, 3 µm (Alltech). The best separation along with shorter retention times and sharper peaks were obtained with the Prevail C18 column. Different compositions of the mobile phase based on water–methanol and water–acetonitrile were tested. The intensities of the analytes were generally higher by using methanol–water as mobile phase. Our aim was not to include any additives in the mobile phase to avoid contamination of the mass-spectrometer in time and possible ion suppression.

Although Electrospray Ionisation (ESI) is mostly applied ionization technique for the analysis of steroid hormones by LC–MS, we achieved higher intensities for the detected analytes using APCI. Analysis was carried out in the multiple reaction monitoring (MRM) mode to monitor the product ions of the steroid hormones and to obtain a high specificity and sensitivity. Table 1 lists the retention time (Rt), precursor and most abundant product ions at corresponding collision energy of the analysed growth-promoters.

Table 1. Precursor and most abundant product ions for the growth promoters with their retention time (Rt) and collision energy (CE).

Compound (Rt min)	Precursor ion	Product ions	CE
Flumethasone (5.23)	411.15(+)	**253.19***/235.08	25/31
Dexamethasone (5.61)	393.15(+)	**237.23**/147.16	30/25
Triamcinolone acetonide (5.71)	435.18(+)	**213.16**/321.20	34/19
α/β-trenbolone (6.47/6.23)	271.17(+)	**199.10**/253.12	28/22
α/β-zearalanol (6.57/5.53)	323.20(+)	**149.10/**189.16	36/32
Diethylstilbestrol (6.99)	273.15(-)	**222.12**/237.137	41/38
Ethynylestradiol (7.03)	295.19(-)	**145.30**/159.11	36/39
α/β-boldenone (7.06/6.36)	287.20(+)	**121.11**/135.25	30/15
Dienestrol (7.15)	265.10(-)	**93.03**/236.17	35/27
β-estradiol (7.16)	271.18(-)	**145.17**/183.20	39/49
Hexestrol (7.25)	269.15(-)	**119.01**/133.02	39/20
α/β-nortestosterone (7.25/6.68)	275.20(+)	**109.12**/145.16	21/28
Methyltestosterone (7.65)	303.00(+)	**109.11**/97.08	22/32
Chlormadinone acetate (8.04)	405.11(+)	**309.17**/345.08	18/12
Megestrol acetate (8.08)	385.24(+)	**224.32**/267.35	30/26
Medroxyprogesterone acetate (8.18)	387.24(+)	**123.20**/285.47	30/23
Melengestrol acetate (8.27)	397.24(+)	**279.32**/236.26	23/37

**Bold most abundant ion for quantification*

Method validation

The developed method has been validated according to the European Commission Decision 2002/657/EC by assessing linearity, specificity, precision, accuracy, decision limit (CCα), detection capability (CCβ) and measurement uncertainty. The validation procedure was carried out on three different days at 1, 1.5 and 2 times the validation level and six replicates per concentration. A concentration of 1 ng g^{-1} was selected as a reasonable validation level to be closer to already recommended concentrations levels for growth promoters in other matrices.

Method linearity was evaluated with matrix-free calibration curve using 9 calibration points at concentrations 0, 0.5, 1, 1.5, 2, 3, 4, 6 and 10ng g^{-1}. The concentration of the mixture of internal standards including methyltestosterone-d3, testosterone-d3, 17β-estradiol-d3, diethylstilbestrol-d6, triamcinolone acetonide-d6, megestrol acetate-d3 and α/β-zearalanol-d4 was set at 2 ng g^{-1}. The regression coefficients (r^2) were greater than 0.995 for all analytes.

In order to determine the specificity of the method, 20 different fat samples from independent sources were analysed and additionally fortified with steroid hormones at a concentration of 1 ng g^{-1} and re-analysed. Results showed no inferences, false positive or false negative identification.

Method's accuracy and precision was determined based on the analysis of the 18 replicate fat samples per concentration performed on three different days. The recoveries ranged between 80.9% and 121.2%. From the repeated analysis of the spiked samples under reproducible conditions the RSDs were calculated and ranged from 0.7% to 12.0% for the repeatability and from 0.8% to 10.2% for the within-lab reproducibility. The results indicated that the method has satisfactory precision and recoveries to be used on a routine basis.

The CCα and CCβ were obtained using the calibration curves of the spiked samples. The decision limits CCα ranged from 0.10 to 0.30 ng g^{-1} while the detection capabilities CCβ ranged from 0.17 to 0.48 ng g^{-1} showing the high sensitive of the developed method (Table 2).

The measurement uncertainty *U* was calculated by multiplying the combined standard uncertainty *u* with the coverage factor *k*. The value of 2.33 was used for the factor *k* to cover a Gaussian distribution of 99% for substances such as the growth-promoters. The combined standard uncertainty *u* was calculated based on the reproducibility variance from the three different days. The repeatability from the specificity experiment was also included to express the variance of the matrix effect of the different fat samples. The measurement uncertainties *U* ranged from 6.42% to 33.08% (Table 2).

In addition, all spiked samples were confirmed based on the requirements of the European Commission Decision 2002/657/EC. The relative retention time (RRT) of the analytes was in the acceptable tolerance of +2.5% and the ion ratios (relative intensities) of the two product ions of all analytes did not exceed the tolerance.

Table 2. CCα, CCβ and U(%) values for the growth promoters.

Compound (Rt in min)	CCα (ng g^{-1})	CCβ (ng g^{-1})	*U* (%)
Flumethasone (5.23)	0.20	0.34	33.08
Dexamethasone (5.61)	0.13	0.22	15.89
Triamcinolone acetonide (5.71)	0.18	0.30	10.29
α/β-trenbolone (6.47/6.23)	0.10/0.13	0.17/0.23	6.72/9.49
α/β-zearalanol (6.57/5.53)	0.17/0.16	0.28/0.28	6.42/11.58
Diethylstilbestrol (6.99)	0.17	0.28	17.85
Ethynylestradiol (7.03)	0.24	0.40	13.38
α/β-boldenone (7.06/6.36)	0.12/0.11	0.20/0.18	14.01/11.62
Dienestrol (7.15)	0.18	0.31	14.80
β-estradiol (7.16)	0.12	0.21	8.59
Hexestrol (7.25)	0.17	0.28	16.71
α/β-nortestosterone (7.25/6.68)	0.15/0.11	0.25/0.18	12.58/8.15
Methyltestosterone (7.65)	0.10	0.17	7.79
Chlormadinone acetate (8.04)	0.20	0.33	12.26
Megestrol acetate (8.08)	0.26	0.44	11.41
Medroxyprogesterone acetate (8.18)	0.30	0.48	12.59
Melengestrol acetate (8.27)	0.20	0.34	11.66

Conclusions

A simple, rapid and automated method was developed for the determination and confirmation of 22 growth-promoters in kidney fat covering stilbenes, steroids, RALs, corticosteroids and gestagens. The GPC clean-up procedure proved to be efficient providing a clear extract of the analytes completely separated from the fat extract. The developed method can be considered rapid achieving satisfactory chromatographic separation in 8.5 min for all analytes. The validation of the method was based on the European Commission Decision 2002/657/EC proving sufficient analytical performance, satisfactory accuracy and precision, and excellent linearity. The method is therefore suitable for laboratories involved in official residue control analyses and animal administration studies with growth-promoters.

References

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PROFICIENCY TESTING IN FOOD CONTROL – ADDED VALUE BY OFFERING TRACEABILITY TO SI-UNITS?

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Abstract
In Europe, laboratories working within the framework of official food control have to be accredited according to ISO 17025. One of the requirements of ISO 17025 is that results should be traceable to SI (international system of units) whenever possible. The reference laboratory system for food control in the EU obligates the EURLs and NRLs to regularly organise inter-laboratory comparisons for the official residue control labs in order to harmonise residue control and to verify laboratory performance. This paper shows an example of how an inter-laboratory comparison can be used in two ways. On the one hand the comparability of measurements within the group of participants is checked and can be verified, on the other hand the worldwide comparability of the measurements via traceability to SI can be proven.
The results of an inter-laboratory comparison for chloramphenicol (CAP) in plasma for the official German routine laboratories were evaluated using a classical consensus value with z-scores and an SI-traceable reference value set by the BVL. All participating laboratories identified CAP in the matrix samples correctly, all z-scores were in the range of ± 2. The SI-traceable reference value and the calculated robust mean value in this study were in very good agreement. Using the uncertainty budget of the individual results provided by the laboratories, the equivalence to the SI-traceable reference value and its associated uncertainty could be shown.

Introduction
In Europe, laboratories working within the framework of official food control have to be accredited according to ISO 17025. One of the requirements of ISO 17025 is that test results should be traceable to SI (international system of units) whenever possible. The reference laboratory system for food control in the EU obligates the EURLs and NRLs to regularly organise proficiency tests (PTs) for the official residue control labs in order to harmonise residue control and to verify laboratory performance. Even though these PTs help to prove (or improve) the comparability of measurements within this community, the question of the traceability of measurements to SI (and with this, of worldwide comparability) is not touched.

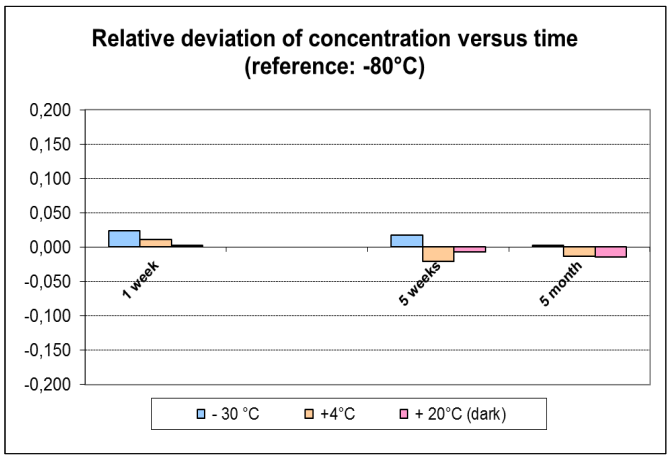
A peer-reviewed traceability route to the SI can be offered by National Metrology Institutes (NMIs) or designated Institutes (DIs), who have published calibration and measurement capabilities (CMC entries) in the CIPM MRA key comparison data base (KCDB) [1,2,3]. The BVL is the designated institute for organic residues in food in Germany. Based on its proven competence for the analysis of CAP in plasma (service listed in the CMC data base), the BVL can provide reference values to test materials for ring trials as one of the possibilities of providing traceability [4]. Hence a potential added value for PT participants can arise from linking proficiency testing and the provision of traceability to SI. An example of how this can be realised is given on the basis of a proficiency test organised for the German residue control labs.

Materials and Methods

PT-material
The test material was produced in the BVL / BfR facilities and originates from a pig which was medicated with chloramphenicol. For the inter-laboratory comparison one incurred test sample was prepared. An incurred plasma sample was pre-tested and subsequently diluted with blank plasma in order get a final CAP content of about 0.3 ng g^{-1}. The material was produced by thoroughly mixing blank and incurred plasma followed by lyophilisation. Subsequently, the lyophilised sample material was homogenised by grinding and mixing in an overhead tumbler and filled into amber glass vials (0.8 g of lyophilised plasma each, corresponding to approximately 10 mL fresh plasma). In addition, an ethanolic standard solution of CAP was prepared gravimetrically.

Tests for Homogeneity and Stability
The test sample was tested for homogeneity and stability. For homogeneity testing twelve aliquots were randomly selected and analysed twice. The homogeneity was checked according to ISO 13528. For the test material a relative between-bottle standard deviation of 1.7% was calculated, the material proved to be homogenous.

The stability of the lyophilised material was tested for a duration of 5 months at temperatures of -30°C, +4°C, +25°C and -80°C as reference temperature. The samples proved to be stable even at + 25°C for at least five months.



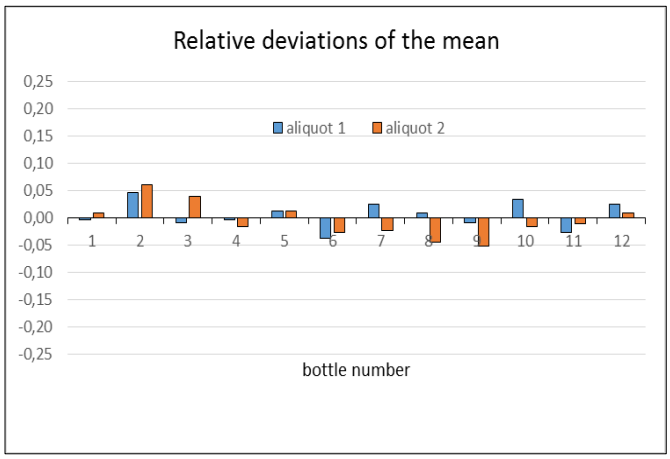


Figure 1. Stability and homogeneity data of the test material.

Inter-laboratory Study Protocol

Thirteen official residue control laboratories including BVL participated in the proficiency test. Each proficiency test participant received a set of three code-number-labelled samples (two incurred samples from the same batch, but not labelled as identical sample material, one standard solution). The samples were to be examined for their total chloramphenicol content. For each sample the results of two parallel determinations were to be submitted. A general study protocol, an instruction sheet for the reconstitution of the lyophilised samples and a report template were provided to the participants. In addition, the laboratories were asked to provide an uncertainty budget for their measurements.

Data Evaluation

The results of the proficiency test were evaluated by two approaches.

(1) Classical z-score evaluation.

The target value (assigned value) was determined by means of the robust estimation of the mean according to Hampel from the results of all laboratories (in accordance with DIN 38402-45).

As target standard deviation, the reproducibility standard deviation was used. The reproducibility standard deviation (sR) refers to the scatter of the measurements from the different participants. The reproducibility standard deviation was calculated by applying the Q-method as described in DIN 38402-45. The individual z-scores were calculated as the difference between the individual measurement result and the target value divided by the reproducibility standard deviation.

The HORRAT-value – the quotient from the value determined for the reproducibility standard deviation (sR) and the Horwitz standard deviation (s) - provides a certain indication of the overall performance level of the laboratories. The AOAC expects Horrat values of 0.5 to 1.5 in Annex D of the “Guidelines for Collaborative Studies”.

(2) Use of a reference value.

The study material was tested and calibrated at the BVL. For the material a reference value and a respective measurement uncertainty were calculated. The assignment of the reference value was done by means of the isotope dilution technique applying the method BVL/CAP_013 as listed in the calibration and measurement database (“CMC”-database) of the BIPM. The assigned value of the study material is traceable to a chloramphenicol standard material provided by the National Metrology Institute of Turkey (UME, TÜBITAK Ulusal Metroloji Enstitüsü).

The reference value was calculated as 0.404 ng g^{-1}, the uncertainty was estimated taking into account the uncertainties of the method (within-laboratory reproducibility, the uncertainty of the standard solution and of the spiking of the samples as well as that due to the inhomogeneity of the material). The relative extended uncertainty (k=2) was calculate as 8.7%.

The calculation of the degrees of equivalence (DoE, see Figure 3) allows the participating laboratories to see the equivalence between their own measurement and the SI-traceable reference value with the stated uncertainties [5]. The DoE and its uncertainty (with a value component and an uncertainty component) between a participant’s result and the reference value is calculated according to the following equations:

a) the value component is $d_i = x_i$ - xref

where d_i is the degree of equivalence between the participant’s result x_i and the reference value x_{ref}. The best possible d_i is zero, when the result is identical to x_{ref}.

b) the uncertainty component is U_i (d_i) = k * u(d_i)

where the expanded uncertainty U_i is calculated by combining the expanded uncertainties $k_i u_i$ of x_i and $k_{ref} u_{ref}$ of x_{ref} as $U_i = [(k_i^2 u_i^2 + k_{ref}^2 u_{ref}^2)]^{1/2}$, using k_{ref}, u_{ref} calculated as described above and k_i, u_i as reported by the participating laboratories.

Measurement Uncertainty

Four of the thirteen laboratories did not indicate the measurement uncertainty as requested, even though it is required for testing laboratories accredited according to ISO17025 to have a concept for the estimation of the measurement uncertainty in place and to provide the uncertainty of their testing results. The reported expanded uncertainties ranged from ± 0.03 to ± 0.12 ng g^{-1}. The basis of the calculation is not always traceable. The availability of a reasonable stated measurement uncertainty is the basis for the calculation of the equivalence between the measurement value and a reference value.

Results

All participating laboratories correctly identified CAP in the matrix samples. Since in this study samples CAP1 and CAP2 were identical materials, an overall assigned value (CAP1+2) was calculated for the calculation of the z-score evaluation (Table 1).

Table 1. Assigned value (consensus value), reproducibility standard deviation and Horrat ratio.

Sample	Assigned value [ng g^{-1}]	S_R absolute [ng g^{-1}]	Relative [%]	Horrat
CAP1	0.388	0.042	10.74 %	0.2
CAP2	0.396	0.058	14.55 %	0.3
CAP standard	13.786	1.946	14.12 %	0.5
CAP 1 + 2	0.392	0.048	12.40 %	0.2

Table 2. Results of participants, stated measurement uncertainties and z-scores based on all reported values (CAP1 and CAP2) for the calculation of target value and reproducibility standard deviation.

Lab code	1st value [µg kg^{-1}]	2nd value [µg kg^{-1}]	3rd value [µg kg^{-1}]	u [µg kg^{-1}]	k	U [µg kg^{-1}]	Mean	Std. dev.	z-Score
LC01	0.391	0.388	0.377	0.04	x	x	0.385	0.007	-0.1
LC01a	0.401	0.373	0.383	0.04	x	x	0.386	0.014	-0.1
LC02	0.377	0.352		x	x	x	0.364	0.018	-0.6
LC02a	0.367	0.352		x	x	x	0.359	0.011	-0.7
LC03	0.340	0.330		0.04	2.33	0.093	0.335	0.007	-1.2
LC03a	0.330	0.330		0.04	2.33	0.093	0.330	0.000	-1.3
LC04	0.345	0.359		0.061	2	0.122	0.352	0.010	-0.8
LC04a	0.361	0.380		0.055	2	0.11	0.370	0.013	-0.4
LC05	0.351	0.362		0.034	2.33	0.079	0.356	0.008	-0.7
LC05a	0.341	0.337		0.034	2.33	0.079	0.339	0.003	-1.1
LC07	0.410	0.407		0.044	2	0.088	0.408	0.002	0.3
LC07a	0.403	0.405		0.044	2	0.088	0.404	0.001	0.2
LC08	0.422	0.422		x	x	~ 30 %	0.422	0.000	0.6
LC08a	0.429	0.428		x	x	~ 30 %	0.428	0.001	0.7
LC09	0.471	0.451		0.036	2	0.072	0.461	0.014	1.4
LC09a	0.461	0.459		0.036	2	0.072	0.460	0.001	1.4
LC10	0.340	0.370	0.370	0.055	2	0.11	0.360	0.017	-0.7
LC10a	0.370	0.380	0.360	0.058	2	0.11	0.370	0.010	-0.4
LC11	0.360	0.400		x	x	x	0.380	0.028	-0.2
LC11a	0.470	0.490		x	x	x	0.480	0.014	1.8
LC12	0.395	0.354		0.05	2	0.1	0.375	0.028	-0.4
LC12a	0.390	0.363		0.05	2	0.1	0.377	0.018	-0.3
LC13	0.433	0.459		x	x	x	0.446	0.018	1.1
LC13a	0.448	0.432		x	x	x	0.440	0.011	1.0
LC14	0.399	0.408		0.0161	2	0.032	0.403	0.006	0.2
LC14a	0.397	0.412		0.0162	2	0.032	0.404	0.011	0.3

Table 2 shows the evaluation of the reported quantitative results and the calculation of the z-scores. All z-scores were in the range of ± 2. Also the HORRAT of below 0.5 proves the very well-established analysis of CAP in the official food control laboratories of Germany. Surprisingly the analysis of the standard solution (not reported here in detail) was of slightly poorer quality compared to the matrix material.

Figure 2 shows the comparison of the two ways of data evaluation. In Figure 2a, all results are in the z-score range of ± 2. If a z-score evaluation using the reference value and its uncertainty was done, a few of the results would be out of the range of ± 2 as can be seen in Figure 2b.

The calculation of the degrees of equivalence takes into account the reported result as well as the reported measurement uncertainty. This is shown in Figure 3, proving that for all laboratories having stated measurement uncertainties some overlap with the zero level is given, *i.e.* the equivalence of the own measurement and the SI-traceable reference value is shown.

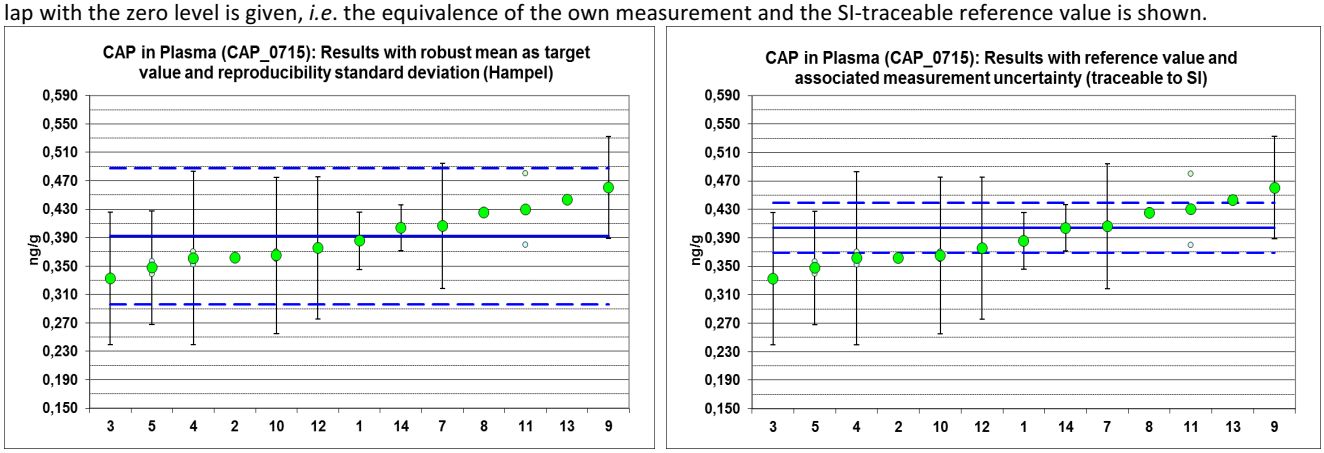


Figure 2. Comparison of evaluation by robust mean and by a given reference value as target value (target values, standard deviation of the target value with k=2, mean measurement values and respective extended measurement uncertainties as reported).

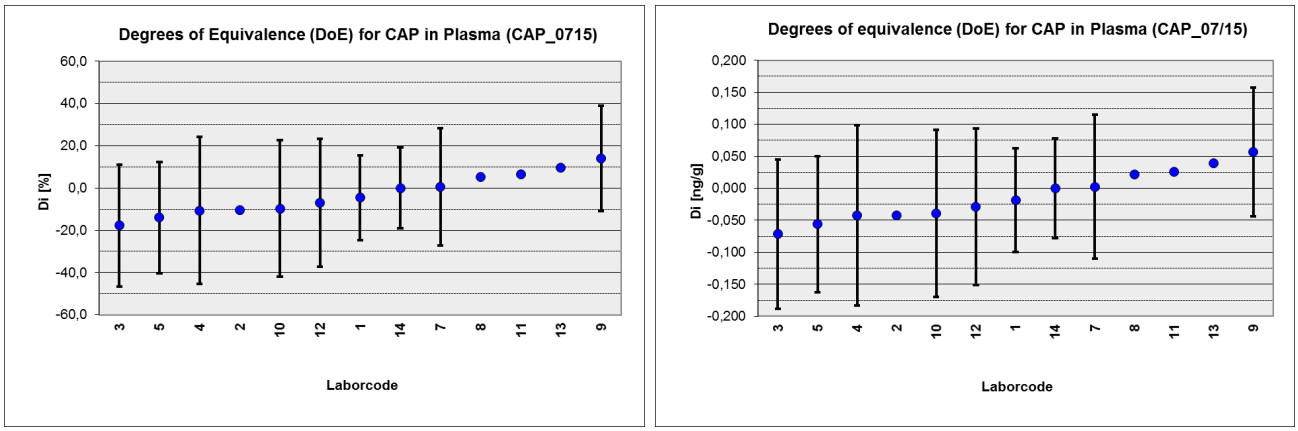


Figure 3. Example of the calculation of absolute and relative degrees of equivalence using the reference value and the associated uncertainty (according to evaluation of key comparison as described in [5]).

Discussion and Conclusions

The SI-traceable reference value and the calculated robust mean value in this study are in very good agreement. This may not always be the case and certainly depends on the analyte/matrix/method combination and the expertise of the participating laboratories. Anyhow already in this study the difference between the uncertainty of the assigned value using the reproducibility standard deviation and the reference value and its uncertainty is relatively high and may lead to a discussion on the question which one should be chosen as acceptance criterion.

However, this example shows that the use of a reference value in an inter-laboratory comparison offers a way of providing traceability to SI. A prerequisite for the calculation of the equivalence of the laboratories' results with an SI-traceable reference value would be that the laboratories were able to provide a reasonable measurement uncertainty budget for their measurement results. This is not always the case, even though an ISO 17025 accreditation would require this.

Acknowledgements

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– Calibration and measurement capabilities database (http://kcdb.bipm.org/appendixC/default.asp)
– International equivalence of measurements: the CIPM MRA, http://www.bipm.org/en/cipm-mra/
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- CQM Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence, Version: 10, Date: 2013-04-12 (http://www.bipm.org/cc/CCQM/Allowed/19/CCQM13-22_Consensus_KCRV_v10.pdf)

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INVESTIGATION OF MATRIX EFFECTS ON SELECTED VETERINARY DRUGS

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Abstract

In animal products for human consumption a sensitive and selective detection of veterinary drug residues is required to ensure human food safety. This central requirement on analytical methods is covered by LC-MS. A drawback of LC-MS is the high probability that matrix effects affect the ionisation of the analytes, which may result in an analyte under- or over-estimation. Therefore, the investigation of matrix effects plays an emerging role in both method development and validation and requests advanced methodological approaches.

The aim of our study was to investigate the dependency of matrix effects on different types of matrices and species for ß-agonists. In addition, a methodological approach which considers matrix effects as part of method validation was verified. These studies were based on the determination of the matrix effect by means of the matrix-matched standard approach, where extracts of blank samples were fortified after the last step of the sample preparation procedure and compared to the standard in solvent. Our studies reveal a significant influence of the type of matrix and species on the matrix effects and indicate that checking matrix effects should be included as general part of method development and validation.

Introduction

Liquid-chromatography mass-chromatography (LC-MS or alternatively LC-MS/MS) is one of the most powerful analytical techniques. LC-MS and particularly LC/MS/MS are characterised by very high sensitivity, selectivity and throughput. They are commonly used to determine many different varieties of analytes in different varieties of biological and non-biological matrices.

However, besides the high sensitivity and selectivity, matrix effects have become a serious concern in quantitative and qualitative LC-MS and LC-MS/MS. Matric effects are observed when other matrix compounds interfere with analytes. These interferences may occur on the LC part of the instrument, which may produce shifts of retention times. In most cases interferences between separate matrix components and analytes occur during the ionization process, causing ionization suppression or enhancement (1-3).

Two main approaches have been proposed for the detection and assessment of matrix effects: post-column infusion methods and post-extraction methods (4-5). Post-column infusion allows a qualitative assessment of matrix effects with respect to the retention time areas where ionization suppression or enhancement are observed. In a typical post-column infusion experiment, a constant flow of a solution containing the target analyte(s) is pumped behind the LC column via a T-piece, using *e.g*. a syringe pump, and is recorded in the mass spectrometer. At the same time a blank sample prepared according to the method in question is injected into the LC-system. The ion suppression or enhancement caused by matrix components can be seen in a decrease or increase of the response of the recorded analyte signal (6). This approach is relatively time- and equipment-consuming. It is mainly used during method development.

In contrast, the post-extraction spike method does not need any further instruments and can be easily included into routine sequences or validation studies. In a post-extraction spike experiment the response of analytes in a standard solution is compared with the response of these analytes in a blank sample spiked into the final extract after extraction. The matrix effect (ME) can be calculated by means of the formula (1):

$$ME\ (\%) = (B/A-1)*100 \qquad [1]$$

A: response (area) of the analyte in standard solution, B: response (area) of the analyte in a blank matrix sample spiked after extraction. A value below 0 indicates ion suppression and, *vice versa*, a value of ME above 0 indicates ion enhancement.

Materials and Methods

Materials

Bambuterol hydrochloride, brombuterol hydrochloride, bromchlorbuterol hydrochloride, cimaterol, cimbuterol, clenbuterol hydrochloride, clencyclohexerol hydrochloride, clenisopenterol hydrochloride, clenhexerol hydrochloride, clenpenterol hydrochloride, clenproperol hydrochloride, hydroxymethylclenbuterol hydrochloride, mabuterol hydrochloride, mapenterol hydrochloride, phenyl ethanolamine A, ractopamine hydrochloride, tulobuterol hydrochloride and zilpaterol hydrochloride were purchased from Witega Laboratorien Berlin Adlershof GmbH (Berlin, Germany). Bamethane hemisulfate, formoterol fumarate, isoxsuprine hydrochloride, ritodrine hydrochloride, fenoterol hydrobromide, salbutamol, salmeterol xinafoate and

terbutaline hemisulfate were bought from Sigma-Aldrich (Munich, Germany). Carbuterol hemisulfate was purchased from Toronto Research Chemicals Inc. (Toronto, Canada).

Stable isotopically labelled compounds used as internal standards were purchased from Witega Laboratorien Berlin Adlershof GmbH (allo erythro isoxsuprine-$^{13}C_6$ hydrochloride, bambuterol-D9 hydrochloride, cimaterol-D7, cimbuterol-D9, clenbuterol-D9 hydrochloride, clenpenterol-D5 hydrochloride, clenproperol-D7, mabuterol-D9 hydrochloride, mapenterol-D11 hydrochloride, phenyl ethanolamine A-D3, pirbuterol-D9 acetate, salbutamol-D9-acetate, salmeterol-$^{13}C_6$ xinafoate, terbutaline-D9 acetate and zilpaterol-$^{13}C_3$ hydrochloride) and from Toronto Research Chemicals Inc. (ractopamine-D6 hydrochloride and Carbuterol-D9 hemi sulphate).

Water was taken from an ultrapure water system Milli-Q Advantage A10, Merck Chemicals GmbH. Methanol (ULC/MS) and formic acid (99%, ULC/MS) were purchased from Biosolve Chimie SARL (Valkenswaard, The Netherlands). Ammonium formiate (LC-MS grade) was bought from Sigma-Aldrich. The used glucuronidase (Glucuronidase-Sulfatase of *Helix pomatia*, Merck 1.04114) was a product of Merck Chemicals GmbH. SPE was carried out on Clean Screen® Dau extraction columns (UCT, Bristol PA, USA).

Potassium phosphate-buffer pH 5 was prepared by dissolving 13.6 g KH_2PO_4 in 950 mL water adjusted to pH 5 using 1 M HCl and filling up to 1,000 mL with water. Potassium phosphate-buffer pH 6 was prepared in the same way, adjusting the pH using 1 M KOH. Na-acetate-buffer pH 5 was prepared as follows: 27 g Na-acetate and 1.7 g ascorbic acid were dissolved in 900 mL water, pH was adjusting to 5 using 25% HCl and volume to 1,000 mL. The SPE elution mixture was a mixture of ethyl acetate and ammonia (32% in water) in a ratio of 97:3. This mixture has to be prepared fresh daily.

LC-MS/MS conditions

Tandem mass-spectrometry was carried out using a waters Xevo TQ S® mass-spectrometer with positive ion electrospray ionization. The MS was coupled with an UPLC Waters Acquity® system. As LC-column a Kinetex F5 (2.1 x 100, 2.6µ) from Phenomenex (Aschaffenburg, Germany) was applied. The column temperature was set to 30°C. Mobile phase A was 5 mM ammonium formate plus 0.1 % of formic acid in water. Mobile phase B was methanol containing 0.1% of formic acid. The following gradient was used for the chromatographic separation: 0 – 0.5 min 99 % A, - 4 min 95 % A, - 20 min 50 % A, - 24 min 50 % A, - 25 min 99 % A, - 30 min 99 % A. The LC-flow was set to 0.5 mL min^{-1}. The desolvation gas flow and desolvation temperature were set were set to 1000 L h^{-1} and 600°C. The cone gas flow was set to 150 L h^{-1}. The source temperature was set to 150°C. The capillary voltage was set to 0.5 kV. Cone voltage and collision energy were optimised for each analyte. The dwell times for the MRM transitions of the analytes were set automatically by MassLynx.

Sample preparation methods

Liver. Ten g of liver from pig, *bovine*, chicken or turkey was chopped finely, *e.g*. in a Moulinette, and filled into 50-mL Sarstedt tubes and 20 mL phosphate buffer pH 5 were added. The slurry was thermostated in a water bath at 40°C and shaken for 20 min at 40 °C. Afterwards the slurry was cooled down to room temperature and centrifuged for 15 min at 4°C. The supernatant was transferred into a further centrifuge tube. The residue was extracted again with 10 mL phosphate buffer (pH 5). After centrifugation the supernatants were combined and 50 µL glucuronidase/sulfatase-solution were added. The samples were then shaken in a water bath for 60 min at 40°C. After cooling to room temperature the pH of the extract was adjusted to 6 using 5 M KOH.

Urine. Ten mL of centrifuged urine of cattle, pig and bull are filled into centrifuge flasks, and 5 mL Na-acetate buffer pH 5 and 50 µL glucuronidase/sulfatase-solution were added. The samples were homogenized using a vortex and incubated overnight at 37°C. The samples were then cooled down to room temperature and, as for the liver extracts, the pH was adjusted to 6.

Solid-phase extraction

The sorbent was conditioned using 2 mL methanol, 2 mL water and 2 mL of phosphate buffer (pH 6). The liver and urine extracts were transferred to the cartridges. The cartridge effluents were discarded. The cartridges were washed with 1 mL 1 M acetic acid. The cartridges were evaporated to dryness for 20 min. Afterwards the cartridges were washed with 2 mL methanol and evaporated to dryness again. To elute the β-agonists, 6 mL of elution solution SPE were added to the cartridge and the effluent was collected. The eluates were evaporated to dryness. In order to investigate the matrix effects, the evaporated eluates were reconstituted in 200 µL mobile phase A/B = 95/5 containing analytes and internal standards.

Results and Discussion

Tables 1 and 2 provide an overview of the matrix effects for some selected ß-agonists in urine and liver of different species and at two different concentration levels. Figures 1 and 2 illustrate these effects for the concentration level of 0.5 µg kg^{-1} in urine and liver of different species. For most compounds, matrix effects cause ion suppression, in particular for analytes in the first third of the chromatogram. Compounds with higher retention times like clenbuterol and brombuterol show an ion enhancement due to the matrix. Isoxsuprine is an exception to this rule. Moreover, the tables and figures give an impression about the really great differences of matrix effects on the different compounds in different species.

Table 1. Matrix effects of ß-agonists in urine of cattle, pig and bull

Analyte	Rt (min)	0.025 µg kg^{-1} ME (%)	0.5 µg kg^{-1} ME (%)	0.025 µg kg^{-1} ME (%)	0.5 µg kg^{-1} ME (%)	0.025 µg kg^{-1} ME (%)	0.5 µg kg^{-1} ME (%)
		Cattle		Pig		Bull	
Cimaterol	2.79	-69	-74	-39	-39	-40	-53
Terbutaline	3.02	-166	-75	-21	-69	-60	-78
Zilpaterol	3.19	-71	-73	-68	-63	-65	-62
Salbutamol	3.46	-42	-42	-25	-27	-26	-38
Ritodrine	5.97	-26	-43	-40	-13	-14	-29
Ractopamine	9.11	-32	-39	-22	-30	-22	-30
Clenbuterol	10.14	57	21	88	43	157	34
Brombuterol	11.46	42	0.90	55	10	65	3.0
Isoxsuprine	11.69	-25	-9.0	-2.7	-7.0	55	-10

Table 2. Matrix effects of ß-agonists in liver of pig, bovine*, chicken and turkey*

Analyte	0.025 µg kg^{-1} ME (%)	0.5 µg kg^{-1} ME (%)	0.025 µg kg^{-1} ME (%)	0.5 µg kg^{-1} ME (%)	0.025 µg kg^{-1} ME (%)	0.5 µg kg^{-1} ME (%)	0.025 µg kg^{-1} ME (%)	0.5 µg kg^{-1} ME (%)
	Pig		*Bovine*		Chicken		Turkey	
Cimaterol	-6.1	-6.1	-47	-49	-3.7	-18	-5.0	-7.1
Terbutaline	-23	-16	-61	-62	-8.2	-18	-10	-13
Zilpaterol	-34	-33	-57	-52	-15	-9.5	-25	-30
Salbutamol	-26	-27	-37	-34	-16	-21	-19	-25
Ritodrine	-1.6	-1.2	-18	-13	-0.49	-10	-6.5	-0.05
Ractopamine	-3.8	-15	-6.5	-11	-4.8	-15	0.15	-6.9
Clenbuterol	62	24	68	19	70	14	78	20
Brombuterol	60	16	65	10	62	8.4	78	18
Isoxsuprine	-2.9	-6.1	-6.9	-8.0	1.3	-7.3	-10	-13

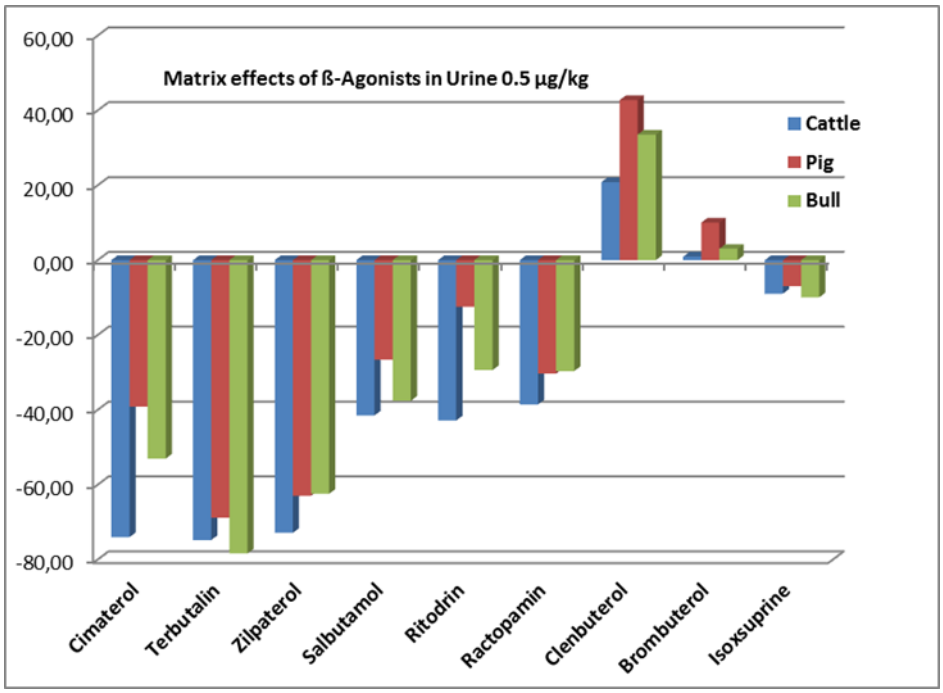

Figure 1. Matrix effects of ß-agonists in urine of cattle, pig and bull

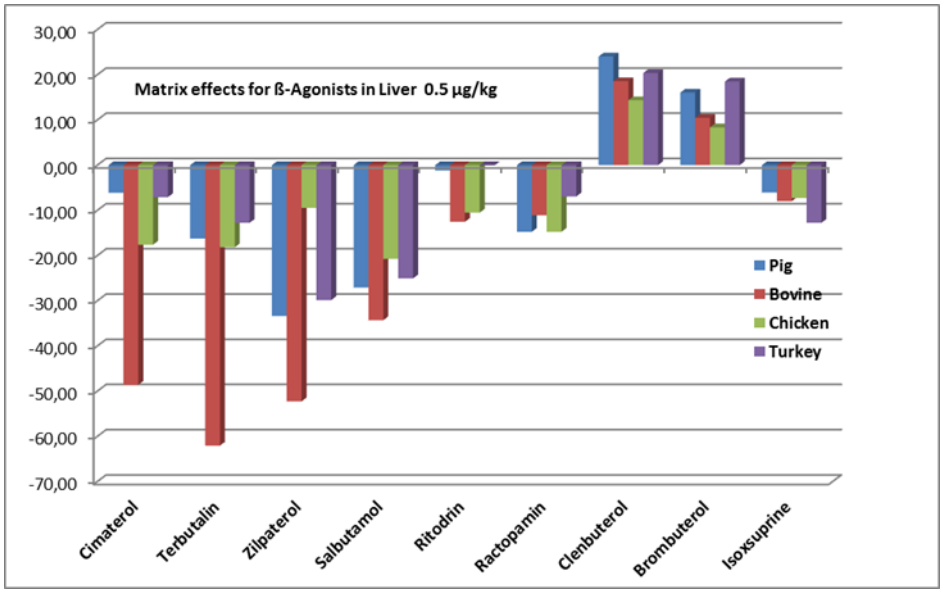

Figure 2. Matrix effects of ß-agonists in liver of pig, bovine, chicken and turkey

Among the selected ß-agonists, cattle urine has the greatest effect on cimaterol, salbutamol, ritodrine and ractopamine. Pig urine has a strong influence on the ß-agonists clenbuterol and brombuterol, which are ß-agonists of the aniline type.

With respect to liver samples a similar behaviour can be observed: a strong ion suppression for compounds in the first third of the chromatogram, *i.e*. with a higher water content of the mobile phase and ion-enhancement for compounds with higher retention times. *Bovine* liver has a remarkably strong influence in comparison to pig-, chicken- and turkey liver on cimaterol, terbutaline, zilpaterol and salbutamol. In contrast to cattle liver, the matrix effects of chicken liver have a similar level of magnitude for all analysed ß-agonists, but with opposite signs for clenbuterol and brombuterol.

Conclusions

Different species cause different matrix effects. Although the pattern of the matrix effects is the same for liver and urine samples of different species, the observed matrix effects are not the same for the different analytes. That is not surprising for multi-methods covering different analytes with different chemical structures and properties. Additional steps in sample preparation, *e.g*. an additional liquid-liquid extraction or other procedures, may reduce matrix effects for some compounds, but have no or inverse effects on other analytes. Therefore, for multi-methods compromises are inevitable.

Although the influence of the matrix effects is the similar for urine and liver samples, it is not clear whether the matrix components which trigger these effects are alike. Further investigations should investigate differences or similarities of these components.

Acknowledgements

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INTESTINAL AND PLASMA CONCENTRATIONS OF FLORFENICOL IN PIGS AFTER (NON-)CONVENTIONAL ORAL AND INTRAMUSCULAR TREATMENT, WITHIN THE CONTEXT OF RESISTANCE SELECTION.

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Abstract

A fast and effective extraction method for florfenicol in pig manure, followed by quantification using LC-MS/MS, has been developed. After diluting manure samples tenfold with PBS and adjusting sample pH with addition of NaOH, liquid-liquid extraction was performed using ethyl acetate. The obtained supernatant was evaporated to dryness and subsequently reconstituted, before injecting an aliquot onto the LC-MS/MS instrument operating in ESI negative mode. Chromatographic separation was achieved by use of a reversed-phase Hypersil Gold™ column. A linear range of $10.00 \cdot 10^{-3}$ – 1.00 µg g^{-1} was achieved, whilst fulfilling validation parameters such as goodness-of-fit (all values ≤10%) and correlation coefficient (all values ≥0.99). The detection limit was $2.90 \cdot 10^{-3}$ µg g^{-1} and the limit of quantification for florfenicol in manure was $10.00\ 10^{-3}$ µg g^{-1}. Hence, this method allows for accurate and precise quantification of florfenicol in pig manure samples. Following, this methodology will be used to assess the exposure of the gut microbiota to florfenicol after antimicrobial therapy. The effect of the detected concentrations on antimicrobial resistance selection in the intestinal microbiota will then be assessed.

Introduction

The current posology of veterinary antimicrobial drugs is mainly established solely on clinical efficacy, without taking resistance selection of pathogenic or commensal bacteria into account. Moreover, dosage regimens of antimicrobials in animal husbandry commonly show considerable variability, even between manufacturers (1). In order to assess the effect of different dosage regimens (administration route and dosage) on resistance selection in the intestinal commensal microbiota, data on the exposure are mandatory.

Florfenicol is a broad-spectrum antimicrobial belonging to the class of the amphenicols. It is increasingly used in veterinary medicine because of its efficacy and currently low levels of resistance (2). When administered in pigs, florfenicol is mainly renally excreted, even though a significant part (up to 24%) is excreted via the faeces (3). Next to the oral bioavailability, the route of elimination is of major importance with regards to exposure of the gut microbiota to the antimicrobial. Indeed, different concentrations can have a different influence on resistance selection in the gut microbiota (4).

Hence, the objective of this study was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify florfenicol in intestinal content and manure of pigs. Because of the complexity of manure as a matrix, similar studies describing analytical methods for florfenicol remain scarce (5-7). This study provides an effective sample pre-treatment method of manure, by using a fast and simple liquid-liquid extraction (LLE) procedure. This was combined with an LC-MS/MS method for quantitative determination of florfenicol in pig manure.

Materials and Methods

Chemicals

For the analytical procedures, HPLC-grade standards of florfenicol (Sigma-Aldrich, Diegem, Belgium) and its internal standard florfenicol-d_3 (Bio-Connect BV, Huissen, The Netherlands) were purchased. Furthermore, all solvents used were of appropriate analytical grade: acetonitrile (ACN), methanol (MeOH) and water (H_2O) were purchased from Fisher Scientific (Erembodegem, Belgium). Glacial acetic acid and ethyl acetate (EtOAc) were obtained from VWR (Leuven, Belgium). Phosphate buffered saline (PBS) was obtained from Sigma-Aldrich (Diegem, Belgium). Next, standard solutions of the antimicrobial compounds were prepared in a H_2O/MeOH solution (50/50; v/v). These solutions were stored airtight and protected from light at ≤ 8°C in agreement with the analytical certificates. For filtering of the extracts polyvinylidene fluoride (PVDF) filters (Merck Millipore, Overijse, Belgium) were used.

Sample pre-treatment

A rapid and effective sample pre-treatment for the faecal samples was developed based on LLE. One gram of manure was homogenized through vortex mixing and diluted tenfold in PBS. Next, a 1-g subsample of this dilution was taken, to which 25 µL of the internal standard solution (10.00 µg mL^{-1}) were added. Based on the logarithmic acid dissociation constant (pK_a) of florfenicol (10.73), 20 µL of 1M sodium hydroxide (NaOH) was also added to the samples. This led to a shift towards pH 10,

which was optimal for the extraction of florfenicol given its pK_a. Next, the samples were vortex mixed and allowed to equilibrate during 15 min. In a next step, 7.5 mL of EtOAc were added, followed by roller mixing (Stuart Scientific, Surrey, UK) during 10 min and centrifugation (4,000 rpm, 4°C, 10 min). A clear supernatant was obtained and transferred to glass tubes, which were subjected to a nitrogen stream (40 ± 5°C) until dryness. The extract was reconstituted using 0.5 mL of a 0.1% acetic acid solution in H_2O/ACN (80/20 v/v). Finally, the sample was transferred to a glass vial after filtering (PVDF filter). An aliquot of 10.00 µL was injected onto the LC-MS/MS instrument.

Instrumentation

Liquid chromatography was performed using a Hypersil Gold™ column (reversed phase, 50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column (10 mm x 2.1 mm i.d., dp: 5µm), both obtained from Thermofisher Scientific (Breda, The Netherlands). Column oven temperature was 40.0°C and the autosampler tray was kept at 8.0°C. Mobile phases for chromatographic separation consisted of 0.1% acetic acid in H_2O (A) and ACN (B). The following gradient elution program was applied: 0-1 min (85% A, 15% B), 1.5-6 min (10% A, 90% B), 7-14 min (85% A, 15% B). Flow rate was set at 300 µL min^{-1}. Liquid chromatography was performed on a Thermofisher Scientific system (Breda, The Netherlands).

This LC system was connected to a TSQ® Quantum Ultra triple quadrupole mass spectrometer (also from Thermofisher). Ion source heated electrospray ionization (ESI) was used operating in negative electrospray ionization mode (ESI). System parameters for MS analysis were optimized using direct infusion of working solutions, *i.e*. 0.10 µg mL^{-1} florfenicol, and its internal standard in a H_2O/ACN mixture (80/20 v/v). Following parameters were obtained after tuning: spray voltage: 4,000 V, vaporizer temperature: 300°C, sheath gas pressure: 49 au (arbitrary units), ion sweep gas pressure 2.0 au, auxiliary gas pressure 25 au, capillary temperature: 250°C, collision pressure: 1.5 mTorr. Acquisition was performed in the selected reaction monitoring (SRM) mode. For florfenicol and its internal standard, following transitions were followed (*quantification ion): *m/z* 356.00 > 185.00/336.00*, florfenicol-d_3: *m/z* 359.00 > 188.00, 339.00*.

Method validation

All validation procedures were carried out based on an in-house developed validation protocol which is compliant with European and international guidelines (8-10). All crucial validation parameters were evaluated, namely linearity, accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), extraction recovery (R_E) and signal suppression and enhancement (SSE).

The LOQ is the smallest measured content of an analyte above which the determination of the analyte can be made with a specified degree of accuracy and precision. This parameter was determined by analysing six independently spiked samples. The LOD is the smallest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. Moreover, this was determined based on the signal-to-noise (S/N) ratio of the florfenicol peaks in the LOQ samples (LOD = S/N ratio ≥ 3).

Accuracy and precision were determined at different concentration levels (each n=6); namely the LOQ-level, an intermediate level and a high level ($1.00 \cdot 10^{-2}$, $1.00 \cdot 10^{-1}$ and 1.00 µg g^{-1}).

Results and discussion

In terms of sample pre-treatment, several procedures were evaluated using different extraction solvents. Because of the time-consuming nature and higher cost, a clean-up step using solid-phase extraction (SPE) was discarded from the start. Thus the focus lay mainly on finding an efficient LLE protocol. Extraction efficiency of several organic solvents such as ACN, ammonium acetate and EtOAc was assessed (data not given). Based on previous experimental procedures for the extraction of several classes of antimicrobials in manure, EtOAc came forward as the most effective extraction solvent. In order to make the procedure more molecule-specific for florfenicol, it was opted to alkalinize samples with 1 M NaOH. This allowed for higher extraction efficiency, because of a decreased ionization of florfenicol at a more basic pH.

A matrix-matched approach was used for calibration of the method in manure. The calibration curve for florfenicol ($1/x^2$ weighing) was linear over the working concentration range ($1.00 \cdot 10^{-2}$ – 1.00 µg g^{-1}) as displayed in Figure 1. This linearity was evaluated based on two parameters: correlation coefficient (r) and goodness-of-fit (g) as given in Table 1.

Next, the LOQ and LOD were determined (Table 1). A chromatogram of a manure sample spiked at the LOQ level is illustrated in Figure 2.

Table 2 shows the results of the accuracy and precision determination.

Table 1. Overview of following validation parameters: LOD, LOQ and linearity.

Matrix	LOD (µg g^{-1})	LOQ (µg g^{-1})	Range of r-values	Range of g-values (%)
Manure	$2.90 \cdot 10^{-3}$	$10.00 \cdot 10^{-3}$	0.9936 – 0.9991	4.18 – 7.77

The acceptance criteria for r were: ≥0.99 and for g ≤10%.

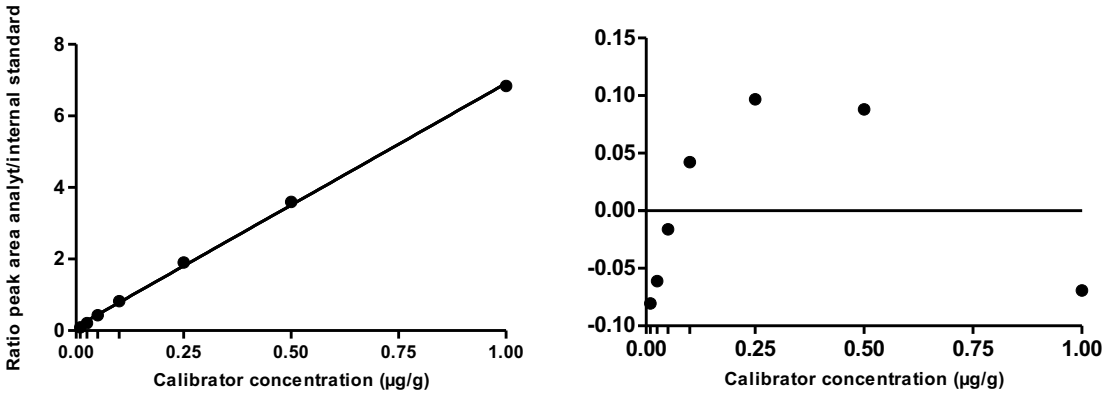

Figure 1. On the left: Representative calibration curve ($1/x^2$) for florfenicol with linearity achieved over the range of 0.010 – 1.00 µg g^{-1} with r^2 equal to 0.9991. On the right: The g value of 6.62% for this curve was based on the given residuals plot.

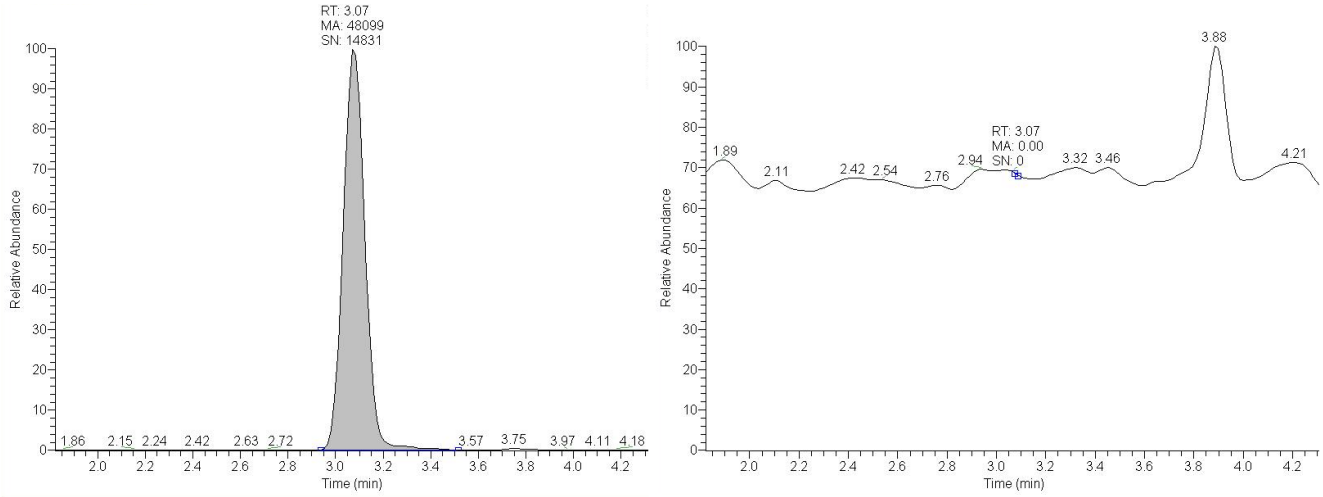

Figure 2. Chromatogram of a manure sample spiked at LOQ level (10.00·10^{-3} µg g^{-1}, left) versus a blank sample (on the right).

Table 2. Validation results for accuracy and precision.

Florfenicol in manure	Theoretical concentration (µg g^{-1})	Mean concentration ± SD (µg g^{-1})	Precision (RSD %)	Accuracy (%)
	[a] 1.00·10^{-2}	1.03·10^{-2} ± 1.23·10^{-3}	10.9	3.0
	[a] 1.00·10^{-1}	1.10·10^{-1} ± 4.88·10^{-3}	4.4	9.9
	[a] 1.00	0.942 ± 0.51·10^{-1}	5.4	-5.8
	[b] 1.00·10^{-2}	9.90·10^{-2} ± 1.01·10^{-3}	10.1	-1.3
	[b] 1.00	0.901 ± 0.58·10^{-1}	6.5	-9.9

[a] Between-run accuracy and precision (n=6) on three different days of analysis; [b] Within-run accuracy and precision (n=6) SD: standard deviation; RSD: relative standard deviation.
Acceptance criteria: accuracy: -20% to +10%, within-run precision (RSD_{max}): ≥ 10 ng g^{-1} < 100 ng g^{-1}: 15%; ≥ 100 ng g^{-1}: 10%, between-run precision: $RSD_{max} = 2^{(1-0.5\log \text{concentration})}$

Conclusions

A fast, accurate and precise method was developed for the quantitative determination of florfenicol in pig manure. This method will be further used to assess intestinal florfenicol concentrations in duodenum, jejunum, ileum, cecum, colon and rectum, after antimicrobial therapy in pigs. Because of the rapid nature of this method, it is possible to process ample amounts of samples within a relative short time span.

Furthermore, determining the concentrations of the drug in intestinal samples is interesting with regards to the exposure of the gut microbiota to florfenicol and possible antimicrobial resistance selection. Even though it has to be noted that the linear range of the method may not suffice for analysing samples from pigs treated with florfenicol. It is expected that concentrations in the faeces could exceed the 1.00 µg g^{-1} upper limit of the calibration curve, despite the tenfold dilutions during

sample pre-treatment. However, this problem can easily be overcome by appropriate dilution of the samples. Moreover, any influence on SSE will be compensated by the use of the internal standard florfenicol-d_3.

Acknowledgements
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DETERMINATION OF AMPICILLIN RESIDUES BELOW THEIR EU-REGULATORY LIMITS IN MUSCLE, LIVER AND PLASMA OF CHICKEN BY LC-MS/MS

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Abstract
Differently to the regulatory monitoring, a full assessment of exposure to residues of veterinary drugs at any level in food-stuff requires analytical methods allowing reliable quantification at levels far below the Maximum Residue Limits (MRL). A new specific method was developed to determine ampicillin, a penicillin antibiotic (β-lactam), in muscle, plasma and liver of chicken below the MRL set at 50 µg kg^{-1}. Ampicillin was firstly extracted from chicken samples with a phosphate buffer solution. For muscle and liver samples only, an additional subsequent extraction step with acetone was implemented in order to remove lipids from these matrices. Then, a purification procedure by solid phase extraction was performed for samples from all matrices followed by the analysis using liquid chromatography coupled to a tandem-mass spectrometer (LC–MS/MS). The performance of the method was then assessed. Linearity and accuracy (recoveries higher than 92 %) meet the requirements of Decision 2002/657/EC. Precision was also estimated with a relative standard deviation less than 16 %. Hence, the method was considered reliable for the quantification of ampicillin in chicken at concentration levels 50 and 100 times lower than the MRL (CCα estimated at 1 µg kg^{-1} in muscle and liver and 0.50 µg kg^{-1} in plasma).

Introduction
Ampicillin is a β-lactam antibiotic belonging to the penicillin sub-family and widely used in veterinary medicine. To protect human and animal health and to mitigate antibiotic resistance the control of antibiotic residues in edible animal tissues is mandatory in the European Union (EU) through the Directive No (EC) 96/23. The maximum residue limit (MRL) of ampicillin is defined in the foodstuffs from chicken origin at 50 µg kg^{-1}.

Therefore, analytical methods developed in France by the National Reference Laboratory (NRL) of Fougères (Anses) for the confirmation of the presence of veterinary drugs in products from animal origin are based on the regulatory MRLs.

However, there is a need to provide scientific data about levels of contamination below these established MRLs for antibiotics in food-producing animals. A particular interest is set on comparing the different modes of animal breeding in regard to the chemical contamination status (French Funded Project SOMEAT: Safety of Organic MEAT). With this aim, the NRL of Fougères took part in the SOMEAT project with the objective to determine the levels of contamination of ampicillin residues in a dozen of diet-contaminated and control chicken animals. For this purpose, a new analytical method allowing the quantitation of ampicillin at concentrations far below the MRL had to be developed.

The present manuscript describes the development of a rapid and sensitive method for the dosage by liquid chromatography coupled to tandem-mass spectrometric detection (LC–MS/MS) of ampicillin in muscle, liver and plasma of chicken at concentrations below the MRL. Performances of the method were also evaluated in line with the Decision No (EC) 2002/657.

Materials and Methods

Standards, reagents and solutions
Analytical grade ampicillin and cephalexin (internal standard) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). LC-MS grade Optima acetonitrile (ACN), acetone, and methanol were obtained from Fisher Scientific (Illkirch, France). 98–100% formic acid solution, and sodium chloride salt and disodium hydrogen phosphate were purchased from Merck (Fontenay-sous-bois, France). Water was deionised by a Milli-Q system of purification (Millipore, St. Quentin en Yvelines, France).

Individual primary stock solution of ampicillin and cephalexin were prepared at 0.5 g L^{-1} in 50:50 acetonitrile/water (V/V). Working solutions of ampicillin and cephalexin at 500 µg L^{-1} and 50 µg L^{-1} were prepared by appropriate dilution in water of stock solutions. Stock solution of ampicillin and cephalexin were satisfactorily stored at 4°C during 6 months and one year, respectively. The 500 µg L^{-1} and 50 µg L^{-1} working solutions of ampicillin and cephalexin were stored at +4°C one month, and 1 week, respectively. The 50 µg L^{-1} solutions were used for spiking samples of muscle, liver and plasma at five levels.

A 0.1 M phosphate buffer was prepared for solid-phase extraction (SPE) by dissolving 7.1 g of disodium hydrogen phosphate in 500 mL of water adjusted to pH 8.1 with 1 N phosphoric acid solution. A 5% sodium chloride solution was prepared by dissolving 25 g sodium chloride in 500 mL water.

Sample preparation

Muscle and liver samples were minced and homogenized. An amount of 1 g muscle, 1 g liver and 1 mL plasma were transferred in three tubes before adding 100 µL of the 50 µg mL^{-1} internal standard solution (cephalexin). Samples were then vortexed for 10 s. A volume of 5 mL 0.1 M phosphate buffer at pH 8.1 was added to the tubes and vortexed again for 10 s. Exclusively for muscle and liver samples, 5 mL of acetone was added in order to extract lipophilic compounds from these matrices to prevent clogging in the SPE step and matrix effects during mass spectrometric analysis.

All samples (muscle and liver tissues, and plasma fluid) were then extracted using a rotary tumbler for 10 min. The the organic phase of the supernatant of each sample obtained after centrifugation at 14,000 *g* for 5 min was evaporation of under a gentle flow of nitrogen.

The remaining aqueous phase (about 5 mL) of each sample tube was then extracted on a vacuum system onto C18 Bond Elut SPE cartridges (500 mg, 6cc, Agilent, Les Ulis, France). Prior to use, SPE cartridges were conditioned with successively 5 mL methanol, 5 mL water, 5 mL 5% sodium chloride solution, and finally 5 mL of the phosphate buffer pH 8.1. The about 5 mL of sample were then loaded onto the pre-conditioned cartridges before a washing step of the cartridges with 3 mL 5% sodium chloride solution followed by 3 mL water. Cartridges were vacuum-dried during 5 min, and the elution of the extracts was carried out with 5 mL acetonitrile. Sample extracts were then evaporated until dryness at 40°C. The dry residue was reconstituted in 300 µL water, vortex-mixed, centrifuged at 20,000 *g* for 5 min at 4°C, filtered over a Millex HV PVDF filters (Millipore, St. Quentin en Yvelines, France), transferred into screw cap vials with silicone-PTFE (polytetrafluoroethylene) septa from Interchim (Montluçon, France), and then analysed by LC-MS/MS.

Liquid chromatography – tandem mass spectrometry conditions

A Thermo Fisher Scientific Accela High-Performance Liquid Chromatography coupled to a Thermo Fisher Scientific triple quadrupole mass spectrometer TSQ Vantage equipped with an electrospray source was used to perform sample analysis. Ampicillin was separated at a flow rate of 0.4 mL min^{-1} using a reverse phase Luna C18 (150 x 2 mm, 3 µm) column from Phenomenex® (Le Pecq, France), with a pre-column Phenomenex® C18 4x2 mm AJO-4286.

A volume of 20 µL sample extract was injected into the column, which was eluted within one single run gradient of 7 min using a binary mobile phase. The mobile phase consisted of purified water with 0.2% of formic acid (A), and acetonitrile (B). B was held at 5% for the first minute, with a linear gradient to 50% from 1.0 to 2.0 min, held at 50 % from 2 to 3.5 min, returning to initial conditions (*i.e.* 5% B) from 3.5 to 4.0 min by a linear gradient, and held at 5 % from 4.0 to 7.0 min for a re-equilibration time.

Mass spectrometric detection was performed using ESI in positive mode, and data acquisition was operated in multiple reaction monitoring (MRM) mode (Table 1). The ESI interface conditions and tandem MS conditions were as follows: Capillary temperature = 250°C; vaporiser temperature = 300°C; sheath gas pressure = 30 psi; ion sweep gas pressure = 10 psi; CID pressure (argon) = 1.3 mTorr; spray voltage = 3,500 V; resolution Q1 and Q3 =0.7; scan width = 0.01 *m/z*.

Table 1. MS/MS parameters for the determination of ampicillin in muscle, liver and plasma of chickens

Analyte	Ion mode	Precursor ion *m/z*	Product ions *m/z*	Collision Energy (eV)	S-Lens (V)
Ampicillin	Positive	350.1 $[M+H]^+$	106.1	15	84
			114.0	34	84
Cephalexin (internal standard)	Positive	348.1 $[M+H]^+$	158.0	15	73

Design of the assessment of the method performances

Determination of the criteria of performances (i.e. selectivity, trueness, repeatability) was performed for the three matrices (plasma, muscle and liver).

For each matrix the experiment consisted in one batch including one blank sample of the matrix, samples prepared for the matrix calibration curve and validation standards.

For the calibration curve, four blank matrix samples were spiked with appropriate volume of working solution of ampicillin and cephalexin (internal standard) in order to obtain a series of five levels of concentration of ampicillin: 0, 1, 4, 7, 10 µg kg^{-1} for plasma matrix, 0, 2.5, 5, 7.5, 10 µg kg^{-1} for muscle matrix, and 0, 2.5, 5, 7.5, 10 µg kg^{-1} for liver matrix. After spiking these samples were prepared in the same way as the samples to analyse. All calibration standards were then analysed twice by LC-MS/MS.

Blank matrix plasma, muscle, and liver were used as validation standards which were prepared as described after spiking at low and high level of known concentrations of ampicillin. Precisely, for each matrix one series of 12 blank matrix samples were prepared at two levels of concentration of ampicillin. Hence, series were made as follows: *i*) 6 spiked blank samples at 1 µg kg^{-1} and 6 spiked blank samples at 10 µg kg^{-1} for plasma, *ii*) 6 spiked blank samples at 2.5 µg kg^{-1} and 6 spiked blank samples at 10 µg kg^{-1} for muscle, and *iii*) 6 spiked blank samples at 2.5 µg kg^{-1} and 6 spiked blank samples at 10 µg kg^{-1} for liver.

Results and discussion

The results of the performance assessment of the method are displayed in Table 2.

Performance of detection

No interference peak was noticed in the window of the retention time for ampicillin in any blank sample of chicken plasma, muscle or liver.

Signal-to-noise ratio was estimated higher than three at all levels of concentration in spiked samples, even at the lowest studied concentrations in all three matrices. Figure 1 shows representative chromatograms of spiked samples of plasma, muscle and liver with ampicillin at 1 (plasma), 2.5 (muscle and liver) and 10 µg kg^{-1} (plasma, muscle and liver). Identification of ampicillin met the tolerance stated in the Decision No (EC) 2002/657 (*i.e.* 2.5%) as the relative retention time (RRT *i.e.* ratio of the retention time of ampicillin to the cephalexin (internal standard)) was found less than 0.7%. An ion ratio criterion relative to the quantifier ion and to the qualifier ions was satisfactorily met for calibration standards in all the three matrices as its coefficient of variation never exceeded the 25% required by the Decision No (EC) 2002/657.

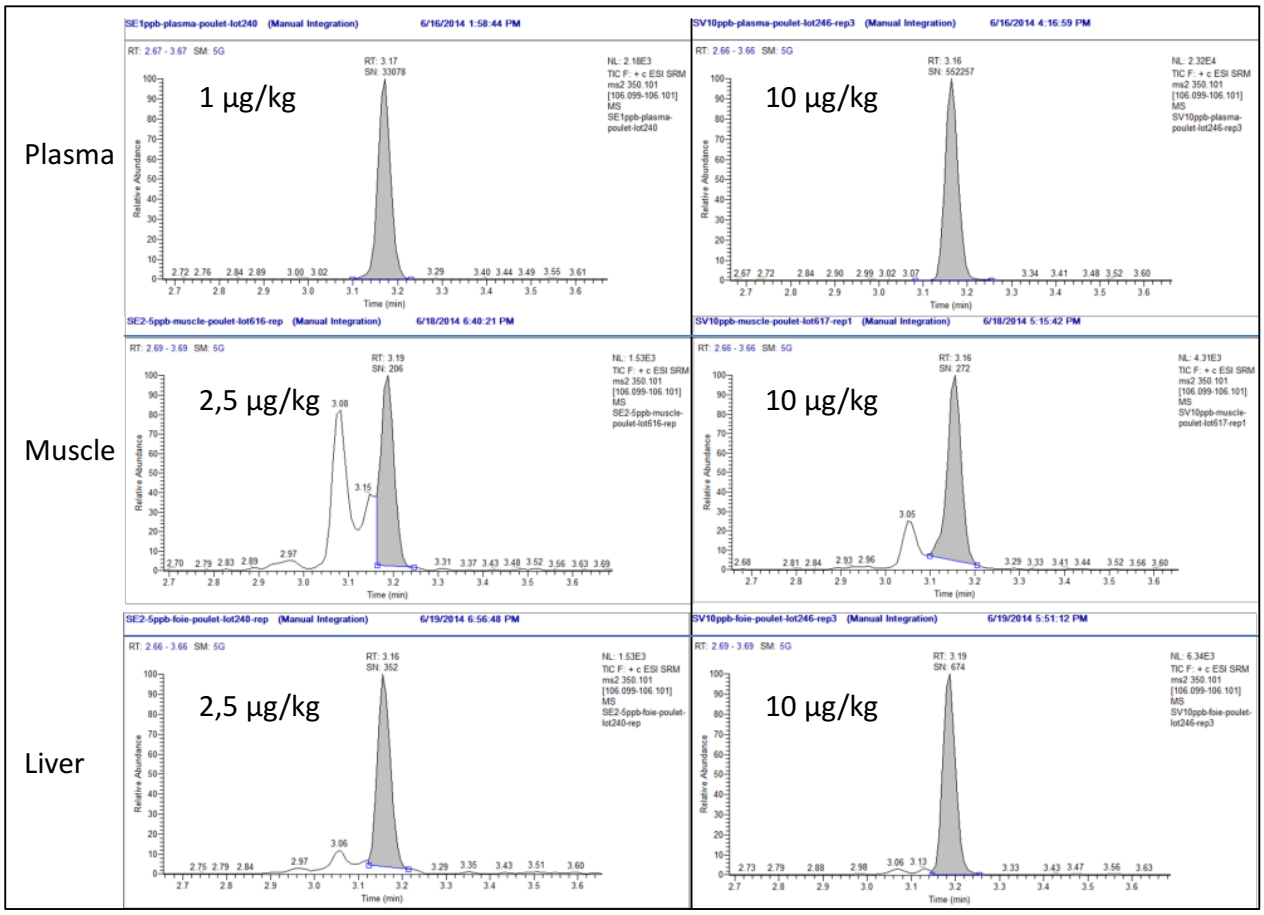

Figure 1. MRM chromatogram (transition m/z 350>106) obtained from spiked sample at 1 (plasma), 2.5 (muscle and liver), and 10 µg kg^{-1} (plasma, muscle and liver) of ampicillin.

Performance of quantification

The linear response function was chosen for the quantitation of ampicillin in samples as it complied with the Decision No (EC) 657/2002 in terms of trueness and repeatability.

Good linearity was observed with a correlation coefficient (R Pearson) ≥ 0.99 for all matrix calibration curves. The method was considered satisfactory for the dosage of traces of ampicillin within the range of 1 to 10 µg kg^{-1} in chicken plasma, and

2.5 to 10 µg kg^{-1} in chicken muscle and liver. The limits of quantification were defined at the lowest level of concentration of the dynamic range of dosage for each matrix, *i.e*. 1 µg kg^{-1} for plasma and 2.5 µg kg^{-1} for muscle and liver. The objective of the study was fully addressed as the method allows quantifying concentrations 20 to 50 times below the MRL.

Trueness was satisfactory as the recoveries (%) of ampicillin for each matrix of interest met the 70-110% range according to the Decision No (EC) 2002/657. More precisely recoveries were all higher than 92% and lower than 102% in all three matrices.

The method precision (intra-day repeatability) was estimated at the lowest and the highest concentration level for each matrix under repeatability level (six replicates, one operator). Precision of the method was considered satisfactory for all matrices as the relative standard deviation (RSD%) was ≤ 5% at both levels of concentration, except for muscle for which a RSD% at low level (2.5 µg kg^{-1}) eventually reached 15%. Because the method was not originally dedicated to the regulatory control, the precision results in muscle tissues were considered acceptable for the analysis of ampicillin traces in this matrix.

The method was considered reliable enough for a unique planned analysis of traces of ampicillin in a dozen samples within the framework of the French national SOMEAT project. However, intermediate precision (at least inter-day reproducibility) would need to be assessed for a further laboratory routine use of the method.

Decision limits CCα

Decision limits CCα were also calculated based on the transition of ampicillin having the worst sensitivity (350.1>114) for each matrix and according to the NF ISO 11843-2 guideline applied to forbidden substances. Results are presented in Table 2.

The CCα's obtained were about 0.5 µg kg^{-1} in plasma, and about 1.0 µg kg^{-1} in muscle and liver; these levels are 50 and 100 times below the MRL (50 µg kg^{-1}), respectively. Calculated CCα is also lower than a previous reported method (Macarov *et al.* 2012). This difference regarding the target level of concentration to quantify in samples can be explained by the notable scientific objectives represented by the MRL regulatory control (Macarov *et al.* 2012) on the one hand and an exposure assessment to contaminants on the other hand. The present results provide a guarantee of good performances for the determination of ampicillin traces in the different matrices of interest.

Table 2. Performance parameters of the analysis of ampicillin in muscle, liver and plasma

Matrix	Spiking level (µg kg^{-1})		Trueness		Precision		Decision Limits
	I	II	Recovery (%)		Repeatability (CV%)		CCα (µg kg^{-1})
Muscle	2.5	10	92	101	15.1	1.1	0.94
Liver	2.5	10	94	99	5.0	4.2	0.96
Plasma	1.0	10	96	102	3.6	5.0	0.50

Conclusions

A simple, fast and sensitive method based on SPE extraction followed by HPLC-MS/MS analysis was successfully developed for the quantification of ampicillin in muscle, liver and plasma samples from chicken. Performances of the method were checked in line with Decision (EC) 2002/657. Data obtained show a satisfactory precision (repeatability) and trueness. The method was considered reliable for the analysis of low levels of ampicillin residues for a one-day planned analysis of traces of ampicillin in a dozen samples in the framework of the French national SOMEAT project. Intermediate precision would need to be assessed for a further laboratory implementation of the method in routine use cases.

Acknowledgements

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IN-HOUSE VALIDATION OF AN ANALYTICAL METHODOLOGY FOR DETECTION OF OXYTETRACYCLINE (OTC), CHLORTETRACYCLINE (CTC) RESIDUES AND THEIR METABOLITES IN FEATHERS BY LIQUID CHROMATOGRAPHY TANDEM-MASS SPECTOMETRY (LC-MS/MS)

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Abstract

In order to study oxytetracycline (OTC) and chlortetracycline (CTC) residue behaviour in feathers, it is essential to validate the available method for the determination of these analytes and their metabolites: 4-epi-OTC and 4-epi-CTC. The methodology described by Berendsen *et al*. (2013) was used as reference. Antibiotic-free broiler feathers were fortified with certified standards at five levels (20-40-60-80-100 µg kg^{-1}), and tetracycline-d6 as the internal standard. Extraction of the analytes was carried out using EDTA-McIlvain buffer plus acetone and OASIS HLB® SPE columns. Extracts were analysed by LC-MS/MS. Detection limit of 20 µg kg^{-1} and quantification limit of 22.6 µg kg^{-1} (OTC), 21.5 µg kg^{-1} (CTC), 24.2 µg kg^{-1} (4-epi-OTC) and 23.0 µg kg^{-1} (4-epi-CTC) were calculated. Validation parameters such as linearity, recovery and precision were evaluated according to the Guideline 657/2002/EC. For linearity, the determination coefficient obtained by the method was more than 0.97 for all analytes. Recovery ranged from 92% to 107% for all the concentrations in the four studied analytes. The method met the acceptance criteria revealing that the method is a precise and reliable for determination of these analytes in feathers.

Introduction

The presence of veterinary drug residues in food above their respective MRLs constitutes a risk to public health. They can cause different adverse effects on human health, including toxic and immunogenic effects (allergic reactions), mutagenicity, carcinogenicity, teratogenicity and effects on intestinal microflora. Low concentrations of these drugs contribute to sustained pressure for selection of resistant bacteria that colonize animal tissues and produce disturbances in the normal flora (Martínez and Baquero, 2002; Anadón and Martínez-Larrañaga, 2012). Moreover, there is enough evidence for the transfer of resistant bacteria and resistance genes from animals to humans. In particular, zoonotic bacteria such as *Campylobacter* spp. and *Salmonella* spp. are of concern as chickens and turkeys are a reservoir for these germs (Anderson *et al*., 2003). Fairchild *et al.* (2005) evaluate the effects of the administration of tetracyclines on bacteria isolated from commercial birds, such as *Enterococcus spp*, *E. coli* and *Campylobacter spp*. They showed that *Enterococcus spp.* and *E. coli* were resistant to tetracycline and found resistance genes in samples obtained from both treated and non-treated chickens.

Tetracyclines were discovered in 1948, as a natural product of *Streptomyces* species. One of the characteristics of these antimicrobials is the presence of a keto-enol functional group that allows chelating divalent cations. Chlortetracycline (CTC) and oxytetracycline (OTC) are used in veterinary and human medicine as a treatment for respiratory and digestive diseases, since their activity spectrum includes different types of bacteria, such as Gram positive, aerobic Gram negative, anaerobic, spirochetes, *Actinomyces*, *Rickettsia*, *Chlamydia*, *Mycoplasma* and some protozoa (Prats *et al.,* 2005; Cristofani *et al.,* 2009). The bioavailability of tetracycline when administrated orally is variable, being 30% for chlortetracycline and 60 to 80% for oxytetracycline and tetracycline. These antibiotics are widely distributed throughout the organism, and they can be found in urine, prostatic fluid and reticuloendothelial cells of the liver and spleen and also, in bone marrow, bone, dentin and tooth enamel (Patiño and Campos, 2008; Rang *et al*, 2012) as well as in human skin and nails accumulation has been described (Geria *et al.,* 2009). Therefore, residues of this antibiotic can be found also in feathers of treated birds, since feathers, nails and hair are complementary structures of the integumentary system (Bragulla and Homberger, 2009).

Currently, the bioaccumulation of antimicrobial residues in feathers has been observed to occur at higher concentrations and for longer periods than in edible tissues. San Martin *et al.* (2007) found higher concentrations of enrofloxacin and ciprofloxacin in feathers of chickens treated with this antibiotic when compared to edible tissues (muscle, liver and kidney). Likewise, a study by Cornejo *et al*. (2011) showed higher concentrations of three formulations of flumequine in feathers when compared to liver and muscle following the corresponding WTD. Besides, the depletion of flumequine in feathers was slower than other tissues. Moreover, Cornejo *et al*. (2012) showed that enrofloxacin and its metabolite (ciprofloxacin) were transferred to feathers from treated chickens, and also resided there for longer periods and at higher levels than in edible tissues. These authors detected concentrations of 100 mg kg^{-1} 9 days post-treatment, whereas, residue concentrations were not detected in edible tissues at that moment with a limit of detection (LOD) of 1 µg kg^{-1}.

Additionally, Heinrich *et al.* (2013) and Berendsen *et al.* (2013) conducted research studies with ceftiofur and oxytetracycline, respectively. The results showed that their residues bioaccumulate in feathers from treated chickens, even when the drug concentrations in muscle and liver were at non-detectable levels.

Therefore, antimicrobials can be transferred to feathers, which poses a risk to public health because feathers are reincorporated into the food chain through diets of other animals, such as fish (Arunlertaree and Moolthongnoi, 2008), since it is an economic source of amino acids for diets intended for production animals (Divakala *et al*., 2009).

Different tetracycline depletion studies for other matrices are available. For example, Anadón *et al*. (2013) quantified chlortetracycline in liver, kidney and muscle of broiler chickens, while Muñoz *et al.* (2014) detected oxytetracycline in eggs from White Leghorn hens. Nevertheless, depletion studies for tetracyclines in feathers are scarce.

The information presented above shows the need to study the behaviour of drugs in this matrix. However, before any depletion study can be undertaken, validation of the analytical method should be performed to asses the reliability, accuracy and selectivity of the results.

Materials and Methods

Experimental animals

Following the recommendations of the Directive 2010/63/EU on the protection of animals used for scientific purposes, 20 one-day-old male broiler chickens were raised in breeding cages. Chickens were kept under controlled environmental conditions (25 ± 5°C, 50-60% relative humidity), *ad libitum* access to water and non-medicated feed. The cages had an elevated wire floor, in order to avoid contamination with faecal content. Chickens were sacrificed according to Regulation (EC) No. 1099/2009 on the protection of animals at the time of the slaughtering.

Sampling

Samples of feathers were obtained immediately after sacrifice. All samples were stored individually in labelled plastic bags. Processing was performed in a food processor (Robot-coupe R4) after a cryogenic treatment of feathers with liquid nitrogen.

Reagents and Standards

Certified OTC, CTC, 4-epi-OTC and 4-epi-CTC standards were supplied by Dr. Ehrenstorfer Gmbh. Certified deuterated tetracycline (TC-d6) was purchased from Toronto Research Chemicals (Canada) and was used as internal standard (IS). All used solvents were high-performance liquid chromatography (HPLC) grade.

Extraction Process

Analyte extraction from feathers was based in that described by Berendsen *et al*. (2013). Acetone and EDTA-McIlvaine buffer were added to 5 ± 0.05 g samples. The suspension was stirred, sonicated and centrifuged. The supernatant was then filtered through a SPE column OASISTM HLB® (6 mL), which was conditioned with methanol, HPLC-grade water and McIlvaine EDTA buffer prior to use. Methanol was used as elution solvent. The eluate was evaporated at 40-50°C under a mild nitrogen flow. The residual extract was reconstituted in 250 µL of a mixture of 0.01 M oxalic acid/ACN (5: 1).

Parameters for validation of analytical methods

An internal validation protocol was generated, which included the parameters retention time of analytes, specificity, linearity, recovery and precision according to the recommendation by the European Union (Commission Decision 2002/657/EC). Limit of detection (LOD), limit of quantification (LOQ), was performed according the FDA (Food and Drug Administration) VICH GL49 validation of analytical methods used in residue depletion studies.

Instrumental analysis

A liquid chromatograph Agilent series 3200 coupled to a mass triple quadrupole spectrometer (API 4000, ABSCIEX) was used. A Sunfire C18 (3.5 mm x 2.1 mm x 150 mm) obtained from Waters® was used as analytical column. Analyst v1.6.2 software was used for sample integration. Chromatographic separation was achieved through a gradient of mobile phase: 0.1% formic acid in water (phase A) and 0.1% formic acid in methanol (phase B). The flow was set at 0.2 mL min^{-1}, the injection volume was 25 µL and the column oven temperature of 30°C.

Results

To determine whether the analytical method is able to detect analytes OTC, CTC and their active metabolites from feathers, analytes were identified by their ion masses and specific retention times (Table 2). In Figure 1 chromatograms from the injection of a pure standard, a blank sample and a control sample in feather matrix for all analytes of interest are shown.

Table 2. Parent ion mass, product and average retention times for OTC, CTC, 4-epi-OTC, 4-epi-CTC and TC D6.

Analyte	Parent ion mass	Product ion mass	Retention time
OTC	461.0	426.0	12.7 min.
CTC	479.0	444.0	14.6 min.
4-epi-OTC	461.0	426.0	8.2 min.
4-epi-CTC	479.0	444.0	10.1 min.
TC D6	451.0	416.0	11.7 min.

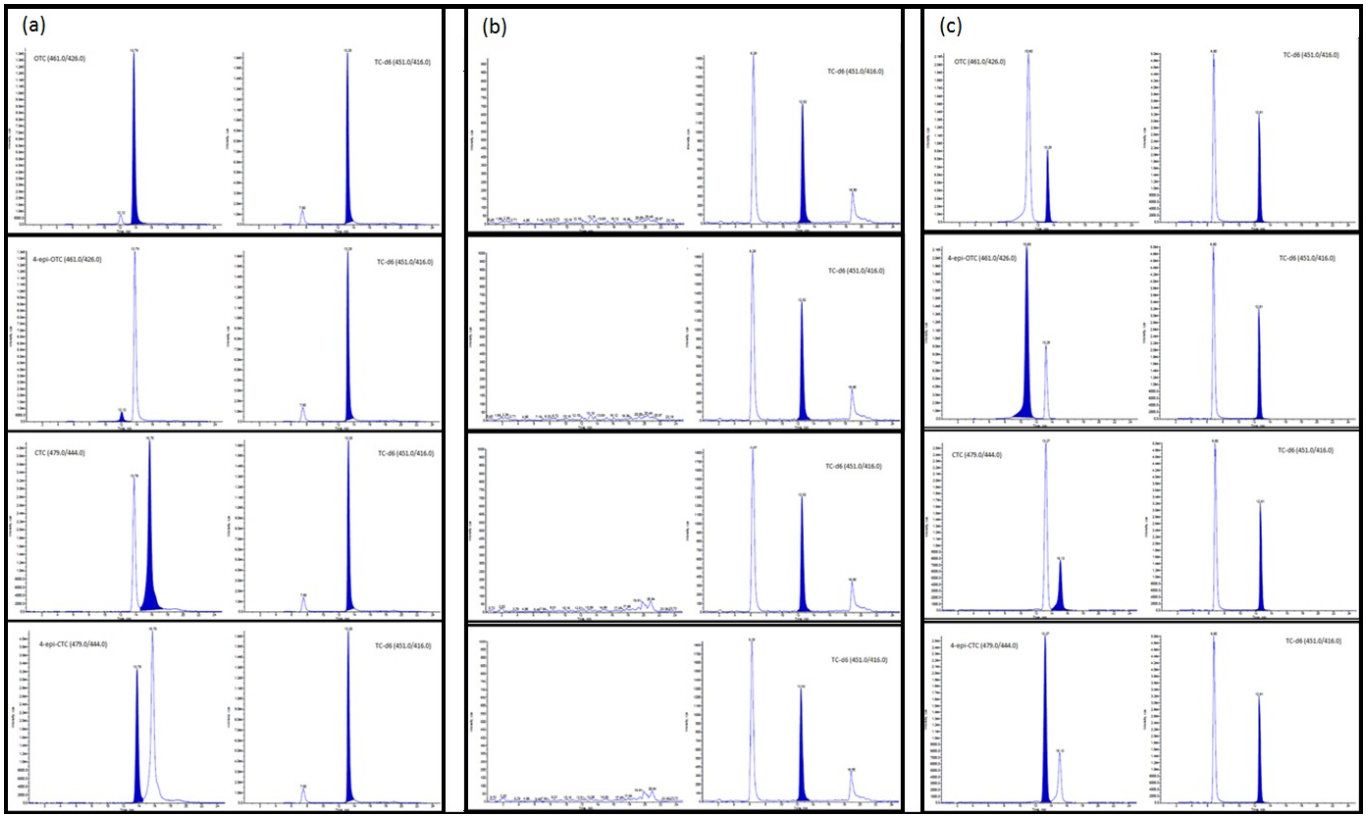

*Figure 1. Chromatograms of OTC, 4-epi-OTC, CTC and 4-epi-CTC: **(a) as** pure standards, (**b**) blank feather samples and (**c**) spiked feather samples.*

Validation of analytical methodology

Analyte retention time. The coefficient of variation (CV) of the retention time following six repetitive injections of pure drug was determined (Table 3). The criterion to accept a retention time was a CV% lower than 5% of variation, which was met for all analytes.

Table 3. Average and CV of retention times from the 6 injections of pure OTC, CTC, 4-epi-OTC and 4-epi-CTC standard.

Analyte	Average retention time	CV
OTC (461.0/426.0)	12.7 min.	0.3 %
CTC (479.0/444.0)	14.6 min.	0.3 %
4-epi-OTC (461.0/426.0)	8.2 min.	2.4 %
4-epi-CTC (479.0/444.0)	10.1 min.	2.3 %

Specificity. Interferences at the retention time of OTC, CTC 4-epi-OTC and 4-epi-CTC were determined through the analysis of the 20 antimicrobials-free samples. The results show that there are no interferences at the specific retention times of the analytes in residue-free feathers samples. Figure 1 shows chromatograms from the injection of pure drug, and residue-free feather samples.

Limit of detection (LOD). In order to establish the limit of detection, the selected concentration need to be acquired a signal-to-noise (SNR) ratio in excess of 3:1. For this reason, the level for the limit of detection was set in 20 µg kg^{-1} for the feather

matrix. With the aim to validate the parameter, 20 repetitions at this concentration were carried out in the fortified matrix. Average concentrations, standard deviation and CV of the 20 repetitions were determined and were accepted when the variation was lower than 25% (Table 4).

Table 4. Average, standard deviation and CV of the detected OTC, CTC, 4-epi-OTC and 4-epi-CTC concentrations from 20 LOD (20 µg kg^{-1}) fortified repetitions in biological feather matrix.

Analyte	Average concentrations	SD	CV
OTC (461.0/426.0)	20.6 µg kg^{-1}	1.6	8%
CTC (479.0/444.0)	19.0 µg kg^{-1}	0.9	5%
4-epi-OTC (461.0/426.0)	14.8 µg kg^{-1}	2.6	17%
4-epi-CTC (479.0/444.0)	22.3 µg kg^{-1}	1.8	8%

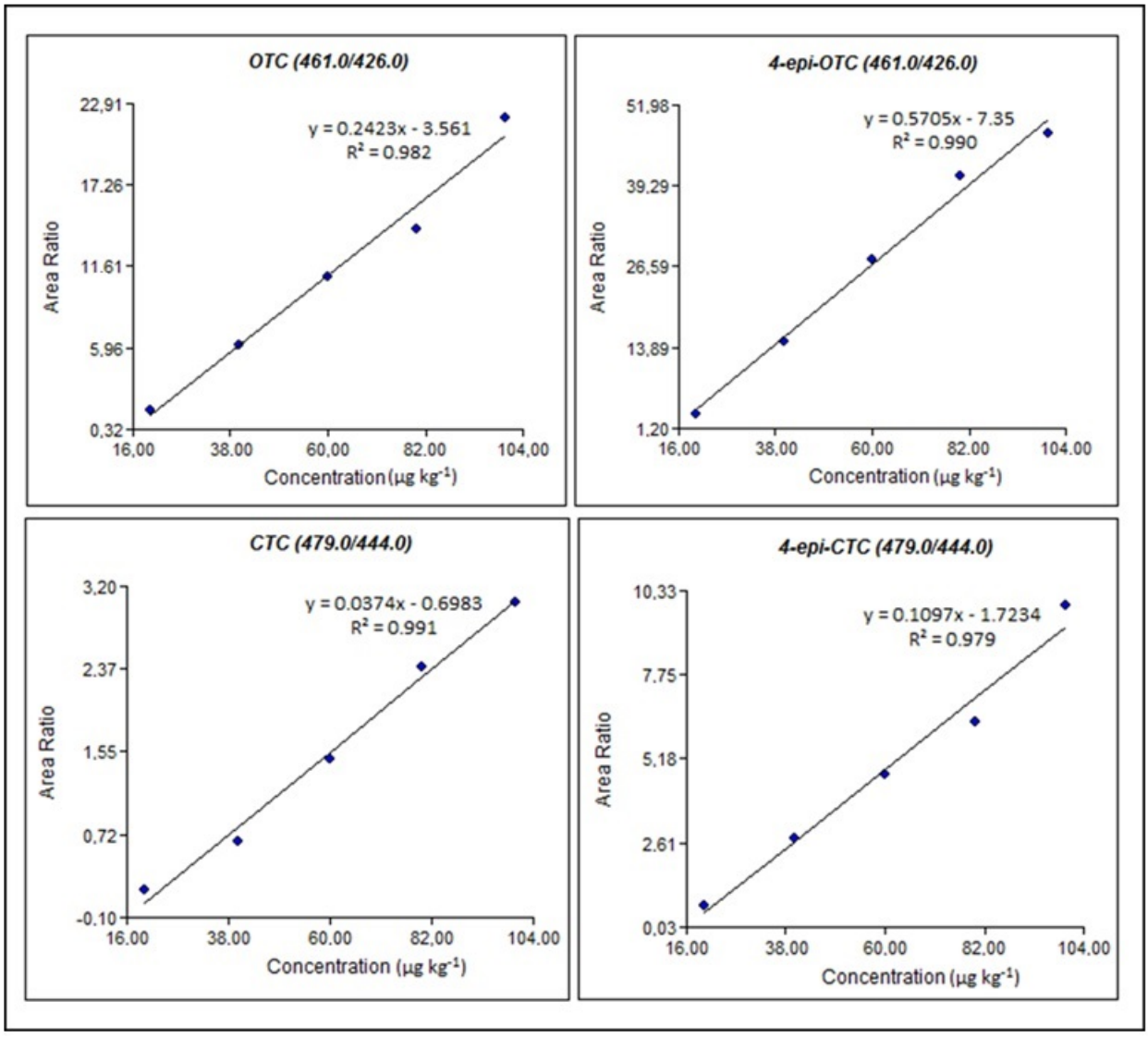

Figure 2. Calibration curves of Oxytetracycline (OTC), 4-epi-oxytetracycline (4-epi-OTC), chlortetracycline (CTC), 4-epi-clortetracycline (4-epi-CTC) at five fortification levels (20, 40, 60, 80 y 100 µg kg^{-1}) in feathers.

Limit of Quantification (LOQ). To determine the LOQ, the concentration established for LOD was increased with 1.64 times the standard deviation of the quantified concentrations from the 20 repetitive analyses of the fortified matrix. Parameter was accepted as the SNR was higher than 10:1. The LOQs determined for OTC, CTC and their active metabolites are listed in Table 5.

Table 5. Linearity, recovery, repeatability, reproducibility, LOD and LOQ for OTC, CTC, 4-epi-OTC and 4-epi-CTC in feathers.

Parameter	Linearity	Recovery at LOD	Reproducibility LOD	Repeatability LOD (%)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
Criteria	$R^2 \geq 0.95$ / CV < 25%	80 – 110%	CV < 35%	CV < Reproducibility	-	-
OTC (461.0/426.0)	$R^2 > 0.98$ / CV=0.6%	96%	19%	16.0	20	22.5
CTC (479.0/444.0)	$R^2 > 0.97$/ CV=0.9%	107%	14%	8.1	20	21.5
4-epi-OTC (461.0/426.0)	$R^2 > 0.96$/ CV=1.1%	92%	16%	13.0	20	24.2
4-epi-CTC (479.0/444.0)	$R^2 > 0.98$/ CV=0.4%	103%	13%	8.6	20	22.9

Table 6. Averages, standard deviation and CV from OTC, CTC, 4-epi-OTC and 4-epi-CTC analyte recovery from biological feather matrix, according to working concentrations at 20, 60 and 100 µg kg^{-1}.

Parameter	Concentration (µg kg-^{1})	Recovery average (%)	Standard deviation	CV (%)
FF (356,0/336,0)	20	100	0.10	10
	100	100	0.04	4
	200	100	0.01	1
FFA (248,0/230,0)	20	102	0.16	16
	100	99	0.06	6
	200	100	0.01	1

Linearity of calibration curves (CC): three 5 level calibration curves were carried out at concentrations 20, 40, 60, 80 and 100 µg kg^{-1}. Concentrations were defined by considering the LOD and MRL for muscle (Commission Regulation (EU) No 37/2010). Linearity of all calibration curves was accepted since R^2 was higher than 0.96 (Figure 2). Moreover, the CV of the calibration curves was lower than 25% of variation. (Table 5).

Recovery. Recovery was calculated using blank samples fortified at three concentration levels 20, 60 and 100 µg kg^{-1}. In Table 6 average concentrations, standard deviation and CV for all analytes are summarised.

Table 7. Average of quantified concentrations, SD, repeatability CV and intra-laboratory reproducibility according to fortification concentrations of 20, 60 y 100 µg kg^{-1} in feathers.

		Repeatability			Reproducibility		
Parameter	fortified concentration	Quantified concentration average	SD	CV (%)	Quantified concentration average	SD	CV (%)
OTC (461.0/426.0)	20 µg kg^{-1}	18.2 µg kg^{-1}	2.9	16.0	19.2 µg kg^{-1}	3.6	19
	60 µg kg^{-1}	63.7 µg kg^{-1}	0.09	9.1	61.6 µg kg^{-1}	7.31	11.8
	100 µg kg^{-1}	98.1 µg kg^{-1}	0.03	3.0	99.2 µg kg^{-1}	3.6	3.7
CTC (479.0/444.0)	20 µg kg^{-1}	22.1 µg kg^{-1}	1.8	8.1	21.4 µg kg^{-1}	3.0	14
	60 µg kg^{-1}	55.9 µg kg^{-1}	3.6	6.4	57.1 µg kg^{-1}	6.0	10.5
	100 µg kg^{-1}	102.1 µg kg^{-1}	1.8	1.8	101.5 µg kg^{-1}	3.0	3.0
4-epi-OTC (461.0/426.0)	20 µg kg^{-1}	20.4 µg kg^{-1}	2.7	13.0	18.4 µg kg^{-1}	2.9	16
	60 µg kg^{-1}	59.1 µg kg^{-1}	5.3	9.0	63.2 µg kg^{-1}	5.7	9.
	100 µg kg^{-1}	100.3 µg kg^{-1}	2.8	2.8	98.4 µg kg^{-1}	2.9	2.9
4-epi-CTC (479.0/444.0)	20 µg kg^{-1}	21.8 µg kg^{-1}	1.9	8.6	20.5 µg kg^{-1}	2.7	13
	60 µg kg^{-1}	56.5 µg kg^{-1}	3.8	6.6	58.9 µg kg^{-1}	5.4	9.1
	100 µg kg^{-1}	101.8 µg kg^{-1}	1.9	1.8	100.5 µg kg^{-1}	2.7	2.7

Precision. Precision was assessed through repeatability and intra-laboratory reproducibility. Results are compared in Table 7. Intra-laboratory reproducibility CVs were lower than 35% of variation for fortification concentrations at 20, 60, 100 µg kg^{-1} for all analytes. At the same time, the repeatability CVs were lower than the intra-laboratory reproducibility CVs.

Discussion and Conclusions

The implemented method was performed according to an internal validation protocol based on the European Union (Commission Decision 2002/657/EC) and the FDA (Food and Drug Administration) VICH GL49 validation of analytical methods used in residue depletion studies. Results of validation parameters show that the analytical method is reliable and accurate for detection and quantification of these analytes in feathers.

The importance of studying antimicrobial concentrations present in the feathers is that these products are incorporated as feather meal in feed for other animals for human consumption. It was calculated that approximately 37% of a chicken is not consumed directly by humans and becomes a source of raw material for feed (Meeker and Hamilton, 2006; Divakala *et al*., 2009). In this way, the feathers are a source of re-entry of antimicrobial residues in the food chain, becoming a risk to public health, especially for the development of antimicrobial resistance.

Considering the published evidence, further research in the pathway of residue re-entry throughout the food chain is needed. The study of the bioaccumulation of tetracyclines in feathers and their relationship with the concentrations found in the tissues contributes to fulfil these need. However, further research in feathers is recommended.

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RAPID ANALYSIS OF SEDATIVES, BASIC AND ACIDIC NSAIDs IN KIDNEY AND MUSCLE BY LC-MS/MS

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Abstract

A rapid method for quantitative and confirmative analysis of sedatives, basic and acidic NSAIDs (non-steroidal anti-inflammatory drugs) in mammal kidney and basic and acidic NSAIDs in poultry muscle using liquid-chromatography tandem mass-spectrometry (LC/MS/MS) was developed and validated. Seven sedatives in kidney and 20 NSAIDs in kidney and muscle were included in the method. The samples were shaken with ammonium acetate, water and acetonitrile followed by centrifugation. One aliquot of the supernatant was diluted and analysed by LC-MS/MS (Waters Xevo TQS), whereas another part of the supernatant was evaporated to dryness, dissolved and then analysed by LC/MS/MS. The compounds were separated on a reversed-phase column (Waters ACQUITY) and detected by electrospray ionization followed by multiple reaction monitoring (MRM). The validation was performed according to the Commission Decision 2002/657/EC.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are extensively used as anti-inflammatory, analgesic, and anti-pyretic drugs in both human and veterinary medicine. NSAIDs are routinely administered to food producing animals. A common indicator is mastitis in dairy cows. NSAIDs are often used in combination with antibiotic treatment.

Sedatives are designed to relax the central nervous system. They are soothing and can be used to reduce stress during transport of animals for slaughter, preventing mortality and poor meat quality. Promazins are banned from use in food-producing animals.

The NSAIDs included in the method are carprofen, celecoxib, diclofenac, firocoxib, flunixin, ibuprofen, ketoprofen, mefenamic acid, meloxicam, naproxen, oxyphenbutazone, phenylbutazone, rofecoxib, salicylic acid, tolfenamic acid, vedaprofen and four metamizole-metabolites (MAA, FAA, AcAA and AA). The following sedatives are included in method acepromazine, azaperol, azaperone, carazolol, propionylpromazine, xylazin and 2-(1-hydroxyetyl) promazin sulfoxid (HEPS).

Materials and Methods

Standards and chemicals

Acepromazine, azaperol, azaperone, carazolol, firocoxib, rofecoxib vedaprofen, 4-methylaminophenazone hydrochloride (MAA), 4-formylaminophenazone (FAA), 4-acetylaminophenazone (AcAA), and 4-aminophenazone (AA) were purchased from Witega. Carprofen, celecoxib, diclofenac sodium, flunixin, ibuprofen, ketoprofen, meloxicam sodium salt hydrate, naproxen, phenylbutazone, propionylpromazine, tolfenamic acid and xylazin were purchased from Sigma-Aldrich. Oxyphenbutazone monohydrate was purchased from LGC. Mefenamic acid was received from BVL, Berlin, Germany. 2-(1-hydroxyetyl) promazin sulfoxide (HEPS) was purchased from TRC, Toronto Research Chemicals, Canada.

Phenylbutazone-D_{10}, $^{13}C_6$-diclofenac sodium hydrate, carprofen-D_3 and firocoxib-D_6 were received from BVL, Berlin, Germany. $^{13}C_6$-tolfenamic acid, 4-methylaminophenazone-D_3 (MAA-d_3), acepromazine-D_6, azaperol-D_4, azaperone-D_4, carazolol-D_7, flunixin-D_3, meloxicam-D_3, propionylpromazine-D_6 hydrochlorid and xylazin were purchased from Witega.

Methanol, LiChrosolve (gradient grade), formic acid (98-100%) and ammonium acetate (reagent grade) were all purchased from Merck. Ammonium formate was obtained from Sigma-Aldrich. Acetonitrile, HPLC-grade, was obtained from Lab Scan.

Instrumentation

A Waters Xevo TQ-S instrument with a reversed phase ACQUITY BEH UPLC C18 column (2.1 x 100 mm, 1.7 µm) was used. The column temperature was 40°C and the flow rate was 0.45 mL min^{-1}. The injection volume was 20 µL or 100 µL, details and MRM are shown in Tables 1 and 2. A gradient of (A) methanol:acetonitrile 80:20 (v:v) and (B) 10mM ammonium formate pH 5.0 was used. The initial gradient condition, 5% A and 95% B, was maintained for one min. The amount of A was then increased to 35% at 1.05 min and to 95% at 7 min. From 7 min to 10 min the amount of A was maintained at 95%, and thereafter the system was equilibrated at the initial conditions with 5% A for about 2 min. The total run time was 12 min. The mass-spectrometer was operated in both positive (ESI+) and negative (ESI-) mode. Two transitions were monitored for each compound in order to fulfil the requirements for confirmatory methods. The strongest transition was used for quantification and the weaker for confirmation.

Table 1. MRM-table NSAIDs.

Compound	Kidney inj vol (µL)	Poultry muscle inj vol (µL)	Retention time (min)	Electrospray ionization mode	Parent ion (*m/z*)	Daughter ions (first fragment used for quantification)	Cone voltage (V)	Collision energi (eV)
MAA	20	20	3.1	positive	218	56, 97	42	14, 14
FAA	20	20	2.5	positive	232	83, 104	44	22, 22
AcAA	20	20	2.5	positive	246	83, 104	28	28, 22
AA	20	20	2.6	positive	204	56, 86	36	14, 12
Carprofen	20	20	5.4	negative	272	228, 226	18	12, 25
Celecoxib	100	100	5.9	positive	382	362, 282	60	26, 34
Diclofenac	100	100	5.1	positive	296	214, 250	30	26, 12
Firocoxib	100	100	4.5	positive	337	283, 130	32	8, 28
Flunixin	20	20	4.7	positive	297	279, 264	28	22, 32
Ibuprofen	100	100	5.4	negative	205	161	28	7
Ketoprofen	100	100	4.2	positive	255	209, 105	46	12, 22
Mefenamic acid	100	100	5.9	negative	240	196, 192	22	20, 25
Meloxicam	20	20	3.6	positive	352	115, 73	48	20, 46
Naproxen	100	100	4.4	positive	231	185, 170	22	14, 24
Oxyphenbutazone	100	100	4.1	positive	325	160, 204	48	20, 12
Phenylbutazone	100	100	4.7	positive	309	160, 77	60	18, 50
Rofecoxib	100	100	3.8	positive	315	269, 192	48	20, 34
Salicylic acid		20	2.2	negative	137	93, 65	35	14, 24
Tolfenamic acid	20	100	5.9	positive	262	244, 209	24	14, 26
Vedaprofen (adduct)	20	20	6.8	positive	300	155, 201	25	25, 15
MAA-d3	20	20	3.1	positive	221	100, 59	42	14, 14
$^{13}C_6$-Diclofenac	100	100	5.1	positive	302	284, 220	12	8, 30
$^{13}C_6$-Tolfenamic acid	20	100	5.9	positive	268	250, 215	24	14, 28
$^{13}C_6$-Tolfenamic acid	100	100	5.9	negative	266	222	14	16
Carprofen-D_3	20	20	5.4	negative	275	231,228	16	12, 26
Firocoxib-D_6	100	100	4.5	positive	343	289, 136	22	8, 32
Flunixin-D_3	20	20	4.7	positive	300	282, 264	54	22, 32
Meloxicam-D_3	20	20	3.6	positive	355	115, 141	50	18, 18
Phenylbutazone-D_{10}	100	100	4.7	positive	319	165, 82	12	20, 44

Table 2. MRM-table Sedatives

Compound	Kidney inj vol (µL)	Retention time (min)	Electrospray ionization mode	Parent ion (*m/z*)	Daughter ions (first fragment used for quantification)	Cone voltage (V)	Collision energi (eV)
Acepromazine	20	4.5	positive	327	86, 58	74	16, 26
Azaperol	20	3.7	positive	330	121, 312	50	14, 14
Azaperone	20	4.2	positive	328	121, 165	50	14, 10
Carazolol	20	3.2	positive	299	116, 222	50	18, 18
HEPS	20	3.2	positive	345	86, 242	6	28, 36
Propionylpromazine	20	5.1	positive	341	86, 58	50	18, 24
Xylazine	20	2.9	positive	221	90, 164	50	20, 24
Acepromazine-D_6	20	4.5	positive	333	92	78	18
Azaperol-D_4	20	3.7	positive	334	316	78	14
Azaperone-D_4	20	4.2	positive	332	127	50	36
Carazolol-D_7	20	3.2	positive	306	123	50	18
Propionylpromazine-D_6	20	5.1	positive	347	92	84	18
Xylazine-D_6	20	2.9	positive	227	170	50	24

Sample preparation

An amount of 1 g of kidney or 5 g of muscle was weighed in a 50-mL Falcon tube, spiked with internal standard, mixed and allowed to equilibrate for 15 min. Then, 8 mL of water, 2 g of ammonium acetate and 10 mL of acetonitrile were added and the sample was vortexed for 10 s and thereafter shaken for 10 min. The samples were centrifuged for 20 min at 4,120 *g* at -5°C. One aliquot of 200 µL of the supernatant was diluted with 800 µL eluent directly in a vial and injected (20 µL). A second aliquot of 4 mL of the supernatant was evaporated to dryness using heat and a nitrogen stream and dissolved in 200 µL methanol/ acetonitrile 80:20 (v:v) and 800 µL 10mM ammonium formate pH 5.0 and injected (100 µL).

Validation

The validation was performed according to Commission Decision 2002/657/EC. In order to compensate for matrix effects, a six-point matrix matched standard curve (0.25 to 4.0 of the target concentration) was used. The ratio of the peak area and the internal standard area was plotted versus the concentration of the compound. The target concentration was either the MRL, or chosen in accordance with concentrations proposed in the guidance-paper published by the European Community Reference Laboratories (EURLs), or as low as possible, shown in Tables 3 and 4.

The specificity was determined by analysing 27 blank mammal kidney samples (eight *bovine*, eight *porcine*, five *equine*, three *ovine* and three deer) and ten different blank poultry muscle samples (seven turkey and three chicken). Poultry was validated as a minor species. Accuracy, *i.e*. trueness and precision, was determined by spiking a pooled kidney sample at 0.5, 1 and 1.5 of the target concentration. Six replicates were prepared and analysed at each level (data not shown). For kidney, the trueness and within-laboratory reproducibility (CV_R), was calculated from the analysis of 27 different samples spiked at the target concentration and analysed at three separate occasions by different laboratory technicians, shown in Table 4. For poultry muscle, ten different muscle samples were spiked at the target concentration and analysed at one occasion, shown in Table 3. The limit of detection (LOD) was defined as the concentration with a signal-to-Noise ratio for the weaker fragment of > 3. The absolute recoveries were determined by comparing samples spiked at the target concentration before and after extraction (data not shown). The decision limits (critical concentration at alpha error, CCα) and the detection capability (critical concentration at β-error, CCβ) were calculated using the within –laboratory reproducibility results in accordance with the procedure described in Commission Decision 2002/657/EC.

Table 3. Target concentrations and validation results poultry muscle.

Compound	Target concentration ($\mu g\ kg^{-1}$)	Accuracy (%)	Within-laboratory Reproducibility CV_R (%)	LOD ($\mu g\ kg^{-1}$)	CCα ($\mu g\ kg^{-1}$)	CCβ ($\mu g\ kg^{-1}$)
MAA	5	96	8.0	<0.3	0.9	1.5
FAA	5	104	21	1.25	2.2	4.0
AcAA	5	101	17	<0.3	1.9	3.3
AA	5	99	17	<0.3	1.7	3.0
Carprofen	5	100	8.3	2.5	1.0	1.7
Celecoxib	5	103	10	<0.3	1.4	2.3
Diclofenac	5	102	4.6	<0.3	0.6	1.0
Firocoxib	5	103	5.4	<0.3	0.7	1.1
Flunixin	5	103	4.9	<0.3	0.7	1.1
Ibuprofen	5	101	23	5	-	-
Ketoprofen	5	89	11	<0.3	1.3	2.1
Mefenamic acid	5	104	13	<0.3	1.7	2.7
Meloxicam	5	103	4.5	<0.3	0.6	0.9
Naproxen	5	88	7.6	<0.3	0.8	1.4
Oxyphenbutazone	5	108	6.3	<0.3	2.0	2.6
Phenylbutazone	5	101	5.6	2.5	0.6	1.0
Rofecoxib	5	93	11	<0.3	1.4	2.2
Salicylic acid[a]	400	101	9.5	100	470	533
Tolfenamic acid	5	104	5.5	<0.3	0.7	1.1
Vedaprofen	5	93	6.1	2.5	0.7	1.2

[a] *MRL*

Table 4. Target concentrations and validation results kidney.

Compound	Target concentration (µg kg^{-1})	Accuracy (%)	Within-laboratory reproducibility CV_R (%)	LOD (µg kg^{-1})	CCα (µg kg^{-1})	CCβ (µg kg^{-1})
MAA[a]	100	106	14	<25	125	150
FAA	100	121	40	<25	180	260
AcAA	100	122	40	<25	181	262
AA	100	117	34	<25	165	229
Carprofen	1000	108	12	<250	1211	1421
Celecoxib	10	96	21	<2.5	4.5	7.9
Diclofenac[a]	10	106	11	<2.5	12	14
Firocoxib[a]	10	105	8.1	<2.5	11	13
Flunixin[a]	100/30/200	104	6.6	<7.5	111/33/222	122/37/244
Ibuprofen[b]	10	71	24	5	-	-
Ketoprofen[c]	10	108	15	<2.5	3.3	5.8
Mefenamic acid[b]	10	99	17	<2.5	4.1	6.8
Meloxicam[a]	65	105	6.6	<16	72	80
Naproxen[b]	10	110	12	<2.5	3.1	5.3
Oxyphenbutazone[b]	5	120	22	<1.25	3.2	5.4
Phenylbutazone[b]	5	116	27	<1.25	3.3	5.8
Rofecoxib	10	107	8.8	<2.5	2.2	3.8
Tolfenamic acid[a]	100	95	11	<25	116	133
Vedaprofen[a]	1000	106	17	<250	1297	1594
Acepromazine[b]	50	103	5.6	<12	6.2	11
Azaperol[a]	100	104	5.5	<25	109	119
Azaperone[a]	100	100	7.7	<25	113	125
Carazolol[a]	15/25	110	15	<3.8	19/32	23/38
HEPS	10	131	55	2.5	-	-
Propionylpromazine[b]	50	106	6.6	<12.5	7.8	13.5
Xylazine[c]	10	106	7.7	<2.5	1.9	3.3

[a] MRL; [b] Recommended concentration (EU-RL requirements); [c] No MRL required

Results and Discussion

Specificity

The specificity of the method is generally good. In kidney no interfering peaks were detected. In muscle, low levels of basic NSAIDs and flunixin were observed, but these levels were so low (less than 10% of the target concentration) that they did not affect the quantification of the compounds.

Precision and linearity

The precision, defined as within-laboratory reproducibility, (CV) is shown in Table 3 and 4. According to the 2002/657/EC the precision should be <30% for analytes at the level of 100 µg kg^{-1}. The within-laboratory reproducibility varies between 6.6 and 40% for NSAIDs in kidney. For a few NSAIDs in kidney, the reproducibility exceeds 30%, *i.e*. AA, FAA and AcAA (34-40%). However, these substances are all metabolites and only included for monitoring. For sedatives in kidney, the reproducibility varies between 5.6 and 15%, except for HEPS (55%) which is a metabolite and only used for monitoring. For NSAIDs in poultry muscle reproducibility is good, with values between 4.5 and 22.6%.

The matrix-matched calibration curves were linear ($r^2 > 0.95$) in the investigated interval, 0.25-4 of target concentration, for all validated compounds except for ibuprofen in poultry muscle ($r^2 = 0.88$).

CC_α, CC_β and LOD

The calculated CC_α, CC_β and LOD values were satisfactory, shown in Table 3 and 4. All compounds are detected at least at ½ of the target concentration, except for ibuprofen in poultry muscle where only target concentration is reached. However, ibuprofen is only included for screening, since only one of the transitions had sufficient signal intensity.

Conclusions

This fast and simple method enables simultaneous detection of seven sedatives and 20 basic and acidic NSAIDs including their metabolites at the levels required by EU legislation. It fulfils the criteria for confirmatory analysis, except for ibuprofen, and can also be used for screening. The proposed method is simple and specific, allowing a single analyst to easily prepare a batch of 40 samples in less than 4 h.

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THE DEPLETION OF DOXYCYCLINE RESIDUES IN POULTRY TISSUES

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Abstract

Doxycycline (DOX), tetracycline of second generation, is active against Gram+ and Gram- bacteria, aerobic and anaerobic. Although there are few pharmacokinetic studies in chickens, it is frequently used for colibacillosis treatment, salmonellosis, staphylococcal infections, avian mycoplasmosis and chlamydia. Our objective was to evaluate the withdrawal time (WT) of DOX formulation at 25% in edible tissues, after PO use in 40 healthy broilers (30-35 days of age). DOX was administered through medicated drinking water for 5 days at 10 mg kg^{-1} (N = 36). Four untreated animals were reserved as controls. Six animals per group were sacrificed by exsanguination, after 24 h until 9 d post treatment when control animals were sacrificed as well. Muscle, liver, kidney and skin/fat samples were obtained. DOX was determined by HPLC with UV detection. DOX concentrations were determined in all tissues examined; generally falling below the MRL at 7 d after administration is terminated. The calculated WTs were 6.58, 8.18, 8.69 and 6.96 d for muscle, liver, kidney and skin/ fat, respectively. After DOX administration at a rate of 10 mg kg^{-1} for 5 days through the drinking water, the WT must be 9 d before treated chickens can be consumed.

Introduction

Doxycycline (DOX) is a semi-synthetic bacteriostatic tetracycline and a broad-spectrum antibiotic against Gram-negative and Gram-positive aerobic and anaerobic bacteria, *Rickettsiae*, *Chlamydiae*, *Mycoplasmas* and some protozoa (Anadón *et al*., 1994; Prats *et al*., 2005). Pharmacokinetics properties of doxycycline are superior over older tetracyclines in terms of higher lipid solubility, complete absorption, better tissue distribution, longer elimination half-life and lower affinity for calcium (Goren *et al*., 1998). The *in vitro* antimicrobial activity of doxycycline is more effective than other tetracyclines for the treatment of respiratory, urinary and gastrointestinal tract diseases (Abd El-Aty *et al*., 2004). Although there are few pharmacokinetic studies in chickens, it is frequently used for the treatment of colibacillosis, salmonellosis, staphylococcal infections, avian mycoplasmosis and chlamydiasis (Anadón *et al*., 1994).

The misuse of DOX, illegal administration to animals, overdosing, and not obeying withdrawal periods can lead to accumulation of residues of this antibiotic in edible animal tissues. Due to persistence of DOX residues in tissues and for consumer's health protection, the European Commission had set a maximum residue limits (MRLs) for this compound at 100 µg kg^{-1} for muscle, 300 µg kg^{-1} for liver and skin/fat, and 600 µg kg^{-1} for kidney. According to Commission Regulation (EU 37/2010) and EMA Summary Report Doxycycline 2, quantification of DOX in animal tissues requires a determination of only the parent compound as the residue marker. In contrast to other tetracyclines for which the MRLs are defined as a sum of the parent drug and its 4-epimer (EU N° 37/2010, EMEA/MRL/270/97, 1997), DOX is not appear with a corresponding 4-epimer.

The antibiotics residue levels reached in organs and the rate of their depletion from tissues depend on the method of administration, animal species, as well as dose and specific given drug (Kung & Wanner, 1994). The differences in the antibiotic concentration and time of its depletion may be also influenced by differences in drinking water intake by animals.

The purpose of the present study was to evaluate the withdrawal time (WT) for DOX formulation at 25% in edible tissues, after its PO use in broilers.

Materials and Methods

Study Design Treatment and Administration

The experiment was conducted with 40 five-week-old chickens; 36 chickens were treated with 10 mg kg^{-1} bw of DOX (25%) once a day for five consecutive days through the drinking water. Solution was prepared by dilution of 400 g of the medicament in 1,000 mL water. The birds were kept in a special space designed for performing experiments on animals. Prior to the treatment, chickens were deprived of water. Antibiotic-free feed was available *ad libitum*. The chickens treated with DOX were euthanized 24 h, 2d, 3d, 5d, 7d and 9d after final drug administration (six animals at each time point) and muscle (breast), liver, skin plus fat and kidney were collected. Four chickens used as controls were euthanized before the experiment commenced, and the same tissues samples were collected from these animals. All samples were stored separately at -20°C until analysis. The animal experiment protocol was in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS-).

Reagents
Doxycycline (DOX) standard was obtained from Sigma-Aldrich Chemical Company (USA). Acetonitrile and methanol were from JT Baker. Oxalic acid dehydrate, trichloroacetic acid and sodium sulphate anhydrous were from Fluka (USA). Solid-phase extraction (SPE) columns (Strata, C18, 100 mg, 1 mL) and analytical column (Luna C18) were obtained from Phenomenex (USA). Doxycycline was formulated as a 25% experimental water soluble powder (DOX 25 g, Tartaric acid 5g and Lactose sqt 100 g).

Standard solutions
Stock standard solution (1 mg mL^{-1}) was prepared by weighing 10.0 ± 0.1 mg of standard and dissolving it in 10 mL methanol. The stock was stored at -20°C in amber glass, and was stable for six months. Secondary standard solutions (100 µg mL^{-1}, 10 µg mL^{-1}) prepared in methanol by diluting suitable aliquots of stock standard were stable for one month and were stored at 2-8°C in amber glass. Working standard solutions in mobile phase were prepared on the day of analysis.

Extraction and clean-up
A portion of 0.4 g of tissue (problem or spiked) was homogenized with 1.4 mL of McIlvaine buffer-EDTA, shaken at high speed and centrifuged at 2,500 *g* at 4°C for 15 min. The upper layer (supernatant S1) was transferred into a new tube. The extraction was repeated thrice. The supernatants S2, S3 and S4 were combined with S1. The mixture was vortexed for 30 s and centrifuged again for 10 min at 2,500 *g* at 4°C.

Clean-up
The supernatant mix (S1-S2-S3-S4) was transferred to SPE C18 cartridges, which were preconditioned with 3 mL methanol and 2 mL ultrapure water. The tube reservoir mix supernatants were washed with 1 mL McIlvaine buffer-EDTA and 1 mL water, after percolation of the whole solution; the columns were washed with these solutions (under vacuum). After drying for 2 min, the doxycycline was eluted with 4 mL of methanol 0.01 M oxalic acid pH 2.0. The cleaned extracts were evaporated to dryness in nitrogen evaporator at 40°C. The dried residues were reconstituted in 200 µL mobile phase. After vortexing and centrifugation, 100 µL were injected into the chromatographic system.

LC-UV analysis
The instrumental analysis was performed using Gilson HPLC system equipped with isocratic pump, autosampler, column oven, and UV/Vis detector (λ = 346 nm), controlled by Unipoint Workstation software. Chromatographic analyses were performed on Luna (Phenomenex) C18 column (5 µm, 150 mm x 4.6 mm) with mobile phase consisting of water-acetonitrile with 0.02 M oxalic acid and 0.5 mM EDTA (72:28, v/v) at 1.2 mL min^{-1} flow rate. The column oven temperature was controlled at 30°C.

Method validation
The following parameters were evaluated for the analysis of each matrix: linearity (concentrations of DOX ranging between 0.1 and 6.0 µg mL^{-1} - µg g^{-1}), precision and accuracy, limit of quantitation, limit of detection and selectivity.

The standard calibration curve was prepared by the injection of standard solutions on seven levels, namely 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 and 6.0 µg mL^{-1} or µg g^{-1}). The correlation coefficient was evaluated.

Samples of tissues were spiked with the DOX working solution to levels corresponding to 0.2, 0.5, 1.0 and 2.0 µg g^{-1}. The six spiked samples with DOX were analysed within three different days. Based on these spiked samples replicates, the precision (repeatability and reproducibility) of the method was determined. The mean accuracy (% recovery) was evaluated by comparing the concentrations in the spiked samples with known amounts of analytes to the concentrations in standard solution which should be within the range 85-115%, while the variation in precision should be ≤ 20%.

The limit of detection (LOD) was estimated through the analysis of 20 aliquots of control tissue (free of DOX). The noise of the base-line was measured; the average and the standard deviation were calculated. The LOD corresponded to three times SD (signal-to-noise ≥ 3:1) and the limit of quantitation (LOQ) corresponded to ten times this SD (signal-to-noise ≥ 10:1).

Withdrawal time
Numerous experimental designs and statistical approach are used to establish the withdrawal time. The EMEA recommends the use of a linear regression analysis of the logarithmic transformed data as the choice method (EMEA, 2002). The withdrawal time is determined as the time when the one-sided, 95% upper tolerance limit of the regression line with a 95% confidence level is below the MRL. Doxycycline concentrations in function of time found in muscle, kidney, liver and skin/fat were plotted and analysed with the program WT version 1.4 in order to recommend a withdrawal time for this experimental formulation.

Results

The development and validation were successfully accomplished. This method performed accurately and reproducibly over a range of 0.1 to 6.0 µg mL^{-1} or µg g^{-1} for DOX.

Precision of the system

One standard solution was prepared containing 0.2 µg mL^{-1} of DOX and the precision of the system was evaluated after the placement of twenty (20) injections in the chromatographic system. In this manner the efficiency of the column and of the system were evaluated. After twenty injections a coefficient of variation (CV) of 5.2% was determined.

Assay linearity

This assay exhibited a linear dynamic range between 0.1 and 6.0 µg mL^{-1}. A linear relationship was obtained across one dynamic range (r values ranged from 0.992 to 0.9997).

Specificity

Six different samples from control tissue (free of DOX) and six tissue samples fortified with DOX were analysed by HPLC and the corresponding chromatograms were compared. No matrix interferences were observed in the chromatograms of the samples with the same retention time as doxycycline. The chromatographic analysis time was short; DOX eluted at 4 min as a sharp and symmetrical peak with no interfering peaks.

Limit of detection (LOD)

The limits of detections (LODs) were 0.040, 0.024, 0.024 and 0.010 µg g^{-1} for DOX in kidney, skin/fat, muscle and liver respectively.

Limit of quantitation (LOQ)

The LOQs were 0.095, 0.074, 0.054 and 0.100 µg g^{-1} for DOX in kidney, skin/fat, muscle and liver, respectively.

Intra-day and inter-day accuracy and precision

The method for the analysis of tissue samples was thoroughly validated and the results are presented in Table 1. To assess the inter-day (over three days) assay accuracy and precision, six sets of tissue samples were prepared containing DOX at 0.2, 0.5, 1.0 and 2.0 µg g^{-1}. The inter-day variation in accuracy (recovery) and precision were assessed. The mean accuracy (recovery) should be within the range 85-115 % and the variation in precision should be ≤ 20%.

To determine the intra-day accuracy and precision, six replicates at four concentrations were analysed along with duplicate standard calibration curves prepared from two separate stock solutions (Table 1).

Table 1. Validation results for the analysis of DOX in chickens tissues

			Intra-day		Inter-day (over 3 days)	
matrix	r	µg g^{-1}	Accuracy (%), n=6	Precision (%), n=6	Accuracy (%)	Precision (%)
Muscle	0.995 (0.2-2.0 µg g^{-1})	0.2	99.9	5.4	99.96	5.0
		0.5	89.7	5.5	91.2	4.6
		1.0	110	4.5	108	2.3
		2.0	97.8	3.3	96.8	2.5
Liver	0.992 (0.2-2.0 µg g^{-1})	0.2	96.4	8.6	97.8	5.0
		0.5	93.1	3.0	101.6	6.6
		1.0	82.3	0.62	90.7	8.3
		2.0	86.4	7.4	93.96	7.4
Kidney	0.993 (0.2-2.0 µg g^{-1})	0.2	97.5	3.5	104	13
		0.5	94.0	5.7	94.4	2.7
		1.0	106	2.1	98.5	8.4
		2.0	97.8	1.1	98.8	1.0
Skin/Fat	0.995 (0.2-2.0 µg g^{-1})	0.2	89.1	0.00	86.4	2.7
		0.5	98.9	0.03	99.0	3.0
		1.0	105	0.07	99.5	5.8
		2.0	98.4	0.10	96.1	2.6

Doxycycline tissue concentrations

DOX was determined in all tissues examined; generally falling below the MRL at 7 d after the end of the administration. The mean ± SD DOX tissue concentrations after the PO administration of doxycycline to chickens in muscle, liver, kidney and skin/fat tissues are presented in Figure 1. After DOX administration to chickens at the dose of 10 mg kg-1 for five consecutive days through the drinking water, the highest content was found in the kidney and liver at one day after the treatment was completed. The maximum determined concentration in the kidney was 6.5 µg g^{-1} and in the liver was 4.79 µg g^{-1}. Whereas in muscle and skin/fat the detected values were lower, with maximum concentration of 1.46 µg g^{-1} and 1.23 µg g^{-1}, respectively. Subsequently a rapid decrease in DOX concentration was observed in all tissues. At 7 d after treatment was completed, the DOX concentration in muscle was above LOQ of the used method.

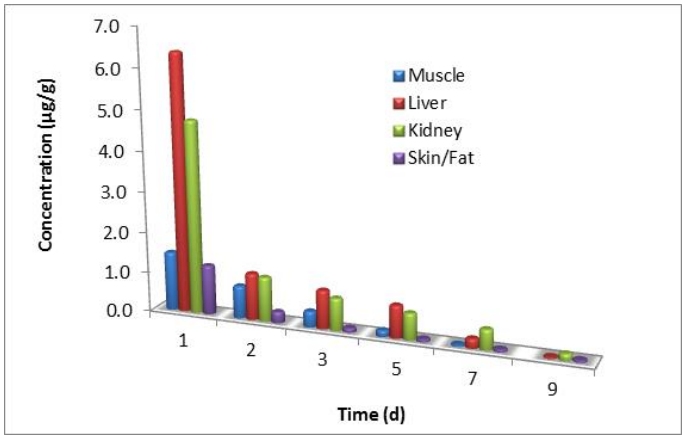

Figure 1. Mean tissue concentrations of doxycycline in chickens slaughtered at day 1, 2, 3, 5, 7 and 9 after oral administration of DOX 25% (dose of 10 mg kg^{-1} body weight during 5 days).

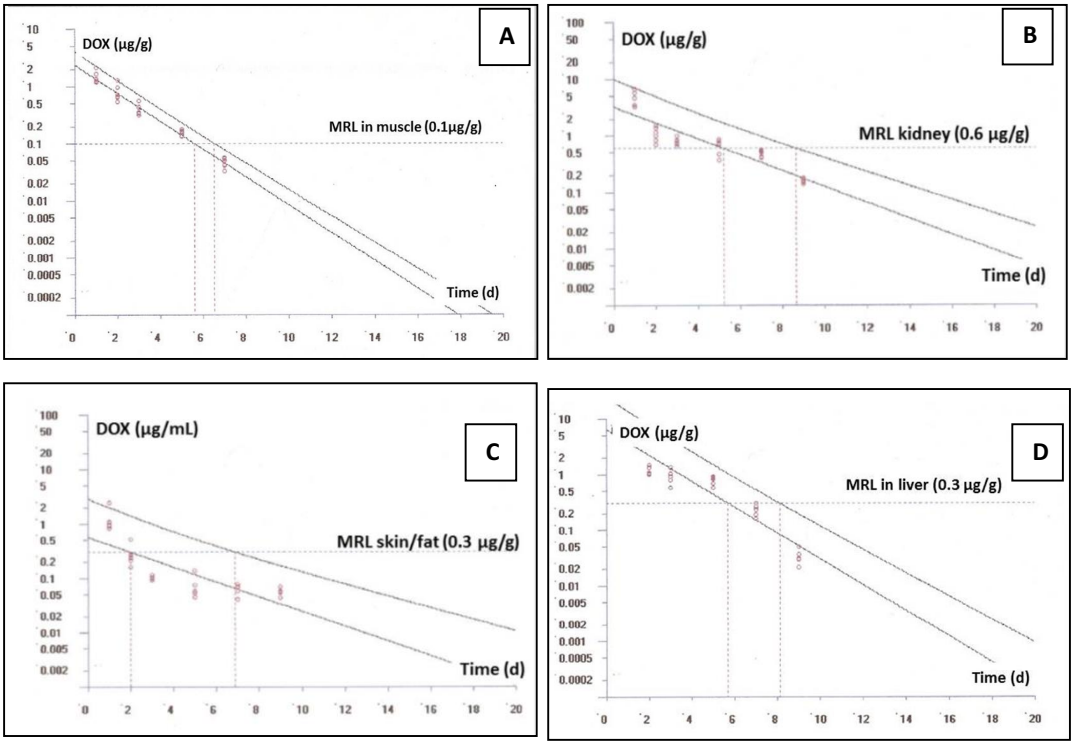

Figure 2: Plot of the withdrawal times calculation for DOX in chicken muscle (A), kidney (B), skin/fat (C) and liver (D) at the time when the one-sided 95% upper tolerance limit is below the EU MRL for doxycycline after its oral administration (5 doses of 10 mg kg^{-1} body weight of DOX 25%). [Residue marker: doxycycline].

Taking into account the MRLs for chickens and considering that the marker residue is doxycycline, the calculated withdrawal times 6.58, 8.18, 8.69 and 6.96 d for muscle, liver, kidney and skin / fat, respectively (Figure 2A, 2B, 2C and 2D).

Discussion

Doxycycline is known as strong lipophilic compound, well absorbed after oral administration, and showing high tissue binding (Jerome del Castillo, 2006). The route of drug administration plays an important role in the effectiveness of the treatment, as well as in the distribution of antibiotics to tissues (Laczay *et al*., 2001). The antibiotics' residue levels reached in organs and the rate of their depletion from tissues depend on the method of administration, animal species, as well as dose and specific formulation drug given. The differences in the antibiotic concentration and time of its depletion may be also influenced by the differences in the intake of drinking water by animals and water quality. Studies on tissue concentrations after different drug formulation administration are essential to control antibiotic residues in food animal products, *i.e*. that an appropriate withdrawal time is recommended.

The data obtained after DOX administration with water shows that at the beginning after treatment, DOX reached high concentrations in all edible tissues. One day after administration of the last dose, DOX concentration rapidly decreased in all assayed tissues. Then, the residues decreased gradually and only trace concentrations were observed on day 9 post-treatment.

Conclusions

The development and validation of the analytical method were successfully accomplished. This method performed accurately and reproducibly over a range of 0.1 to 6.0 μg mL^{-1} or μg g^{-1} for DOX.

Our results demonstrate that DOX oral administration at the 10 mg kg^{-1} for five days jointly with drinking water require withdrawal time of nine days in order to respect the MRL fixed for edible part of broilers.

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USE OF CEPHALOSPORINS IN VETERINARY MEDICINE RESULTS OF THE GERMAN NATIONAL ANTIBIOTIC RESISTANCE MONITORING (GE*RM*-VET)

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Abstract
Cephalosporins (3rd and 4th generation) are classified by the WHO as "highest priority critically important antimicrobials" and by the OIE as "critically important antimicrobials". They are important to treat bacterial infections in veterinary medicine. Based on a statistically valid sampling plan, bacterial isolates were investigated by using the broth-microdilution method according to CLSI documents. The MIC values were assessed with their corresponding clinical veterinary breakpoints (CLSI VET01S). If no breakpoints were available, MIC90 values were used for classification.

Introduction
Cephalosporins (3rd and 4th generation) are classified by the WHO as "highest priority critically important antimicrobials" and by the OIE as "critically important antimicrobials". They are indeed important to treat bacterial infections in veterinary medicine. Since 2001, an annual representative German-wide monitoring study (GE*RM*-Vet) on bacterial isolates from diseased animals generates resistance data amongst others against a set of five different cephalosporins.

Materials and Methods
Based on a statistically valid sampling plan, bacterial isolates were investigated by using the broth-microdilution method according to CLSI document VET01-A4. The MIC values were assessed with their corresponding clinical veterinary breakpoints (CLSI VET01S). If no breakpoints were available, MIC_{90} values were used for classification.

Results
The resistance data were evaluated according the bacterial species, animal species and indications that were investigated.

Mastitis (diary cow)
S. aureus isolates show very low resistance rates against cephalosporins (0.0-0.8%). The MIC_{90} values for *E. coli* were increasing over a period of 2 years (from MIC_{90} 0.12 to 8 mg L^{-1}; ceftiofur under 2% in 2005 to 9% in 2012).

Calves
MIC_{90} values for cephalosporins of the 3rd and 4th generation are high for bacterial strains isolated from calves (for all tested cephalosporins >32 mg L^{-1}). The rate for ESBL positive *E. coli* isolates from calves increased from 7% in 2006 to 20% in 2012.

Poultry
Cephalosporins are not approved for veterinary use in poultry. Nevertheless, we see high MIC_{90} values for broilers although the ESBL rates for *E. coli* are still at 2.4%

Pets
The situation is similar as for poultry: For a short time, MIC_{90} values for *E. coli* and cephalosporins rose. Particularly, bacterial strains isolated from infections of the gastrointestinal tract are of concern.

Discussion
This representative antimicrobial resistance monitoring serves as a valid tool in risk management. With these representative and quantitative data, we are able to monitor and to estimate the development of antimicrobial resistance of veterinary pathogens against 3rd and 4th generation cephalosporins.

Conclusions
An intelligent and rational application of antimicrobial agents is needed to minimise the development and the spread of antimicrobial resistant bacteria and their resistance genes as much as possible. Depending on the affiliation to animal and bacterial species, we see large differences in resistance data and a very different impact on the resistance situation in veterinary medicine.

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DOXYCYCLINE RESIDUES IN EDIBLE TISSUES OF PIGS

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Abstract

Doxycycline (DOX) is a variant of the tetracycline antimicrobial with similar properties, but with a longer action period. It is widely used in swine production. The presence of residues of antibiotics in food products of animal origin has a special toxicological interest due to their potential effects on human health.

Our aim was to evaluate the withdrawal time (WT) of DOX formulation (25%) in edible tissues of swine, after PO administration. Eighteen healthy young pigs (30-35 days old) were used. DOX was administered with drinking water during 5 days at 10 mg kg^{-1}. Two animals, as the control group, were not treated. Four animals per group were sacrificed by exsanguination 24 hours until 11 days post-treatment. Muscle, liver, kidney and skin/fat samples were obtained. DOX was determined by HPLC with UV detection. For muscle tissue, a WT of 4.3 days was determined. In other tissues, DOX concentrations were measured until 7-11 days post-administration. The WT was 7.2, 4.9 and 4.5 days for liver, kidney and skin/fat, respectively. After administration of DOX at 10 mg kg^{-1} for 5 days through medicated drinking water, a WT of 8 days must be set for safe consumption of medicated animals.

Introduction

Doxycycline (DOX) is a derivate of tetracycline with similar properties, but with a longer action period. It is the most active antibiotic of tetracycline group, so the minimum inhibitory doses are low. Is an antimicrobial widely used in swine production against Gram-positive and Gram-negative bacteria, including some anaerobes. Tetracycline agents are bacteriostatic antibiotics, which act by inhibiting the formation of proteins within the bacterial cell. Doxycycline is widely used in the treatment of respiratory and urinary tract infections in swine production. Doxycycline is more lipid soluble than the other tetracyclines, and after application it penetrates body tissues and fluids better. Long persistence of doxycycline in an animal's body could cause unacceptable concentration in animal tissues.

The European Commission has set maximum residue limits (MRLs) for oxytetracycline, tetracycline, and chlortetracycline as a sum of parent compounds with their corresponding 4-epimers. For the doxycycline constituent there is no 4-epimer, and an MRL for only the DOX compound is described. The MRLs are 100 ng g^{-1} for muscle, 300 ng g^{-1} for liver and skin plus fat, and 600 ng g^{-1} for kidney (EU 37/2010).

The antibiotic residue levels reached in organs and the rate of their depletion from tissues depend on the method of administration, animal species, as well as dose and the specific formulation of the drug given (Kung & Wanner, 1994). The differences in the antibiotic concentration and time of its depletion may be also influenced by the differences in the intake of drinking water by animals.

Residue studies of DOX have been conducted in several species, including chickens (Anadón *et al*., 1994), turkeys (Croubels *et al*., 1998), calves (van Dongen & Nouws, 1993) and pigs (Anadón *et al*., 1996). The purpose of this study was to determine the residues of DOX in edible tissues (kidney, liver, skin plus fat and muscle) of pigs after 5 days of oral medication *via* drinking water at 10 mg DOX 25% kg^{-1} body weight (BW) per day. The DOX concentrations in plasma and the stability of DOX in drinking water were also determined. Based on the residues in the tissues, a withdrawal time was calculated according to Guideline N° EMEA/CVMP/036/95 of the Committee for Veterinary Medicinal Products (EMEA, 1995).

Materials and Methods

Study Design Treatment and Administration

The study was conducted with 18 healthy Duroc Jersey pigs (30-35 days old). They were treated once a day with 10 mg kg^{-1} of an experimental formulation of DOX 25% water soluble powder, for five consecutive days through the drinking water. A solution was prepared by dilution of 400 g of the medicament in 1,000 mL of water.

The pigs were housed in four groups of four animals; one group of two pigs was managed as blanks. The animals were fed a pig feed free from antibiotics. Feed and water were available *ad libitum*. The drinking water system consisted of drinking nipples per box connected *via* plastic piping to a plastic storage tank of 100 L. The tanks were provided with a continuous stirring system and provisions for the measurement of daily water intake. After a controlled drug free period of 15 days, medicated drinking water was given for a period of five consecutive days at a daily dose of 10mg kg^{-1} of DOX 25%. Pigs were weighed daily and the water intake per group was recorded daily. Based on the mean daily water intake and the mean body weight per group of four the dosage of DOX was calculated every day.

The pigs treated with DOX were euthanized at 24 h, 3d, 7d and 11 d after the end of the drug administration (four animals at each time point). One whole kidney and about 300 g of liver, skin, fat and muscle were collected separately to avoid contamination and frozen at -20 °C pending analysis. All samples were analysed within two months after sampling. Two pigs used as controls were euthanized before experiment, and the same tissues samples were collected. This experimental animal protocol was accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS).

Reagents
Doxycycline (DOX) standard was obtained from Sigma-Aldrich Chemical Company (USA). Acetonitrile and methanol were from JT Baker. Oxalic acid dehydrate, trichloroacetic acid and sodium sulphate anhydrous were from Fluka (USA). Solid-phase extraction (SPE) columns (Strata, C18, 100 mg, 1 mL) and analytical column (Luna C18) were obtained from Phenomenex (USA). Doxycycline was formulated as a 25% experimental water soluble powder (DOX 25 g, tartaric acid 5g and Lactose sqt 100 g).

Standard solutions
Stock standard solution (1 mg mL^{-1}), was prepared by weighing 10.0 ± 0.1 mg of standard substances and dissolving it in 10 mL methanol. The stock was stored at -20°C in amber glass, and was stable for six months. Secondary standard solutions (100 µg mL^{-1}, 10 µg mL^{-1}) prepared in methanol by diluting suitable aliquot of stock standard were stable for one month, stored at 2-8°C in amber glass. Working standard solutions in mobile phase were prepared on the day of analysis.

Extraction and clean-up
A portion of 0.4 g of tissue (incurred or spiked) was homogenized with 1.4 mL of McIlvaine buffer-EDTA, shaken at high speed, and centrifuged at 2,500 g at 4°C for 15 min. The upper layer (supernatant S1) was transferred into a new tube. The extraction was repeated three times. The supernatants S2, S3 and S4 were combined with the S1. The mixture was vortexed for 30 s, and centrifuged again for 10 min at 2,500 g at 4°C.

Clean-up
The supernatant mixture (S1-S2-S3-S4) was transferred to SPE C18 cartridges, which were preconditioned with 3 mL methanol and 2 mL ultrapure water. The tube reservoir mix supernatants were washed with 1 mL of McIlvaine buffer-EDTA and 1 mL water. After percolation of the whole solution, the columns were washed with these solutions (under vacuum). After drying for 2 min, the doxycycline was eluted with 4 mL methanol 0.01 M oxalic acid pH 2.0. The cleaned eluates were evaporated to dryness in nitrogen evaporator at 40°C. The dried residues were reconstituted in 200 µL mobile phase. Then after vortexing and centrifugation, 100 µL were injected into the chromatographic system.

LC-UV analysis
The instrumental analysis was performed using Gilson HPLC system, equipped with isocratic pump, autosampler, column oven, and UV/Vis detector (λ = 346 nm), controlled by Unipoint Workstation software. Chromatographic analyses were performed on Luna (Phenomenex) C18 column (5 µm, 150 mm x 4.6 mm) with mobile phase consisting of water-acetonitrile with 0.02 M oxalic acid and 0.5 mM EDTA (72:28, v/v) at 1.2 mL min^{-1} flow rate. The column oven temperature was controlled at 30°C.

Method validation
The following parameters were evaluated for the analysis of each matrix: linearity (concentrations of DOX ranging between 0.1 and 6.0 µg mL^{-1}), precision and accuracy, limit of quantitation, limit of detection and selectivity. The standard calibration curve was prepared by injection of standard solutions at seven concentration levels. The correlation coefficient and linearity ranges were evaluated. The detection limits (LOD) and limit of quantitation (LOQ) of the method were calculated. The accuracy was defined as the closeness of agreement between the true (spike) value and the mean result of a series of experiments (n = 6). It was determined by comparing the measured concentration to the spiked concentration. The mean accuracy (recovery %) should be within the range 85-115% and the variation in precision should be ≤ 20%. The limit of detection (LOD) was estimated through the analysis of 20 aliquots of control tissue (free of DOX). The noise of the base-line was measured; the average and the standard deviation were calculated. The LOD corresponds to the average plus three times SD (signal-to-noise ratio ≥ 3/1) and the limit of quantitation (LOQ) corresponds to the average plus ten times the SD (signal-to-noise ratio ≥ 10/1).

Withdrawal time
The withdrawal periods for edible tissues of pigs (muscle, liver, kidney and skin plus fat) were estimated by linear regression analysis of the log transformed tissue concentrations and determined at the time when the upper one-sided 95% tolerance limit for the residue was below the MRLs, with a confidence of 95% (EMEA, 2002). Doxycycline concentrations as a function

of time found in muscle, kidney, liver and skin/fat were plotted and analysed with the program WT version 1.4 in order to recommend a withdrawal time period for this experimental formulation.

Results

This method performed accurately and reproducibly over a range of 0.1 to 6.0 µg mL^{-1} for DOX. The linearity (r) was between 0.9913 to 0.9975 in all tissues assayed. The chromatographic analysis time was short: DOX eluted at 4 min as a sharp and symmetrical peak with no interfering peaks (Figure 1A and 1B).

The LODs were 0.020, 0.020, 0.030 and 0.026 µg g^{-1} for DOX in kidney, skin/fat, muscle and liver, respectively, while LOQs were 0.050, 0.136, 0.050 and 0.225 µg g^{-1} for kidney, skin/fat, muscle and liver, respectively.

The analytical method for tissue samples was thoroughly validated (Table 1), and was found specific for all samples with respect to interference from endogenous compounds. The validated analytical methodology showed satisfactory sensitivity, precision and accuracy that allow its use for the detection and quantification of DOX residues in pig tissue (Figure 2).

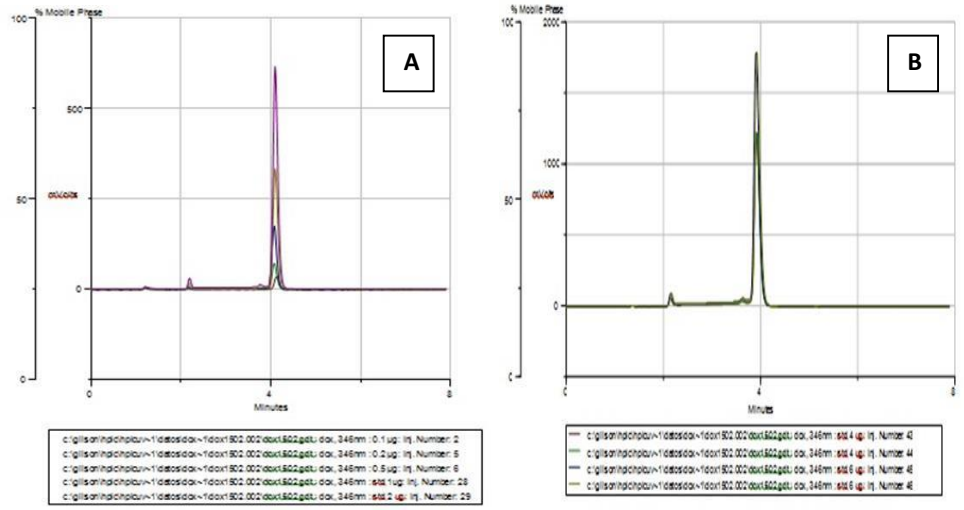

Figure 1. HPLC Chromatograms of DOX standard solution at seven concentrations: A) 0.1; 0.2; 0.5; 1.0 and 2.0, and B) 4.0 and 6.0 µg mL^{-1}.

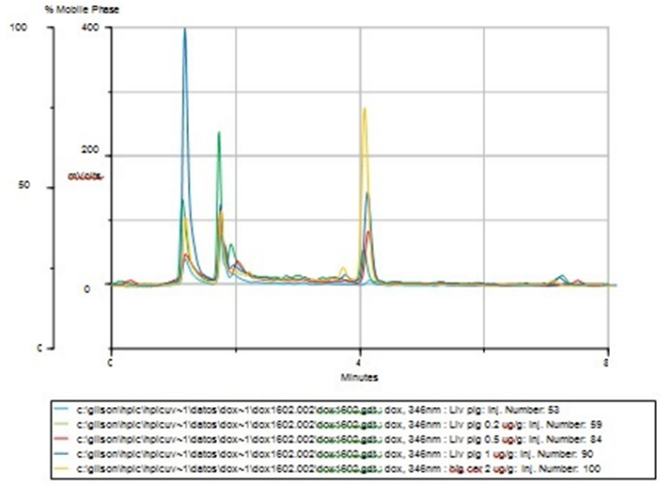

Figure 2. Chromatograms of blank liver and liver spiked at 0.2, 0.5, 1.0 and 2.0 µg g^{-1} DOX.

Doxycycline tissue concentrations

In Figure 3A, 3B, 3C and 3D, the mean tissue concentrations and their SD values for muscle, kidney, liver, skin +fat, respectively, at 1, 3, 7 and 11 days after cessation of medication are presented. Highest residues were found in liver, followed by kidney, muscle and skin + fat. The concentrations in all matrices were near or below the MRL at day 3 after treatment and below the respective LOQ at 11 days after the end of the treatment. In muscle sample, DOX was only detected at 24 h and day 3 post treatment.

Table 1. Analytical validation results for the analysis of DOX in pig tissues.

			Intra-day		Inter-day (over 3 days)	
matrix	r	µg g^{-1}	Accuracy (%), n=6	Precision (%), n=6	Accuracy (%)	Precision (%)
Muscle	0.995 (0.2-2.0 µg g^{-1})	0. 2	97.2	3.0	97.4	2.0
		0.5	92.2	1.2	91.4	1.7
		1.0	109	11.1	104.9	9.2
		2.0	97.9	3.0	97.5	2.5
Liver	0.994 (0.2-2.0 µg g^{-1})	0.2	88.4	4.1	89.5	5.7
		0.5	93.8	1.8	93.3	1.2
		1.0	111	3.0	109	2.4
		2.0	97.2	2.2	95.4	1.7
Kidney	0.998 (0.2-2.0 µg g^{-1})	0.2	93.4	2.1	92.8	2.8
		0.5	100	7.0	100	6.0
		1.0	103	1.2	103	2.5
		2.0	99.0	3.2	97.0	2.7
Skin/Fat	0.991 (0.2-2.0 µg g^{-1})	0.2	89.0	2.3	88.0	3.0
		0.5	97.7	6.2	91.2	7.5
		1.0	109	2.0	106	2.8
		2.0	97.2	2.1	93.6	3.4

Linear regression analysis of the logarithmic transformed data can be considered for the calculation of the withdrawal periods. Using this approach, the withdrawal time is determined as the time when the one-sided, 95% upper tolerance limit of the regression line with a 95% confidence level is below the MRL. The European Agency for the Evaluation of Medicinal Products Guideline recommends that values less than the LOQ should be set at one-half of the LOQ. Using this approach, the withdrawal time could only be calculated for muscle tissue: 4.33 days. In our study, taking into account the MRLs in pigs and considering that the marker residue is doxycycline, the calculated withdrawal times were 7.23, 4.87, 4.50 and 4.33 days for liver, kidney, skin + fat and muscle, respectively.

Discussion

Antibiotics are used in pig farms to enhance growth, feed efficiency and reduce diseases. Additionally, prophylactic treatment is common during periods of stress. Tetracyclines are the most commonly used antimicrobials in food-producing animals. In Argentina doxycycline is frequently used in pigs production; therefore, it is important to control their residues in edible tissues. Doxycycline given to pigs orally raise possibility for residues which, remain in edible tissues, particularly when the animals are slaughtered without respecting the withdrawal period. Such residues may pose public health hazards to consumers including toxicological, microbiological, immunological and pharmacological disorders depending on the type of food and the amount of residue present (Oka *et al*., 2000). Additionally, the use of antibiotics in related food may lead to resistance in bacterial populations that do not respond to treatment commonly used for human illnesses (Marchetti *et al*., 2012). Studies on tissue concentrations after different drug formulation administration are essential to recommend appropriate withdrawal times to secure control over the potential antibiotic residues in animal food products.

The data obtained after DOX administration with water shows that at the beginning after treatment, DOX reached high concentrations in all edible tissues. One day after administration of the last dose, DOX concentration rapidly decreased in all assayed tissues. The residue concentrations decreased gradually thereafter and only trace concentrations were detected on day 8.

Conclusions

This analytical method exhibited good linearity and reproducibility over the calibration range for DOX in edible tissues of pigs.

Our results demonstrate that oral administration of DOX at 10 mg kg^{-1} for five consecutive days through the drinking water require withdrawal time of 8 days in order to respect the fixed MRLs for edible tissues from treated pigs.

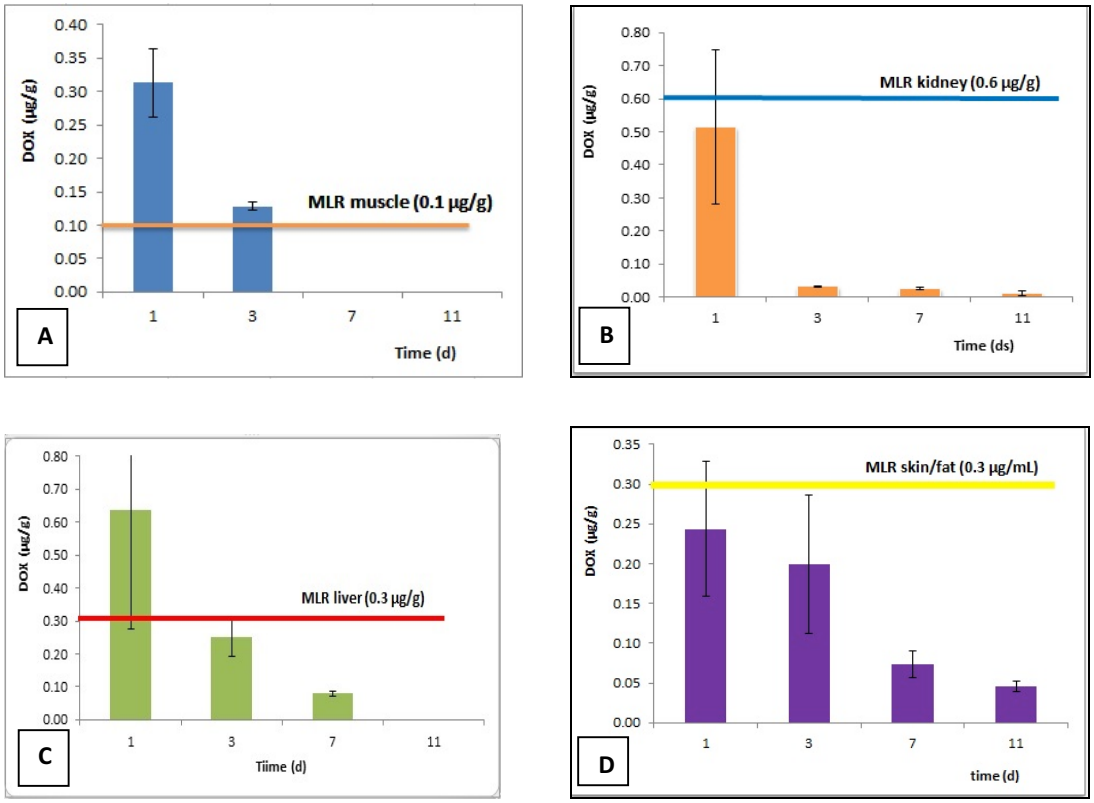

Figure 3. Mean tissue concentrations of doxycycline (A: muscle, B: kidney, C: liver and D: skin + fat) in pigs slaughtered 1, 3, 7 and 11 d after oral administration of DOX 25% (dose of 10 mg kg^{-1} body weight during 5 days).

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RESOLUTION OF A DISPUTED ALBENDAZOLE RESULT IN THE UK OFFICIAL CONTROL SYSTEM – TIME FOR MORE GUIDANCE?

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Abstract
Albendazole, one of the benzimidazole anthelmintics, is used in ruminants and has maximum residue limits in muscle, fat and other tissue owing to reported teratogenicity. Albendazole is extensively metabolized in domestic animals and humans with rapid conversion to a sulfoxide and subsequently a sulfone metabolite. Sulfoxide metabolites are responsible for the systemic biological activity of benzimidazole drugs. Herein we report a case of disputed results for albendazole in a consignment sampled at import in which the Official Analyst certified against the consignment for excess albendazole. A laboratory acting for the importer reported data below the MRL, including a finding of the parent drug which is not included in the residue definition. The Government Chemist has a statutory duty as a route of technical appeal in the UK Official Food Control system and the case was referred for referee analysis. We report our findings based on a LC-MS/MS method along with recommendations on official sampling at import and interpretation of results.

Introduction
Albendazole is one of the benzimidazole anthelmintics the use of which stems from the discovery of thiabendazole in 1961, (McKellar and Scott 1990), and is used in ruminants with a maximum residue limit, MRL, in muscle, fat and other tissue. In human medicine albendazole has been shown to be an effective treatment against helminth parasites (*e.g*. hookworm, roundworm) (Horton 2000) and is commonly used in tropical countries. Human adverse reactions have been reported (Martindale 2002) and the UK licensed preparation, Eskazole is, owing to reported animal teratogenicity, contraindicated in pregnancy and in women thought to be pregnant. Women of childbearing age are advised to take effective precautions against conception during and within one month of completion of treatment with Eskazole (MHRA 2015).

Benzimidazole anthelmintics are extensively metabolized in domestic animals and humans with rapid conversion of albendazole to a sulfoxide metabolite and subsequently a sulfone metabolite. Sulfoxide metabolites are responsible for the systemic biological activity of benzimidazole drugs (Wu *et al*. 2013). Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin allows the administration of albendazole to ruminants but limits the residues of albendazole in meat (muscle tissue and fat) to 100 µg kg^{-1} as the sum of albendazole sulphoxide, albendazole sulphone, and albendazole 2- amino sulphone, expressed as albendazole. Note, the parent drug is not included in the residue definition. Of the 134 RASFF notifications for veterinary residues in the 18 months from 01 January 2014 to 30 June 2015 six were for albendazole residues (RASFF 2015). Of these five were for corned beef from Brazil and one was for frozen minced beef from Brazil. Concentrations of albendazole (as the MRL definition) were reported: 167 µg kg^{-1}, 245 µg kg^{-1}, 256 µg kg^{-1}, 415 µg kg^{-1}, 536 µg kg^{-1} and 780 µg kg^{-1}.

In the UK Official Food and Feed Control System the Government Chemist, based in LGC (Laboratory of the Government Chemist) provides a statutory route of technical appeal, commonly described informally as 'referee analysis', independent expert analysis and / or interpretation to avoid or resolve disputes. There are statutory provisions for referral of retained portions of formal samples to the Government Chemist in regulations made under both the UK Food Safety Act 1990 and the Agriculture Act 1970. The statutory conditions for referral begin with the contemplation or commencement of legal proceedings where the prosecution intends to adduce analytical evidence. The referral may be by the local authority authorised sampling officer, the prosecutor or the court. The defendant may also, subject to agreement to defray some or all of the Government Chemist's costs, request referral. If the above route is not open to a trader, they may request a supplementary expert opinion (SEO) pursuant to Article 11(5) of Regulation 882/2004 on official controls and in defined circumstances SEO may be requested of the Government Chemist. The Government Chemist also acts as a source of advice for government and the wider analytical community on the analytical chemical implications on matters of policy, standards and regulations. Hence referee casework arises by a variety of routes and is a publically funded programme under the auspices of the UK central government Department for Business, Innovation and Skills, (Walker and Gray 2013; https://www.gov.uk/government/organisations/government-chemist)

In mid 2015 a UK Port Health Authority approached the Government Chemist following disputed results from an official sampling exercise carried out on a consignment of 54,000 cans (340 g each), 18.36 tonnes, of Brazilian corned beef. The official laboratory (Public Analyst, http://www.publicanalyst.com/) had reported 245 ± 65 µg kg^{-1} albendazole sulphoxide expressed as albendazole, (no albendazole, albendazole sulphone, or albendazole 2-amino sulphone was detected). A laboratory acting for the consignment owner reported an albendazole concentration of 80 µg kg^{-1} and albendazole sulfoxide 82 µg kg^{-1}, equivalent to 77 µg kg^{-1} as the residue definition, as the albendazole concentration is not taken into account. The Government Chemist was initially asked for advice and subsequently to carry out a referee analysis.

Initially it appeared that no unopened cans from the official sampling exercise remained available for an independent analysis by the Government Chemist and we advised that a further sampling exercise was required. We advised that a representative sample could be obtained by taking the cubed root of the number of units (cans) and, as the consignment was made up of two lots represented by different production dates and $\sqrt[3]{54{,}000} = 38$ (to the nearest whole number), we requested a further 20 cans from each production date to be taken at random. We also offered to reanalyse the samples homogenised and held by the two laboratories involved. In the event, on receipt of the original samples from each laboratory it emerged that they had each retained an unopened can. Thus analysis was carried out on (a) two portions of the homogenised corned beef originally analysed by the laboratories previously involved, (b) two unopened cans from the original sampling exercise and (c) four cans chosen with the aid of random numbers from the second sampling exercise (two from each production date). The remainder of the cans arising from the second sampling exercise were securely held pending the outcome of the above analyses and any decisions thereon.

Materials and Methods

The previously unopened cans were opened and the contents individually homogenised, the portions received already homogenised were thoroughly mixed and multiple replicates were analysed in line with the requirements of recognised official guidance (ECD: 2003) for albendazole and its metabolites, albendazole sulphoxide, albendazole sulphone, and albendazole 2-aminosulphone. The analysis consisted of acetonitrile extraction, liquid / liquid partitioning (acetonitrile / hexane) and solid phase extraction clean-up followed by liquid-chromatography separation and tandem mass-spectrometry detection and quantification (LC-MS/MS). Isotopically labelled albendazole D3 and albendazole sulphoxide D3 were used as internal standards. At least two precursor ion to product ion transitions were examined for each analyte and quantification was performed against appropriate calibration curves established by a series of post-extraction matrix standards. Method performance was monitored including by assessing calibration linearity, appropriate transition ratios, pre-extraction matrix spikes and for each positive sample a further extract was fortified to estimate overspike recovery. Having established results by reference to the isotopically labelled internal standard, the data were not further adjusted by reference to the overspike recovery. The validity of key steps in the analytical procedures was attested by being witnessed by a second designated scientist.

Apparatus

In addition to normal laboratory apparatus and disposables the following were used: automatic pipettes (Gilson) serviced and calibrated externally at 6-month intervals and checked in-house by single-point calibration on a daily basis; LC system Waters Acquity H-Class, utilising ACE Excel 2 C18-AR column, 3mm x 150mm, running a gradient elution of acetonitrile / water. Column temp 40°C, injection volume 5 µL, flow rate 0.6 mL min^{-1} gradient time (min) = 0 (95% Water), time = 1 (95% water), time = 7 (40% water), time = 8 (0% water), time = 10 (0% water), time = 10.5 (95% water). MS: ABSciex 4000 QTrap hybrid triple quadrapole, mode: positive electrospray ionisation, curtain gas: (nitrogen, arbitrary units) 10.00, ion spray (V): 5,000, temperature 650°C, gas 1 (nitrogen) 40.0 arbitrary units, gas 2 (nitrogen) 40.0 arbitrary units, interface heater: off, CAD gas: (nitrogen) medium CEM: 2,200 arbitrary units. The transitions monitored are given in Table 1.

Reagents

Solvents (acetonitrile, DMF, hexane and methanol) were at least HPLC grade, 18MΩ water. Albendazole, ≥ 98 % Sigma-Aldrich St Louis USA, albendazole sulphoxide and albendazole sulphone, >99 % Sigma-Aldrich GmbH, albendazole 2-aminosulphone, 99 %, Dr Ehrenstorfer via LGC Standards, albendazole-(methyl-d3), >99 % Sigma-Aldrich GmbH, and albendazole sulphoxide-d3(methyl), >99 %, Witega, Berlin, were made up initially in DMF/methanol with subsequent dilutions in methanol; SPE: Phenomenex Strata C18E, 1,000mg, 6 mL product code: 8B-S001-JCH.

Table 1. Transitions monitored.

Analyte	Declustering Potential (V)	Precursor (*m/z*)	Collision Energy (V)	Product T1 (*m/z*)	Product T2/T3 (*m/z*)
Albendazole	50	266.0	32	191.1	234.2
Albendazole sulphoxide	50	282.1	39	159.0	208.1/240.1
Albendazole sulphone	80	298.0	22	159.0	224.1/166.1
Albendazole 2-aminosulphone	80	240.2	30	133.2	198.2
Albendazole D3	50	269.1	24	234.1	N/A
Albendazole sulphoxide D3	50	285.1	29	208.1	243.1

Results

The mean results of the analyses for albendazole and its metabolites in each laboratory sample expressed as micrograms per kilogram of sample (µg kg^{-1}) are shown in Table 2 below. Only albendazole sulphoxide was detected in significant amounts. In Table 2 '< 50' implies the compound was not detected, *i.e*. there was no visual presence of a confirmed peak in the chromatogram at the relevant retention time and a detection limit of 50 µg kg^{-1} applies. The uncertainty cited was calculated as a 95% confidence interval, with a coverage factor of 2.571 based on the degrees of freedom available. Overspike recoveries for the positive samples ranged from 70.8 % to 129.5 %, however as stated above data were not further adjusted for these recoveries.

Table 2. Results, means of ≥ 3 replicate determinations.

Sample Ref	Albendazole µg kg^{-1}	Albendazole sulphoxide µg kg^{-1}	Albendazole sulphone µg kg^{-1}	Albendazole 2-aminosulphone µg kg^{-1}
Previously analysed 1	<50	302 ± 36	<50	<50
Previously analysed 2	<50	167 ± 5.3	<50	<50
Can received unopened	<50	1,537 ± 34	<50	<50
Can received unopened	<50	150 ± 13	<50	<50
Can received unopened	<50	<50	<50	<50
Can received unopened	<50	<50	<50	<50
Can received unopened	<50	<50	<50	<50
Can received unopened	<50	<50	<50	<50

Discussion

The results of the referee analysis overlapped with and confirmed those of the official laboratory. Contrary to the findings of the defence laboratory no parent drug was found in the samples analysed. Our findings confirmed that the sample contained albendazole sulphoxide above the MRL taking into account the measurement uncertainty associated with the findings, *i.e*. beyond reasonable doubt as a 95 % confidence interval. Moreover, two of the cans received unopened contained albendazole sulphoxide above the MRL, one markedly so. However, the positive findings were confined to only one of the two production dates present in the consignment.

Article 23 of Regulation **(**EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin stipulates that a food such as corned beef containing residues of albendazole exceeding the maximum residue limit shall be considered not to comply with Community legislation. Article 14 (6) of Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, and laying down procedures in matters of food safety prescribes that where any food which is unsafe is part of a batch, lot or consignment of food of the same class or description, it shall be presumed that all the food in that batch, lot or consignment is also unsafe, unless following a detailed assessment there is no evidence that the rest of the batch, lot or consignment is unsafe. Article 14 (7) of Regulation (EC) No 178/2002 establishes that food that complies with specific Community provisions governing food safety shall be deemed to be safe insofar as the aspects covered by the specific Community provisions are concerned. Hence the converse is true and corned beef containing albendazole above the MRL does not comply with Community provisions, is thus unsafe, and it can be assumed that all the corned beef in the batch, lot or consignment is also unsafe.

Moreover, Council Directive 97/78 (EC 1997) which regulates the veterinary checks carried out at Border Inspection Posts (BIPs) on Products of Animal Origin (POAOs), defines 'consignment' as a quantity of products of the same type, covered by

the same veterinary certificate(s) or veterinary document(s), or other document(s) provided for by veterinary legislation, conveyed by the same means of transport and coming from the same third country or part of such country, Article 2, 2(f).

Thus the corned beef in this case falls to be considered as one consignment despite having two different lots within it. Article 17 (2) of Directive 97/78 requires the competent authority, where an 'irregularity' is revealed by sampling and analysis, either to redispatch or destroy the product. This is supported by official Defra guidance, (Defra 2013).

The interpretation of the above law was tested before Mr. Justice Scott-Baker (as he then was) sitting in the Queen's Bench Division (Administrative Court) in 2002. The claimants, owners of a consignment of beef shipped from Australia to the UK, challenged, by Judicial Review, the decision of one of the authors (DS, acting as Official Veterinary Surgeon, OVS), to redispatch or destroy a consignment part of which was water damaged and did not comply with public health requirements. Rejecting the claimant's argument that DS should not have condemned all the cartons of meat but only those that were damaged, the Court found that "... there is no discretion available under Article 17 [of Directive 97/78] to allow the OVS to split the consignment. It may have been different if there were two or more products. ..." The Court concluded there was no substance to the claimant's case; leave to appeal was refused. Although not binding as case law this judgement is highly persuasive (Angliss 2002).

In view of all the above the consignment in this instance was redispatched for reasons of excess albendazole. The referee case raised some further interesting points.

The parent drug, albendazole, is not included in the residue definition because it is extensively metabolized in domestic animals and humans with rapid conversion to a sulfoxide and subsequently a sulfone metabolite, and sulfoxide metabolites are responsible for the systemic biological activity of the drug (EMEA 1999). However, there are anecdotal reports, supported by the owner's laboratory findings in this case that the parent drug albendazole is sometimes found on analysis. Since the toxicology of albendazole includes that of the parent compound it is recommended that the residue definition is amended to include albendazole itself. Had this been the case in the current instance no dispute would have arisen as to the fate of the consignment in question.

The preference of the Government Chemist in conducting referee analysis is that the official sampling should be as representative as possible of the consignment under investigation and the separate parts of the official sample (for official laboratory, owner's laboratory and referee analysis) should be as equivalent as possible. These aims are no doubt shared by all. However, there are practical difficulties and advice on sampling is limited. To our knowledge little or no research on the operating characteristics of sampling for veterinary residues has been carried out and certainly not to the same extent as that for mycotoxins. However, the results presented here suggest the existence of 'hot spots' of veterinary residue contamination within the consignment. In mycotoxins sampling and analysis the presence of hot spots of contamination is dealt with by extensive incremental sampling, Commission Regulation No 401/2006. It may be useful for official samplers to bear in mind taking the cubed root of the number of units for a statistically representative sample. However, lack of time to do so in a truly representative manner (turning out the entire consignment) may mitigate against its application and the problem of how many of the resulting units to analyse, or combine for analysis is challenging.

Conclusions

We describe herein the resolution of a technical appeal to the Government Chemist against the findings of the official analyst of excess albendazole in a consignment of corned beef from Brazil. The Government Chemist referee analysis upheld the finding of albendazole (as its sulphoxide) in excess of the permitted MRL in the consignment which was subsequently redispatched from the UK. The analysis identified hot spots of albendazole contamination in the consignment. The law, including case law, on the matter is reviewed showing that a finding of excess veterinary residue in some parts of a consignment means that the whole consignment can be taken to be unsafe and must be redispatched or destroyed. One of the laboratories involved in the case reported finding the parent compound, albendazole, which is not included in the residue definition. We recommend that the residue definition should be reconsidered to include albendazole as well as its metabolites. There is a paucity of research on sampling for veterinary residues and little guidance in this difficult area. Given the presence of hot spots of residues reported herein we recommend further research on sampling leading to practical guidance for sampling officers. In the meantime, this case report demonstrates that as a backstop against any possible shortcomings in sampling or analysis, appeal to the Government Chemist is a viable and equitable process.

Acknowledgements

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The paper should not be taken as an authoritative statement or interpretation of the law, as this is a matter for the courts.

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ANALYSIS OF 17ß-NORTESTOSTERONE METABOLITES TO DISTINGUISH BETWEEN ABUSE AND NATURAL OCCURENCE

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Abstract

17β-nortestosterone is a known anabolic steroid which is used for fattening purposes in cattle breeding. In urine, 17β-nortestosterone can also occur endogenously. To gain unambiguous prove 17β-nortestosterone found in *porcine* urine is endogenous or exogenous, methods and criteria are needed. For this purpose, a GC-MS/MS method for 16 17β-nortestosterone metabolites in urine has been developed and validated. Urine of treated and non-treated sows, boars and barrows from different animal studies have been analysed with this method. A metabolite profiling method has been derived from the dataset using the ratio of two of the main metabolites of 17β-nortestosterone. This model can distinguish between treated and non-treated sows and treated and non-treated barrows. Additionally, a survey of Dutch *porcine* urine was performed to gain inside in 17β-nortestosterone. A number of 62 *porcine* urines, female and male, were analysed and the ratios of norepiandrosterone and norandrostenedione were plotted into the model. One urine (sow) fell into the 95% confidence limit, so this animal is suspected for having been treated with 17ß-nortestosterone. To confirm if the animal was treated with 17ß-nortestosterone or if the animal could probably be an intersex or hermaphrodite, the sample should be analysed with GC-c-IRMS.

Introduction

The use of anabolic compounds is prohibited in food producing animals in the European Union. The 17β form of nortestosterone is a powerful steroid that may be used for fattening purposes (Debruyckere and Van Peteghem 1991, Pinel *et al*. 2010). Even though the presence/absence criteria of 17β-nortestosterone (17β-NT) in urine may seem adequate, the occurrence of 17β-nortestosterone in boar urine can be of endogenous origin (Poelmans *et al*. 2005). Also in female pigs, 17β-NT is seen in low concentrations (Groot *et al*. 2012). The origin of these findings can be an indication of abuse or from endogenous production. Intersex or hermaphrodite animals are thought to produce 17β-NT. Therefore, non-ambiguous criteria, possibly based on different metabolites, are required for confirmation of 17β-NT administration in pigs.

This study describes the development of a GC-MS/MS method for the analysis of 17β-NT and 16 known metabolites (*cf*. Table 1). These metabolites include some isomers, so the GC method should be able to separate these isomers. Additionally, the method was validated according Commission decision 2002/657/EC. In order to obtain profiles of treated and non-treated animals, an animal trial was performed where sows were treated with Nandrosol® (17ß-nortestosterone phenylpropionate). The urine was collected and analysed with the developed GC-MS/MS method. Finally, a survey of Dutch sow and boar urine was carried out to gain insight in the current situation of possible abuse of 17β-NT in The Netherlands. The designed model should be able to make a distinction between endogenous and exogenous 17β-NT in *porcine* urine.

Materials and Methods

Materials

Milli-Q water was prepared using a Milli-Q® system at a resistivity of at least 18.2 mΩ.cm^{-1} (Millipore, Billerica, MA, USA). Methanol (MeOH), *iso*-octane, *n*-pentane were obtained from Actu-All (Oss, The Netherlands). *Tert*-butyl-methyl-ether (TBME), sodium hydroxide, hydrochloric acid, acetic acid, sodium acetate and β-glucuronidase/arylsulfatase (*Helix Pomatia*) were obtained from Merck (Darmstadt, Germany).

Tris(hydroxymethyl)-aminomethane (TRIS), ammonium iodide, DL-dithiothreitol were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) from Thermo Scientific. PCB-138 (2,2',3,4,4',5'-hexachloro-biphenyl) from Fluka. The reference standards 5α-estrane-3ß,17α-diol (ABA), 5α-estrane-3ß,17ß-diol (ABB), 5α-estrane-3α,17α-diol (AAA), 5α-estrane-3α,17ß-diol (AAB), 5ß-estrane-3ß,17ß-diol (BBB), 5ß-estrane-3α,17ß-diol (BAB), 5ß-estrane-3α,17α-diol (BAA), 5ß-estrane-3ß,17α-diol (BBA), 5ß-estrane-17ß-ol-3-one, 5α-estrane-17ß-ol-3-one, Noretiocholanolone, norandrostendiol, norandrostanedione, norandrostenedione, norandrosterone, norepiandrosterone, nortestosterone-17α and nortestosterone-17ß were obtained from Steraloids INC. (Newport, USA). The internal reference standards norethiocholanolone-d4 and 19-norandrosterone-d4 were from Steraloids Inc. 17ß-Nortestosterone-d_3 and 17ß-testosterone-d_3 were obtained from Bank of Reference Standards EURL Wageningen UR (RIKILT, The Netherlands).

Stock solutions were prepared in MeOH at 1,000 mg L^{-1}, 17ß-nortestosterone-d_3 and 17ß-testosterone-d_3 at 10 mg L^{-1}.

Sample preparation

Two mL urine sample was transferred into a glass tube and 1 mL sodium acetate (2.0 M, pH 5.2) was added. The pH of the urine was adjusted to 5.2 using dilutions of acetic acid and sodium hydroxide. 25 µL β-glucuronidase/arylsulfatase was added for deconjugation of the conjugated compounds and the sample was incubated for 16 h at 37°C.

Liquid-liquid-extraction (LLE) was performed by adding 2 mL TBME. After extraction and centrifugation, the TBME layer was pipetted in a clean tube, the extraction was repeated and the combined TBME fraction was evaporated to dryness under a gentle nitrogen stream at 45°C. The extract was dissolved in 0.1 mL MeOH and 4.9 mL water before solid-phase extraction (SPE) was carried out.

A Bond Elut (Agilent Technologies, Bremen, Germany) C_{18} 500 mg/6 mL cartridge was conditioned with 5 mL MeOH followed by 5 mL water. The extract was applied onto the cartridge and washed with 5 mL 40% MeOH. The components were eluted from the cartridge using 5 mL 80% MeOH. The eluate was evaporated to dryness under a gentle nitrogen stream at 50°C.

The extract was dissolved in 0.2 mL MeOH and 2.0 mL TRIS buffer (0.1 M, pH 9.5). 5 mL *n*-pentane was added to the sample and after extraction and centrifugation the *n*-pentane layer was pipetted in a clean tube. The extraction was repeated and the combined *n*-pentane fraction was evaporated to dryness under a gentle nitrogen stream at 45°C.

The residue was dissolved in 150 µL MeOH and transferred to a 1.1-mL derivatization vial. This step was repeated with 150 µL MeOH and the combined MeOH extract was evaporated to dryness under a gentle nitrogen stream at 45°C. Derivatization was performed by adding 25 µL of MSTFA++ to the vial which was closed with a cap. The reaction mixture was incubated during 30 min at 60°C. After derivatization the reaction mixture was evaporated to dryness under a gentle nitrogen stream at 45°C and dissolved in 27 µL PCB-138 (0.1 mg L^{-1} in *iso*-octane) injection solution. The final extract was transferred into a GC-MS/MS sample vial with insert and cap.

Table 1. RT (min) and MRM transitions for nortestosterone-17ß and metabolites.

Compound	Abbreviation	RT (min)	MRM-1	MRM-2
5α-estrane-3α,17α-diol	AAA	13.65	407>199	407>241
5β-estrane-3α,17α-diol	BAA	13.97	407>199	407>241
5β-estrane-3β,17α-diol	BBA	14.11	407>199	407>241
5α-estrane-3α,17β-diol	AAB	14.22	407>199	407>241
Norandrosterone		14.21	405>225	405>315
5α-estrane-3β,17α-diol	ABA	14.55	407>199	407>241
5β-estrane-17β-ol-3-one		14.55	420>240	420>330
5β-estrane-3β,17β –diol	BBB	14.68	407>199	407>241
Noretio-D4	NE-D4	14.77	409>229	
Noretiocholanolone		14.81	405>225	405>315
5β-estrane-3α,17β –diol	BAB	14.87	407>199	407>241
5α-estrane-3β,17β –diol	ABB	15.03	407>199	407>241
Norepiandrosterone		15.01	405>225	405>315
Norandrostenediol		15.45	330>225	330>240
5α-estrane-17β-ol-3-one		15.85	420>240	420>330
Norandrostanedione		15.83	403>171	403>223
Nortestosterone-17α	NT-17α	16.42	418>182	418>194
17β-NT-d3	NT-D3	16.95	421>182	
Nortestosterone-17β	NT-17β	16.99	418>182	418>194
Norandrostenedione		16.95	416>220	416>234
17β-Test-D3	T-D3	17.28	435>209	
PCB-138	PCB	16.45	360>290	

GC-MS/MS analysis

The GC-MS/MS system consisted of a gas-chromatograph, automatic injector (SSL injector), and a mass-spectrometer (Bruker, Waldbronn, Germany) in electron impact mode (EI). For the separation of 17β-nortestosterone and its metabolites, an analytical GC column (VF-35MS, 60 m x 0.25 mm ID, film thickness 0.25 µm; Agilent CP8880) was used. Injection of 2 µL of the derivatised vials was carried out in pulsed split-less mode at 260°C. The GC program starts with an initial oven temperature of 110°C (1.0 min), a temperature increased of 20°C min^{-1} to 280°C, remaining time 0.5 min. The program follows with an increase of 1.0°C min^{-1} to 286°C, followed by an increase of 10°C min^{-1} to 325°C, remaining time 1.0 min. The total runtime

is 21 min. The temperature transfer line is set to 330°C at a constant flow mode of helium at 1.0 mL min^{-1}. The mass spectrometer source temperature is set to 250°C and fragmentation of the 17β-nortestosterone and metabolites was carried out in multiple reaction monitoring (MRM). As collision gas argon was used at 1.0 mTorr, the ionisation energy was set at 70 eV, scan width in MRM mode was 0.70 amu and the scan time was 0.18 s. Mass peak width of Quad1 is 2.0 and of Quad3 is 1.5 amu. The retention times (RT) and specific MRM transitions for each component are given in Table 1.

Results

Figure 1 shows a MRM chromatogram of a standard solution of 1.0 µg L^{-1} and a spiked *porcine* urine at 1.0 µg L^{-1} of the GC-MS/MS separation of the eight isomers (ABA, ABB, AAA, AAB, BBB, BAB, BAA, BBA). For quantification and confirmation, the separation on the GC column is important for these eight isomers because they have the same MRM transitions.

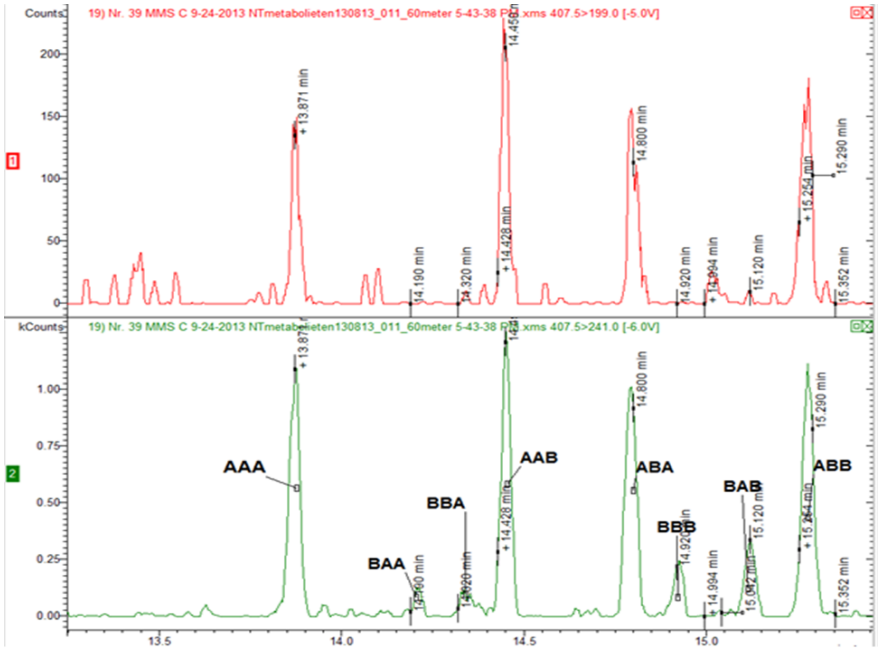

Figure 1. Examples of the MRM chromatograms of a standard solution 1.0 µg L^{-1} (top panel) and a matrix matched standard pig urine spiked at 1.0 µg L^{-1} level (bottom panel) of isomers from nortestosterone-17ß.

Method validation

A one-day validation was carried out according to Commission Decision 2002/657/EC. Seven different *porcine* urine samples (male and female) were spiked at three levels, respectively 0.5x, 1.0x and 1.5x validation level of 1.0 or 2.0 µg L^{-1} for the different components. After clean-up, derivatization and measurement with GC-MS/MS the concentrations of the components were calculated based on a matrix matched standard (MMS) calibration curve in blank *porcine* urine. These matrix-matched standards (MMS) were spiked at 0.0, 0.5, 1.0, 2.0, 3.0 and 5.0 µg L^{-1} and the internal standards at 4.0 µg L^{-1} level.

Based on CRL Guidance paper (2007), the concentration for 17α-NT and 17β-NT to be determined is 1.0 µg L^{-1}. The following parameters were determined: linearity, trueness, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ), selectivity, robustness and stability. These parameters are automatically calculated based on the concentrations for the three levels for each component in a ResVal calculation sheet according to ISO 11843 and 2002/657/EC.

An overview of the validation results (performance, CCα and CCß) is given in Table 2.

Profiling and Survey

Urine from an animal experiment in which sows were treated with Nandrosol® (17ß-nortestosterone phenylpropionate) were analysed using the described method. Measured concentrations of all components (if present) were used to design a profiling method by which treated sows can be distinguished from non-treated sows. This is done by calculating the ratio of the concentrations of norepiandrosterone and norandrostenedione. These components are always present in the urine of treated animals. Figure 2 shows the ratio curve constructed from the treated sow urines. On the Y-axis the ratio of the calculated concentrations of norepiandrosterone and norandrostenedione is plotted. An average line and a 2s (twice the standard deviation) line as a lower and upper limit are constructed based on the ratio values. These 2s lines correspond with the 95% confidence interval. Additionally, the ratios of the non-treated sows from the animal experiment are plotted into the graph (Figure 2). As can be seen, all ratios of non-treated sows fall below the 95% confidence interval. In none of these non-treated sows, 17ß-NT or any of the metabolites (including norepiandrosterone and norandrostenedione) was detected. The model

implies that when a ratio for measured urine falls within the 2s lower and upper lines, the urine is suspected for the exogenous treatment with 17ß-nortestosterone.

Table 2. Performance and validation results of 17ß-nortestosteron and metabolites in porcine *urine.*

Compound	Abbreviation	Performance	CCα ($\mu g\ L^{-1}$)	CCß ($\mu g\ L^{-1}$)
5α-estrane-3β,17α-diol	ABA	quantification/confirmation	0.79	1.57
5α-estrane-3β,17β-diol	ABB	confirmation	1.05	2.09
5α-estrane-3α,17α-diol	AAA	confirmation	0.87	1.74
5α-estrane-3α,17β-diol	AAB	confirmation	0.64	1.28
5β-estrane-3β,17β-diol	BBB	screening		2.87
5β-estrane-3α,17β-diol	BAB	screening		2.51
5β-estrane-3α,17α-diol	BAA	screening		1.93
5β-estrane-3β,17α-diol	BBA	screening		3.15
5β-estrane-17β-ol-3-one		screening		4.69
5α-estrane-17β-ol-3-one		screening		5.05
Norethiocholanolone		quantification/confirmation	0.17	0.34
Norandrostenediol		quantification/confirmation	0.44	0.88
Norandrostanedione		screening		3.20
Norandrostenedione		confirmation	1.04	2.07
Norandrosterone		quantification/confirmation	0.37	0.74
Norepiandrosterone		confirmation	0.63	1.26
17α-Nortestosterone	NT-17α	quantification/confirmation	0.39	0.77
17β-Nortestosterone	NT-17β	quantification/confirmation	0.27	0.54

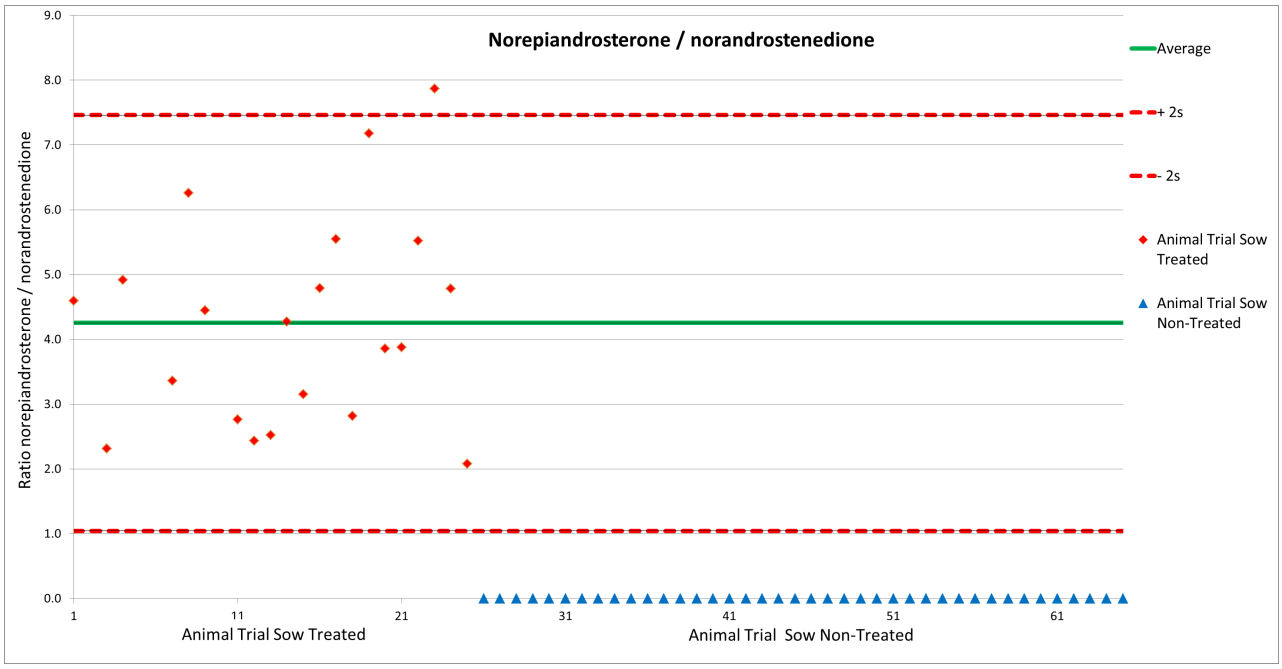

Figure 2. Ratio norepiandrosterone / norandrostenedione of urine of 17ß-nortestosterone treated sows and non-treated sows with the 95% confidence interval for suspicion of 17ß-nortestosterone.

A survey was performed to gain insight in the current situation of possible abuse of 17ß-NT in The Netherlands. With this objective, 62 urine samples of sows (n=32) and boars (n=32) from different Dutch farms were collected. These urine samples were analysed with the presented method and the ratio of the concentrations of norepiandrosterone and norandrostenedione (if present) were calculated and plotted in the ratio curve. Figure 3 shows the ratio curve with the 95% confidence interval from Figure 2. Figure 3 includes the ratio of the calculated concentrations of norepiandrosterone and norandrostenedione of the survey samples. All survey boar urine samples fall outside the 95% confidence limit. This implicates that the ratio

of norepiandrosterone and norandrostenedione, which are both present in the boar urine, deviates from the ratio of norepiandrosterone and norandrostenedione in the treated sow urines. Figure 4 shows one sow urine of which the ratio of norepiandrosterone and norandrostenedione falls within the 95% confidence interval as well. According to this model, the sample is suspected for treatment with 17ß-NT. To either confirm that the animal was treated with 17ß-NT or that the animal could be an intersex animal or hermaphrodite, the sample must be analysed with GC-c-IRMS.

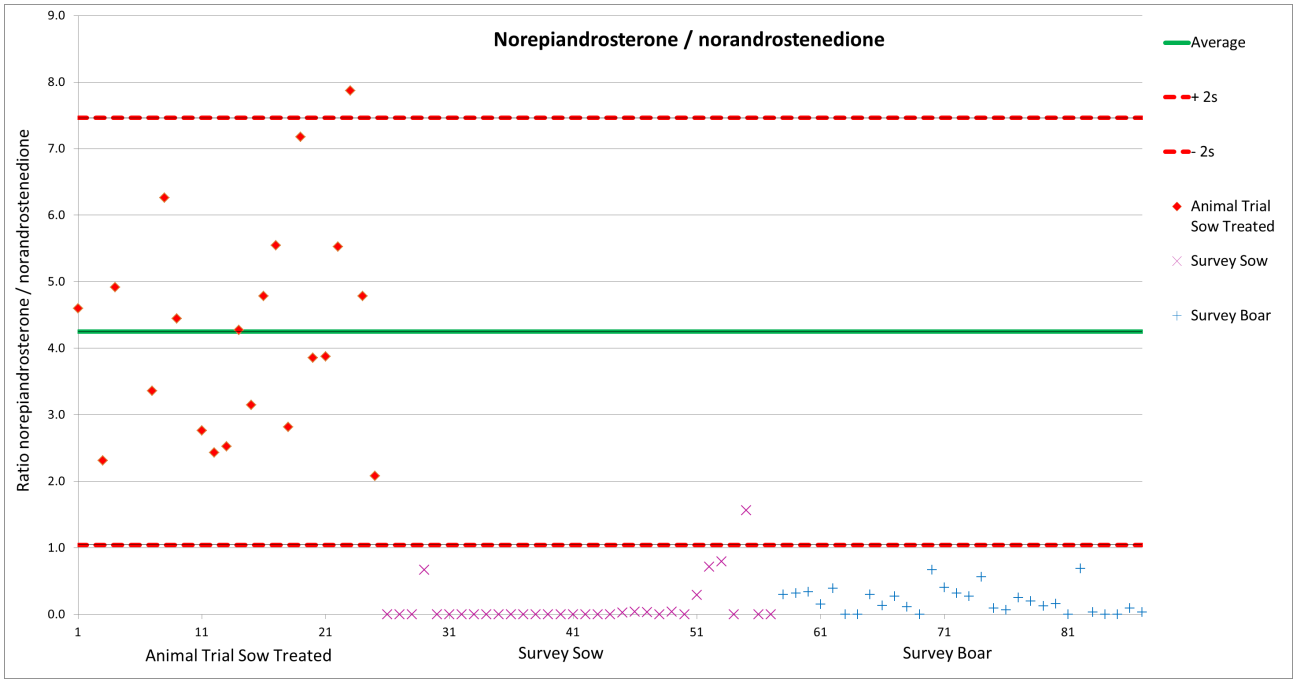

Figure 3. Ratio norepiandrosterone / norandrostenedione of urine of 17ß-nortestosterone treated sows survey samples, sow and boar, of Dutch farms with the 95% confidence interval for suspicion of 17ß-nortestosterone.

Conclusion

A method was developed which is capable of detecting 17ß-NT and 16 known metabolites. These metabolites, which include some isomers, were all separated and detected by GC-MS/MS. A one-day validation was performed for *porcine* urine according to Commission Decision 2002/657/EC. A profiling method was designed on the basis of an animal trial in which sows were treated with 17ß-nortestosterone phenylpropionate (Nandrosol®). This model, which uses the ratio of the concentration of norepiandrosterone and norandrostenedione, can distinction between urine of treated and non-treated sows as well as urine of non-treated boars and barrows. One sow urine out of 62 urine samples (sow and boar) from a survey of Dutch farms was classified by this model as a suspected sample. To confirm that the animal was treated with 17ß-NT or whether the animal is an intersex animal or hermaphrodite, the sample must be analysed by GC-c-IRMS (Ventura *et al*. 2008).

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DISTRIBUTION OF CHLORAMPHENICOL TO TISSUES, PLASMA AND URINE AFTER ORAL INTAKE OF LOW DOSES BY PIGS.

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Abstract
The toxic effects in humans caused by chloramphenicol treatment led to the prohibition of its use in food-producing animals in the EU 1994. To harmonize the level of acceptable residues, a minimum required performance levels (MRPL) was specified for chloramphenicol at 0.3 µg kg^{-1} (Commission Decision 2002/657/EC). This MRPL is now accepted as a Reference Point for Action (RPA) which protects consumer health at that level (Commission Decision 2005/34/EC). In 2012, chloramphenicol was found in urine (0.3 µg kg^{-1}) and muscle (0.14 µg kg^{-1}) from pigs in Sweden. Recently, chloramphenicol was found as naturally occurring substance in straw. Straw is used as bedding material for pigs in Sweden. Therefore, the aim of the present study was to investigate if residues could be found in pigs treated orally with low levels of chloramphenicol (4, 40 and 400 µg/pig) during 14 days and after 7 days withdrawal time. A dose-related increase of chloramphenicol residues was found in muscle, plasma, kidney and urine, but no residues were found in liver. Levels of chloramphenicol above the RPA were found in muscle, kidney and especially urine.

Introduction
In 1947, the soil organism *Streptomyces venezuelae*, which is a Gram-positive bacterium, was shown to produce chloramphenicol (Erlich *et al*, 1947). Chloramphenicol (CAP) is a broad-spectrum antibiotic particularly effective against gram-negative bacteria causing illness in humans and animals. After some years, CAP was shown to cause severe adverse effects in humans, *e.g*. bone marrow toxicity leading to irreversible aplastic anaemia. To prevent residues of CAP from ending up in animal-derived food, the drug was banned for use in food-producing animals in the EU in 1994 (Commission Regulation (EC) No 1430/94) and is listed as a prohibited substance in Table 2 of Commission Regulation (EU) No 37/2010.

To harmonise the limit of detection for analytical methods used in residue surveillance between laboratories in EU, a Minimum Required Performance Levels (MRPL) was established for some prohibited substances. The MRPL for chloramphenicol is 0.3 µg kg^{-1} (Commission Decision 2002/657/EC) in animal products including urine. Later this limit was used as the Reference Point for Action (RPA) for products of animal origin imported from third countries (Commission Decision 2005/34/EC). A scientific opinion on CAP in food and feed was published by EFSA 2014 (EFSA Panel on Contaminants in the Food Chain, 2014) with the conclusion that the current RPA, when applied to food products, but also feed, is sufficiently protective for animal health and for public health.

Chloramphenicol was shown to be present naturally in herbs and grass (Berendsen *et al*., 2010). Furthermore, CAP in the range of 0.1 to 11 µg kg^{-1} was found in samples of straw in The Netherlands (Stolker *et al*., 2012; Berendsen *et al*., 2013). In the Swedish national monitoring program 2012, CAP was found in urine (0.30 µg kg^{-1}) and in muscle (0.14 µg kg^{-1}) from pigs. No illegal use or abuse could be identified at the farms concerned. It was suspected that straw could be the reason as it is compulsory to use straw as bedding material for pigs in Sweden. An investigation on CAP in straw from different parts of Sweden showed that 117 of 209 samples were positive and the concentration in 26 samples was higher than the LOQ (2.0 µg kg^{-1}) (Nordkvist *et al*., 2016).

The aim of the present study was to investigate whether CAP can be detected in tissues, plasma and urine in pigs exposed to low levels of CAP at or above the RPA, and to what extent applying the RPA to urine is a suitable method for detecting abuse of CAP.

Materials and Methods

Animal experiment
Twenty four pigs, weighing 23-27 kg at the start of the experiment, were kept in four groups (A-D, one group per room) of six pigs each, at the National Veterinary Institute of Sweden. The pigs were kept on concrete floor with sterilized wooden chips as bedding and fed commercial feed which had been showed to be free from CAP. The experiment was reviewed and approved by the Board of Agriculture in February 2014 (dnr: 2013/1029). The timeline of the experiment is shown in Figure 1.

During the one-week adaptation period, urine samples were taken. Before exposure, blood samples were taken to confirm that no CAP was present. The individual groups were exposed to 0 (group A), 4 (group B), 40 (group C) or 400 (group D) µg CAP per pig per day for 14 days. Every morning, each animal was given their daily dose of chloramphenicol via a prepared sugar cube. In order to control the exposure of CAP for each individual pig, the sugar cubes were given manually. After the exposure period, 4-5 pigs per group B-D were sacrificed and samples of blood, urine, liver, kidney and muscle were taken. One week after the end of exposure the remaining pigs were euthanized and corresponding samples were taken. All samples were kept at -18°C before analysis of CAP by the National Food Agency (NFA, Uppsala) and RIKILT (Wageningen).

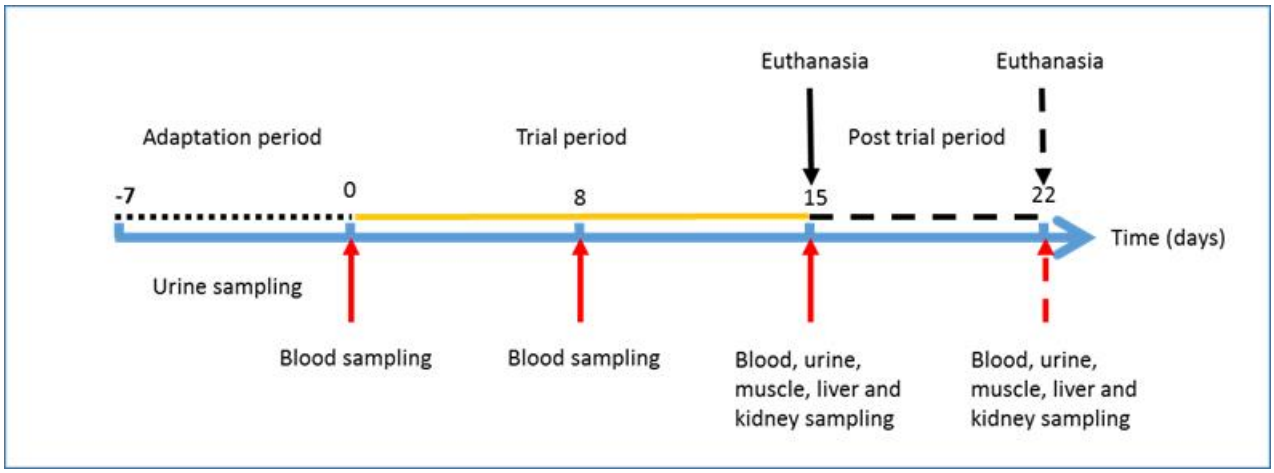

Figure 1. Timeline and sampling scheme for the animal experiment. At day 15, all pigs except for two pigs per group A, B and D, were euthanized and samples of blood, urine, skeletal muscle (ham), liver and kidney were taken and frozen at -20°C. The same procedure was followed at day 22 for the remaining six pigs.

Analysis

Analysis of CAP was performed by LC-MS/MS at both RIKILT and NFA using similar but not identical procedures. In both laboratories, chromatography was performed on an ACQUITY UPLC system (Waters, Manchester, UK) equipped with an ACQUITY UPLC BEH C18 analytical column (Waters). Detection was carried out using a Waters model Xevo TQS triple quadrupole mass spectrometer in the negative electrospray ionization (ESI) mode. Chloramphenicol and chloramphenicol-d5 were fragmented using collision induced dissociation (CID). The main differences were in extraction and sample clean-up. Detailed method comparison issues are, however, beyond the scope of the present paper.

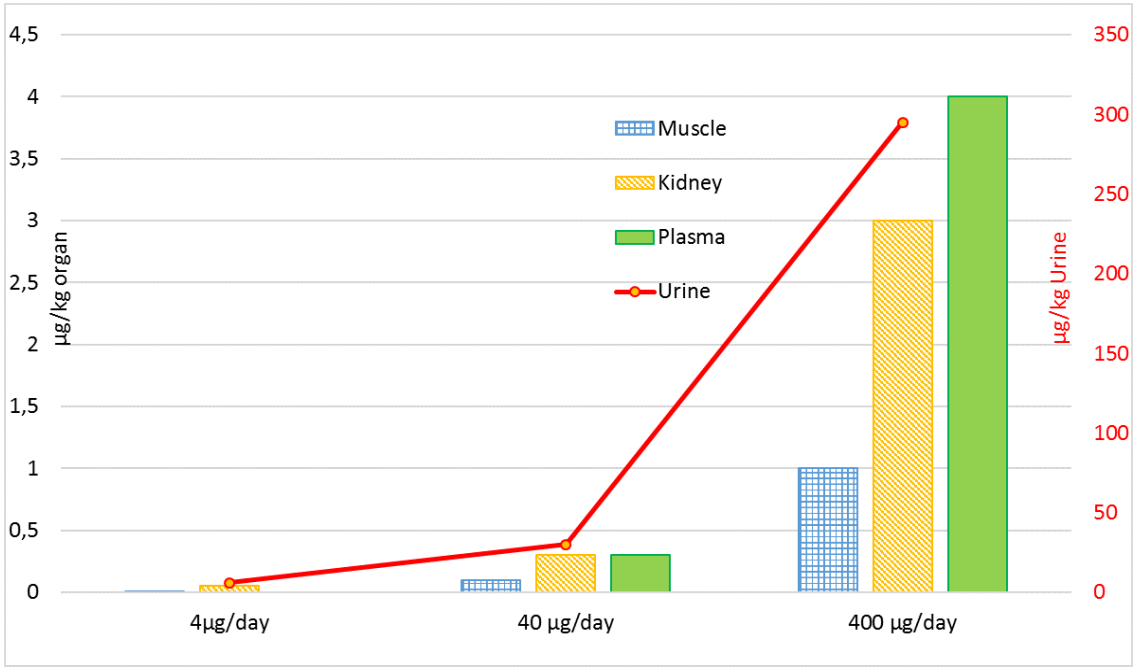

Figure 2. Group median levels of chloramphenicol (µg kg^{-1}) after 14 days of exposure to different low levels of orally administered CAP. Since the level in urine was more than one order of magnitude higher than in the organs this was plotted on a secondary scale (to the right).

Results

The results as median values per group and treatment are presented in Figure 2. It can be seen that a dose related increase of CAP was found in plasma, muscle, kidney and urine. No residues (LOD <0.05 µg kg^{-1}) were found in the livers.

Discussion

A relation between levels of CAP in tissues, plasma and urine from pigs exposed to sub-therapeutic doses of CAP has not been found in literature. Therefore, pigs were treated daily for 14 days with a oral dose of 0, 4, 40 or 400 µg reflecting the values found in straw. Due to small number of pigs, and hence observations per group, robust statistics were used for evaluation of the experiment. Group results are thus presented as median values rather than as averages. The results showed approximately a 10 and 100 times increase of residues in plasma, muscle, kidney and urine in the middle and highest exposure group compared with the lowest dose-group (4 µg).

Some differences among individuals in the same group were found which could partly be explained with biological variations and partly by the fact that some pigs did not like the taste of drug on the sugar cubes and therefore spit the sugar cube out. The level in urine may differ more between individuals in the same group than in muscle and kidney tissue. This depends on how much the pig drank and when they last urinated, which influenced the concentration of CAP in urine. In all dose groups, much higher concentrations of CAP in urine were found than in muscle.

Levels in plasma increased with the number of dosing, which could indicate that CAP is accumulated in the body. However, on the other hand, no residues were found in muscle, kidney and urine 7 days after withdrawal of exposure which indicates that chloramphenicol is excreted rather fast.

CAP is known to be metabolised in various animal species (EFSA, 2014). However, at the low exposure to the drug used in our experiment, we could not find any residues of CAP in the liver, and therefore we did not investigate metabolites.

The influence of bran (dietary fibres) on oral absorption of chloramphenicol palmitate in pigs treated with high doses of CAP (8 g, approximately 200 mg kg^{-1} bw) twice a week has been investigated (Bueno *et al*., 1983). The bioavailability of CAP increased in pigs fed with a fibre-rich diet compared with milk or a standard diet. Thus, when pigs get CAP by eating straw that contains CAP, a higher level of residues might occur than after a normal diet, as was used in our study.

The highest levels of CAP reported so far in straw were approximately 30 µg kg^{-1}. Measurable residues, but lower than the RPA, could be found in plasma, muscle and kidney in pigs exposed to low oral doses (4 µg/pig daily) of CAP. However, residues in urine above the RPA were found at this low dose. The higher doses (40 and 400 µg/pig daily) caused residues above the RPA in plasma, kidney and urine but not in muscle. Since levels in edible products below the RPA were considered to present no risk for consumers by EFSA (2014), and considering the naturally occurrence levels of CAP in straw, it might be questioned whether urine is still a good marker when applying the RPA as cut-off.

Conclusions

A dose-related increase of levels chloramphenicol in muscle and kidney, urine and plasma was found, but no residues could be found in liver. This study proves that chloramphenicol in very low doses (4 µg/pig daily) that may be found naturally, can be absorbed by pigs resulting in positive findings above the RPA in urine. It takes approximately 10 times higher doses to find residues of chloramphenicol higher than the RPA in plasma and kidney but even higher doses to exceed the RPA in muscle.

Acknowledgements

The authors thank the staff at the animal facilities at the NVI, especially Annika Daggfeldt and Per Karlsson. We would also like to thank Team pre-analysis at the NFA, especially Jannika Bergman, Hanna Thorén for the analysis of urine and muscle at the Department of Chemistry, Leif Busk (NFA) for participating in the first planning of the experiment and for making efforts to find funding for the experiments. The analysis carried out at RIKILT was funded by the Dutch Ministry of Economic affairs.

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VALIDATION OF THE BETASTAR S COMBO FOR THE RAPID SCREENING OF MILK FOR RESIDUES OF ß-LACTAMS AND TETRACYCLINES

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Abstract
The Betastar S Combo (Neogen Corporation, Lansing, MI) is a qualitative lateral flow assay for rapid screening for residues of β-lactams and tetracyclines in raw milk. The test strip contains besides a test line for β-lactams and tetracyclines a separate test line for the detection of (desfuroyl)ceftiofur. The test is simplified to just incubating the test strip in 300 µL of milk at 47.5°C for 5 min. The test can be interpreted both visually and by an AccuScan Gold or Pro reader (Neogen Corporation). The Betastar S Combo was validated at ILVO-T&V (Melle, Belgium) on its applicability as a screening test according to Commission Decision 2002/657/EC and CRL Guidelines (*Anon.*, 2010).
All β-lactam marker residues were detected at their respective MRL (Maximum Residue Limit, Commission Regulation 37/2010) except for cefazolin and cefalexin (Accuscan Pro reader). All tetracyclines (including the 4-epimers) present on the MRL-list with an MRL in milk are detected at MRL except for the 4-epimer of chlortetracycline with a 95% detection from 125 µg kg^{-1} on. The CCβ for doxycline is 50 µg kg^{-1}.
Also the robustness of the test was tested: impact of changes in the test protocol, evaluation for influences from compositional components or milk quality, applicability to test milk of different animal species, rate of false positive results, and participation in a national ring trial.

Introduction
BetaStar S Combo (Neogen Corporation, Lansing, MI) is a competitive receptor test in dipstick format for the rapid detection of residues of β-lactams (penicillins and cefalosporins), desfuroylceftiofur (the metabolite of ceftiofur), and tetracyclines in raw milk. The time to result for the assay is 5 min.

A validation study was performed at ILVO-T&V (Technology & Food Science Unit of the Institute for Agricultural and Fisheries Research of the Flemish Community) according to Commission Decision 2002/657/EC and to the guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer) (*Anon.*, 2010).

Materials and methods

Test procedure
Add 300 µL of milk and the test strip to the supplied plastic vial and incubate for 5 min at 47.5°C (Rapid test incubator, Neogen Corporation) and then read the result visually or by using an electronic reader (AccuScan Gold or Pro reader (Neogen Corporation)).

Negative result: sample line is darker than or equal to control spot; no drug residue of respected group detected; ratio sample line / reference line ≥1.00. Positive result: sample line is lighter than control line; drug residue of respected group detected; ratio sample line / reference line <1.00. Despite all testing was performed on both reader types, in this proceeding only Accuscan Pro reader results are discussed.

Test selectivity
The selectivity of BetaStar S Combo was tested by analysing milk doped with compounds belonging to different families of antibiotics or chemotherapeutics other than β-lactams (one per family) and by all β-lactams (on the channel for desfuroylceftiofur and tetracyclines). Raw milk was spiked at a high concentration, namely 100×MRL or 100×MRPL (Minimum Required Performance Limit, Commission Decision 2003/181/EC). All testing was performed in duplicate. Following compounds were tested: all β-lactams, oxytetracycline (tetracyclines), erythromycin (macrolides), enrofloxacin (quinolones), chloramphenicol (amphenicols), neomycin (aminoglycosides), colistin (polymyxins), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), sulfadiazine (sulfonamides), trimethoprim (diamino-pyrimidine derivatives) and dapsone (other chemotherapeutics).

Furthermore, 375 blank individual farm milk and 300 blank tanker milk samples were tested in order to check the rate of false positive results.

Detection capability

The detection capability of the BetaStar S Combo for all β-lactams and tetracyclines listed as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010 were determined. Each compound was individually spiked in blank raw milk at different concentrations. The increment between the different concentrations was depending on the concentration level in relation to the MRL, as indicated in Table 1. The blank raw milk was originating from four cows in mid-lactation that were not treated with antibiotics or chemotherapeutics during the last months.

Table 1. Increment between the concentrations tested.

Concentration (in µg kg^{-1})	Increment (in µg kg^{-1})
1-10	1
10-20	2
20-50	5
50-100	10
100-250	25
250-500	50
500-1,000	100
1,000-5,000	500

According to the CRL guidelines for the validation of screening methods (*Anon*., 2010), each concentration was tested 20, 40 or 60 times, respectively, in a time period of at least three days and by different technicians. The number of replicates is related to the closeness of the tested concentration to the MRL:

concentration tested is <0.5×MRL: 20 replicates
concentration tested is 0.5 - 0.9×MRL: 40 replicates
concentration tested is 0.9 - 1.0×MRL: 60 replicates
concentration tested is >MRL: 20 replicates

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Test robustness

To test the robustness of the BetaStar S Combo, blank and fortified (2 µg kg^{-1} benzylpenicillin, 35 µg kg^{-1} ceftiofur and 50 µg kg^{-1} tetracycline, respectively) raw milk samples were first analysed strictly following the test protocol and using milk of normal composition. Afterwards, other conditions were tested like a different incubation time and delaying the reading of the test strips. Also the impact of the volume of milk was studied, besides the impact of the temperature of the milk. The impact of a high somatic cell count (>10^6 somatic cells per mL), a high bacterial count (>$5 \cdot 10^5$ CFU per mL), a high fat content (>6 g per 100 mL), a low fat content (<2 g per 100 mL), a high protein content (>4 g per 100 mL), a low protein content (<3 g per 100 mL), a high pH (7.5) and a low pH (6.0) of the milk was studied.

In addition, the suitability of the BetaStar S Combo to test heat-treated milk and milk of animal species other than the cow (goat, ewe, mare) was tested. The difference between two lots of reagents was evaluated by testing the same set of blank and spiked samples with two different lots of reagents. Finally, the test was integrated in the monitoring of dairy samples (farm milk, truck milk, consumption milk and milk powders) and a national ring trial to check the number of false positive and false negative results.

Results and Discussion

Test selectivity

No interference by compounds not belonging to the group of β-lactams or tetracyclines was noted except for clavulanic acid. The interaction of clavulanic acid, a β-lactamase inhibitor, is expected since this molecule contains a β-lactam structure resembling that of penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring. When challenged with high concentrations of non-target drugs, each test channel was found to be highly specific for its target drug class. Ceftiofur and desfuroylceftiofur produced positive results in both the β-lactam and desfuroylceftiofur channels, as expected. Cefquinome was also causing positive results on the desfuroylceftiofur channel. Hence the detection capability was determined for cefquinome on the desfuroylceftiofur channel (30 µg kg^{-1}).

No false positive results were obtained in the testing of 375 individual farm milk and 300 tanker milk samples, showing the BetaStar S Combo test to be highly specific in routine testing of raw milk samples. Mean, minimum, and maximum ratios were similar for individual and tanker milk samples.

Detection capability

The detection capability of the BetaStar S Combo for β-lactams and tetracyclines is shown in Figure 1.

All β-lactams with a MRL in milk (EU-Regulation 37/2010 and amendments) are detected by the BetaStar S Combo. All of these compounds are at least in 95% of the replicates detected at the MRL except for two compounds: cefazolin (CCβ = 90 µg kg^{-1}, MRL 50 µg kg^{-1}) and cefalexin (CCβ = 3,000 µg kg^{-1}, MRL 100 µg kg^{-1}). Inclusion of the desfuroylceftiofur line in this test allows ceftiofur and desfuroylceftiofur to be detected at their MRL. All tetracyclines (parent drugs) tested are detected at their MRL, of the 4-epimers only the 4-epimer of chlortetracycline is missed at MRL (CCβ = 125 µg kg^{-1}, MRL 100 µg kg^{-1}). Phenoxymethylpenicillin and doxycycline were detected at 2 and 50 µg kg^{-1}, respectively. No MRL for phenoxymethylpenicillin and doxycycline in milk has been established; phenoxymethylpenicillin is not registered for dairy cows and doxycycline is not for use in animals from which milk is produced for human consumption.

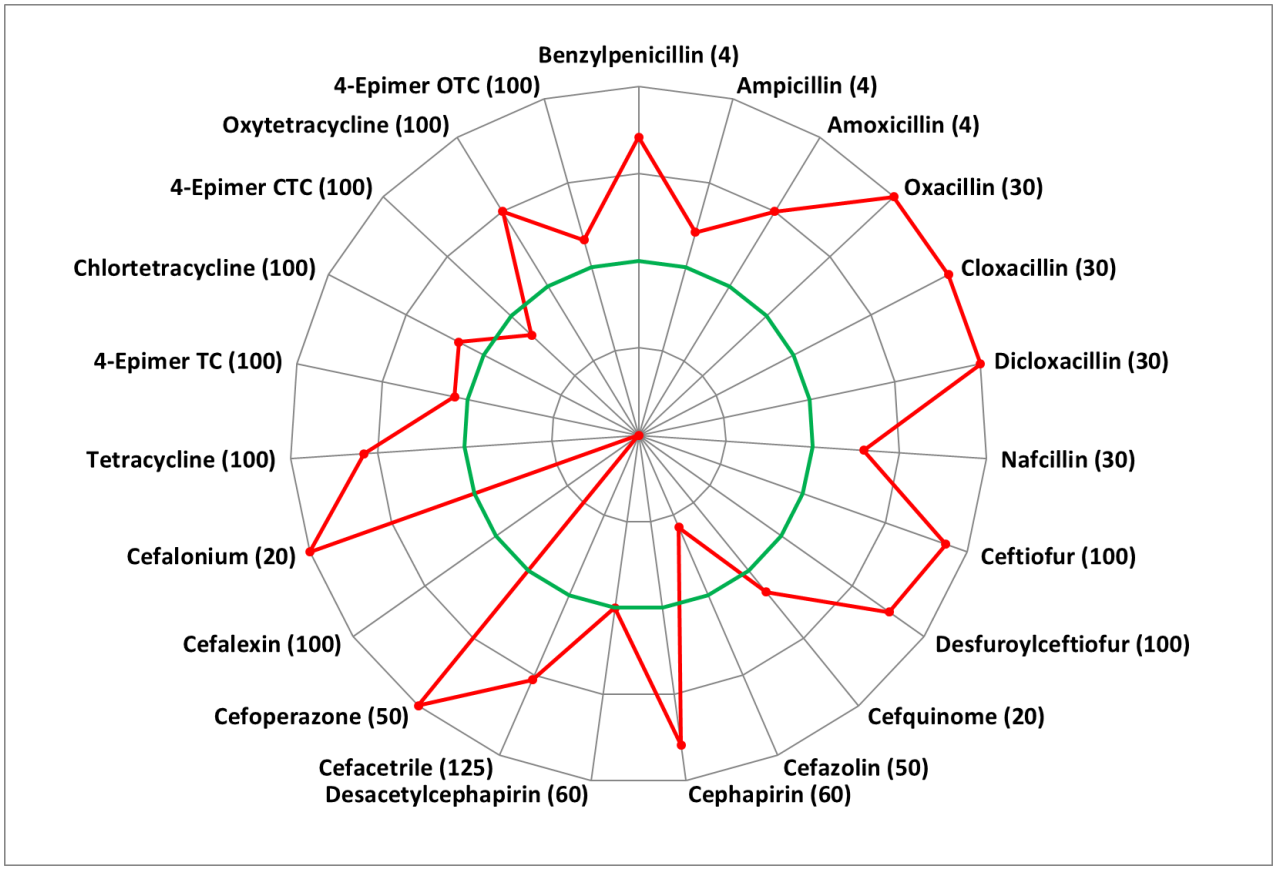

*Figure 1. Detection capability of BetaStar S Combo for β-lactams and tetracyclines related to their respective MRL (Commission Regulation (EU) N° 37/2010 (situation at 01/01/2016)). Inner circle = 2 × MRL; circle 2 = **MRL**; circle 3 = 0.5 × MRL; circle 4 = 0.25 × MRL. MRL (µg kg^{-1}) in cows' milk in between brackets after the name of each substance. Results obtained with Accuscan Pro reader and cut-off = 1.00. TC, tetracycline; CTC, chlortetracycline; OTC, oxytetracycline.*

Test robustness

Performing the BetaStar S Combo assay with incubation times different from the standard 5 min had no impact on test results for blank milk or milk fortified at 2 µg kg^{-1} benzylpenicillin, 35 µg kg^{-1} ceftiofur or 50 µg kg^{-1} tetracycline. There were no false positive results with blank milk or no false negative results with doped milk at either the standard incubation time or incubation times 30 s below or above the specified time.

Longer delay in reading the test strips did not impact the interpretation of positive test results. With blank milk, ratios decrease slightly with a delay in reading of the test strips. However, even after a 15 min-delay, blank milk still produces a clearly negative result for all three test channels.

Varying milk sample volume by +/- 10% (30 µL) from the prescribed volume of 300 µL did not produce a noticeable effect on performance of the BetaStar S Combo test. All blank milk and spiked milk samples produced expected negative or positive results. One blank milk sample produced a lower ratio on the β-lactam channel at 270 µL milk volume, but the result was still negative.

Variation in milk temperature did not affect the performance of the test. For blank milk samples, ratios were somewhat more variable for milk samples with a temperature higher compared to the 4°C reference condition, but all tests still produced

strongly negative results for all three test channels. Variations in milk temperature did not appear to produce changes in ratios for tests of doped, positive milk.

With blank milk, high bacterial count, high fat, high protein content, and especially high somatic cell count resulted in some elevated ratios compared with the reference condition of normal milk. However, correct negative results were produced in all cases. With antibiotic-spiked milk, low fat, high fat and high protein content all produced at least one false negative result on the desfuroylceftiofur channel at the drug level tested. High somatic cell count, high fat, high protein and high pH all produced one or more false negative results on the tetracycline channel at the drug level tested. No abnormal milk condition produced any aberrant results on the β-lactam channel. In these experiments, the level tested was below the MRL for each drug, so the risk of obtaining false negative results in milk at MRL is extremely low.

The applicability of the test for the screening of milk of animal species other than the cow or heat-treated milk was also tested. For blank mares' milk lower ratios compared to the reference condition were obtained, but all tests were negative. Sterilized and ewes' milk spiked with ceftiofur, produced one or more false negative results on the desfuroylceftiofur channel at the level tested. On the tetracycline channel, UHT milk, reconstituted milk powder, goats' milk, and ewes' milk all produced one or more false negative results at the level tested. On the β-lactam channel, UHT milk and sterilized milk produced slightly elevated ratios but all tests were correctly positive. In these experiments, the level tested was below the MRL for each drug, so the risk of obtaining false negative results in milk at MRL is extremely low.

Very good results, in line with the detection capabilities and selectivity study, were obtained with BetaStar S Combo reagents in a proficiency test (Ooghe and Reybroeck, 2015).

Conclusions

The BetaStar S Combo with its easy one-step test protocol could be used for the screening of tanker milk on the presence of β-lactam and tetracycline antibiotics at the entrance at the dairy plant. The test is also giving reliable test results for different milk types and milk of different animal species (cow, goat, ewe, mare).

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DEVELOPMENT, VALIDATION AND APPLICABILITY OF A MULTI-RESIDUE LC-MS/MS METHOD FOR THE DETECTION OF ANTHELMINTICS IN MILK

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Abstract
Anthelmintics are a diverse group of substances often used to prevent or cure intestinal infestations by parasites. They are classified as group B substances by the European Council and MRLs have been set for some, while others are prohibited. These legislative limits necessitate the need for sensitive analytical methods to confirm the absence or identify and quantify the presence of such residues.
The aim of this study was to develop and validate a multi-residue method for the simultaneous analysis of 23 anthelmintics (plus metabolites) by means of a sensitive, selective and fast LC-MS/MS analysis. The mass-spectrometric parameters were optimized on a Xevo TQ-S® (Waters) in order to allow the detection of minimum two product ions per precursor ion for each compound. Subsequently, the chromatographic conditions were optimized on the Acquity™ UPLC® (Waters) system equipped with BEH C_{18} (2.1x100 mm, 1.7 µm) column protected by a guard column. The extraction was performed using acetonitrile. The final optimized method was validated according to Commission Decision 2002/657/EC. Performance characteristics such as specificity, linearity (R^2), recovery (R), repeatability (RSD_r), intra-laboratory reproducibility (RSD_R) and the decision limit (CCα) were determined. The method was used in a proficiency test organized by the EU-RL Berlin and it can be concluded that this method can be used in routine to check on the compliance of milk samples for anthelmintics.

Introduction
Anthelmintics are a diverse group of substances that are often used to prevent or cure intestinal infestations by parasites. These infestations have a negative effect on the production, on the animal welfare and on public health (1). This therapeutic approach, necessary due to breeding circumstances and economic implications, is possible because resistance occurs less frequently then in bacteria. Several groups of substances can be used: benzimidazoles and pro-benzimidazoles (*e.g*. albendazole, febantel, flubendazole, triclabendazole), amino-acetonitrile derivatives (*e.g*. monepantel), tetrahydropyrimidines (*e.g*. pyrantel), quinolines (*e.g*. praziquantel), derivatives of phenol and salicylanilides (*e.g*. closantel, nitroxinil, oxyclozanide), imidazothiazoles (*e.g*. levamisole), organophosphates (*e.g*. phoxim), macrocyclic lactones and coccidiostats (1). For benzimidazoles a review was published on the methodology to detect them (2) and more recently a multi-residue method able to detect 88 veterinary drugs in milk, including benzimidazoles, was published. However, no other anthelmintics were included (3). The aim of this study was to develop and validate a multi-residue method for the simultaneous analysis of 23 anthelmintics, including metabolites belonging to the groups mentioned above (excluding macrocyclic lactones and coccidiostats).

Materials and Methods

Reagents and Materials
Levamisole, thiabendazole, oxibendazole, albendazole, fenbendazole, mebendazole, oxfendazole, febantel, triclabendazole, oxyclozanide, morantel, cambendazole, flubendazole, parbendazole, praziquantel, pyrantel pamoate and rafoxanide were from Fluka Vetranal/Pestanal (Sigma-Aldrich, Diegem, Belgium). Closantel, clorsulon, phoxim, nitroxinil, amino flubendazole, triclabendazole sulphone were from Sigma (Sigma-Aldrich). Albendazole sulphone, albendazole sulphoxide, albendazole 2 amino sulphone hydrochloride, oxfendazole-d_3, closantel $^{13}C_6$, nitroxinil $^{13}C_6$, rafoxanide $^{13}C_6$, oxyclozanide $^{13}C_6$, cambendazole d_7, albendazole 2 amino sulphone d_3, febantel d_6, oxibendazole-d_7, mebendazole-d_3, flubendazole-d_3, fenbendazole-d_3, albendazole sulphone-d_3, albendazole sulphoxide-d_3, albendazole-d_3, thiabendazole $^{13}C_6$, praziquantel-d_{11} and triclabendazole-d_3 were from Witega (Berlin, Germany). Netobimin and parbendazole-d_3 were provided by CRL Berlin and oxfendazole sulphone by RIKILT. Hydroxy flubendazole was kindly provided by Elanco. Monepantel sulfone was from Alsachim (Illkirch Graffenstaden, France). Dimethylsulfoxide was from Merck. Methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were LC-MS grade and provided by Biosolve (Valkenswaard, The Netherlands). Ethanol was from BDH (VWR, Haasrode, Belgium). Water (H_2O) was HPLC grade (generated by a Milli-Q Gradient purification system, Millipore, Brussels, Belgium). Filters were Millex-GV 0.22 µm from Millipore.

Individual stock solutions at a concentration of 1 mg mL^{-1} were prepared in an appropriate solvent, being different per compound, after correction for stock purity, and stored at -20°C. Working solutions were freshly prepared in ACN/H_2O (50/50) (10 ng μL^{-1}) or H_2O (1 ng μL^{-1} and 0.1 ng μL^{-1}).

Liquid chromatography and mass spectrometry

The liquid chromatographic system consisted of an Acquity UPLC® system (Waters, Milford, MA). Separation was achieved on an Acquity UPLC BEH C_{18} 2.1 x 100 mm, 1.7 µm column protected by a guard column of the same material. The column was held at 30°C, the injection volume was 5 µL and the eluent flow was at 0.4 mL min^{-1}. The elution was performed gradually with changing amounts of H_2O/ACN (95/5) + 0.3 % FA (A) and H_2O/ACN (5/95) + 0.3 % FA (B). See Table 1 for the gradient.

The mass-spectrometric equipment consisted of a Xevo TQ-S® (Waters) equipped with a Z-spray system. The analytes were determined with tandem mass-spectrometry with one transition in screening mode and at least two transitions for the confirmation mode. Most compounds were measured in electrospray positive ionisation mode, some of them in the negative ionisation mode. The mass spectrometric conditions are shown in Table 2.

For the proficiency test also following compounds, for which validation is in progress were measured: 4-hydroxy tetramisole, cyclobendazole, fenbendazole amine, aminomebendazole, 5-hydroxymebendazole, ketotriclabendazole, triclabendazole sulphoxide, 5-hydroxy-thiabendazole, niclosamide.

Table 1. Programme of gradient elution.

Time (min)	% A	% B
0.0	100	0
2.0	100	0
8.0	70	30
12.0	0	100
13.0	0	100
13.01	100	0
14.6	100	0

Table 2. Overview of precursor ions, product ions, internal standards used and ionisation mode.

Analyte	**Precursor Ion**	**Product Ions**			**IS**	**Ionisation mode**
albendazole	265.87	158.87	191.05	**233.90**	albendazole-d_3	ES^+
albendazole sulphone	297.88	**158.88**	223.84	265.84	albend. sulphon-d_3	ES^+
albendazole sulphoxide	281.86	**158.94**	207.83	239.82	albend. sulphoxide-d_3	ES^+
albendazole-2-aminosulfone	239.98	**78.96**	105.82	132.99	albend.-2-amino sulphon-d_3	ES^+
febantel	446.90	279.85	**382.82**	414.87	febantel-d_6	ES^+
fenbendazole	299.85	130.91	158.86	**267.89**	fenbendazole-d_3	ES^+
oxfendazole	315.84	**158.93**	190.91	283.88	oxfendazole-d_3	ES^+
oxfendazole sulphone	331.90	**130.91**	158.86	299.87	oxfendazole-d_3	ES^+
flubendazole	313.86	**94.96**	122.92	281.89	flubendazole-d_3	ES^+
amino flubendazole	255.99	**94.96**	122.98	132.93	flubendazole-d_3	ES^+
mebendazole	295.87	77.01	104.96	**263.91**	mebendazole-d_3	ES^+
oxibendazole	249.89	147.89	175.91	**217.92**	oxibendazole-d_7	ES^+
triclabendazole	358.73	170.82	273.79	**343.73**	triclabendazole-d_3	ES^+
triclabendazole sulphone	389.97	**310.90**	245.02	149.09	triclabendazole-d_3	ES^-
thiabendazole	201.81	64.99	130.94	**174.90**	thiabendazole-$^{13}C_6$	ES^+
clorsulon	377.68	141.90	241.75	**341.68**	oxfendazole-d_3	ES^-
closantel	660.56	**126.74**	314.75	344.58	closantel-$^{13}C_6$	ES^-
levamisole	204.85	**91.00**	122.87	177.95	oxfendazole-d_3	ES^+
netobimin	420.92	132.99	**152.89**	388.82	oxfendazole-d_3	ES^+
nitroxinil	288.70	115.90	126.89	**161.80**	nitroxinil-$^{13}C_6$	ES^-
oxyclozanide	397.59	175.78	201.72	**361.66**	oxyclozanide-$^{13}C_6$	ES^-
rafoxanide	623.53	**126.74**	344.56	512.46	rafoxanide-$^{13}C_6$	ES^-
pyrantel	206.86	**96.98**	135.87	149.92	oxfendazole-d_3	ES^+
morantel	220.88	110.96	**122.92**	142.88	oxfendazole-d_3	ES^+

Table 2. (continued).

parbendazole	247.84	**144.89**	159.90	215.95	oxfendazole-d_3 (initial) parbendazole-d_3	ES^+
monepantel sulfone	505.96	103.99	**236.86**	478.87	oxfendazole-d_3	ES^+
hydroxyflubendazole	315.95	**96.97**	159.92	283.91	flubendazole-d_3	ES^+
phoxim	299.02	**77.01**	96.91	129.03	oxfendazole-d_3	ES^+
cambendazole	302.56	**189.88**	216.87	260.84	cambendazole-d_7	ES^+
praziquantel	313.15	83.06	174.03	**203.02**	oxfendazole-d_3 (initial) praziquantel-d_{11}	ES^+
Internal standards						
albendazole-d_3	269.06	234.03				ES^+
albendazole sulphone-d_3	301.05	159.00				ES^+
albendazole sulphoxide-d_3	285.05	243.00				ES^+
albendazole-2-aminosulphone-d_3	243.07	133.07				ES^+
febantel-d_6	453.10	383.03				ES^+
fenbendazole-d_3	303.10	268.02				ES^+
oxfendazole-d_3	318.91	158.93				ES^+
flubendazole-d_3	317.06	282.03				ES^+
mebendazole-d_3	299.07	264.05				ES^+
parbendazole-d_3	251.03	216.14				ES^+
oxibendazole-d_7	257.08	225.11				ES^+
triclabendazole-d_3	361.92	273.98				ES^+
thiabendazole-$^{13}C_6$	207.88	180.98				ES^+
praziquantel-d_{11}	324.15	204.17				ES^+
closantel-$^{13}C_6$	666.69	126.80				ES^-
nitroxinil-$^{13}C_6$	294.76	167.85				ES^-
oxyclozanide-$^{13}C_6$	403.78	367.85				ES^-
rafoxanide-$^{13}C_6$	629.74	126.84				ES^-
cambendazole-d_7	310.11	218.02				ES^+

Product ions in bold: transition followed in screening analysis.

Sample preparation and extraction

Four mL of milk was brought into a glass tube. Relevant solutions (internal standards and eventually standards) were added and the sample was equilibrated for 10 min. Six mL of acetonitrile was added and the tube was vortexed during 30 s after which the tubes were centrifuged during 10 min at 1,912 *g*. The upper solution of the extract was transferred to a graduated glass tube. The tube was placed in a water bath at 60°C and the extract was evaporated under nitrogen until 4 mL. The extract was filtered over a 0.22 µm filter and 5 µL of the extract was injected into the LC-MS/MS instrument.

Validation

The method was validated according to Commission Decision 2002/657/EC as a confirmatory method. Following characteristics were determined: specificity, linearity (R^2), recovery (R), repeatability (RSD_r), intra-laboratory reproducibility (RSD_R) and the decision limit (CCα).

Specificity was tested by injecting 20 different blank samples to check if interferences were present in the area of the retention times of the anthelmintics.

To assess linearity, three sets of calibration curves in matrix were analysed in a relevant concentration range, depending on the MRL/MRPL (internal) status. For the recovery three sets of blank milk samples were spiked at 0.5 x, 1 x and 1.5 x MRL and 1 x, 1.5 x and 2 x internal MRPL. The recovery percentages were calculated using TargetLynx software version 4.1 (Waters). The experiments for recovery and linearity were carried out on three days. From these data the coefficients of variation for RSD_r and RSD_R could be calculated at the different concentration levels by means of a one-way ANOVA. CCα was calculated using the data obtained in the recovery experiments at MRL-level, being MRL + 1.64 times the standard deviation.

Participation in proficiency test ANTH1014 (EURL Berlin)
Seven milk samples were send by the EURL Berlin to the participating laboratories. First a screening analysis was performed in which 1 transition per compound was measured together with a negative and a positive control sample spiked at MRL/ internal MRPL. For the confirmatory analyses on the suspect samples, analysis was done measuring minimum two transitions per compound together with a calibration curve in blank matrix.

Results and discussion
A summary of the validation parameters is shown in Table 3.

Table 3. Overview of validation parameters.

Compound	MRL/internal MRPL (µg L^{-1})	Concentration levels (µg L^{-1})	CCα (µg L^{-1})	RSD_r (%)	$RSDR_R$ (%)	Recovery overall (R) (%)	Linear-ity (R^2)
albendazole	Sum 100	12.5 25.0 37.5	27.2 extrapolated : 108.8	3.2	4.4	92	0.9957
albendazole-2-amino sulphone		25.0 50.0 75.0	56.0 extrapolated : 112.0	4.8	5.7	100	0.9980
albendazole sulphone		12.5 25.0 37.5	28.8 extrapolated : 115.1	4.4	9.2	96	0.9887
albendazole sulphoxide		12.5 25.0 37.5	28.1 extrapolated : 112.5	4.1	9.3	98	0.9958
aminoflubendazole	5*	5.0 7.5 10.0	5.6	6.8	9.8	101	0.9943
cambendazole	7.5*	7.5 11.25 15.0	7.9	3.2	3.6	102	0.9985
clorsulon	16	8.0 16.0 24.0	17.9	7.7	8.8	86	0.9938
closantel	45	22.5 45.0 67.5	52.9 (47.5 on Xevo TQ-MS)***	7.9	8.5	98	0.9937
febantel	10	5.0 10.0 15.0	11.1	4.2	6.1	101	0.9980
fenbendazole	10	5.0 10.0 15.0	10.6	3.1	4.3	102	0.9980
flubendazole	5*	5.0 7.5 10.0	5.6	4.0	5.5	98	0.9980
levamisol	5*	5.0 7.5 10.0	6.2	9.6	14.2	98	0.9935
mebendazole	5*	5.0 7.5 10.0	5.7	5.7	7.5	100	0.9977
monepantel sulfon	170	170.0 225.0 340.0	202.3	11.7	13.8	90	0.9945
morantel	50	25.0 50.0 75.0	68.5	23.4	24.9	110	0.9808
netobimin (see marker for albendazole; here values for netobimin itself))	100	25.0 50.0 75.0	66.2 extrapolated: 132.4	9.1	15.7	100	0.9903

Table 3. (continued).

Compound	MRL/internal MRPL (µg L^{-1})	Concentration levels (µg L^{-1})	CCα (µg L^{-1})	RSD_r (%)	$RSDR_R$ (%)	Recovery overall (R) (%)	Linearity (R^2)
nitroxinil	20	10.0 20.0 30.0	39.2 (21.7 on Xevo TQ-MS)***	40.7	48.6	96	0.9674
oxfendazole	Sum 10	5.0 10.0 15.0	10.6	2.6	3.5	104	0.9982
oxfendazole sulphone		5.0 10.0 15.0	10.6	3.3	3.8	100	0.9989
oxibendazole	1*	1.0 1.5 2.0	1.1	4.9	6.4	98	0.9946
oxyclozanide	10	5.0 10.0 15.0	11.6	8.3	9.4	103	0.9892
parbendazole *(I.S. oxfendazole-d_3)*	30*	30.0 45.0 60.0	35.4	8.2	13.1	99	0.9883
parbendazole *(I.S. parbendazole-d_3)*	30*	30.0	31.9	-	-	-	
phoxim	50*	50.0 75.0 100.0	61.0	11.4	12.3	93	0.9842
praziquantel *(I.S. oxfendazole-d_3)*	20*	20.0 30.0 40.0	21.1	4.6	5.2	99	0.9946
praziquantel *(I.S. praziquantel-d_{11})*	20*	20.0	21.9	-	-	-	
pyrantel	50*	50.0 75.0 100.0	64.3	18.0	25.3	93	0.9934
rafoxanide	10**	10.0 15.0 20.0	12.2	10.3	11.3	100	0.9908
hydroxyflubendazole	20*	20.0 30.0 40.0	22.2	6.8	7.3	106	0.9910
thiabendazole	Sum 100	50.0 100.0 150.0	110.5	4.0	7.9	104	0.9490
triclabendazole	Sum 10	5.0 10.0 15.0	10.9	4.1	5.8	103	0.9936
triclabendazole sulphone		5.0 7.5 10.0	7.0 extrapolated: 14.0	22	26.2	77	0.9609

: internal MRPL; **: initially validated as internal MRPL; in the meantime MRL of 10 µg L^{-1} was set; *: in negative electrospray better results are obtained on a Xevo TQ-MS (in practice final analysis for those compounds will be done on Xevo TQ-MS*

No peaks were detected in blank samples that could give rise to false non-compliant samples (for all substances < 1 µg L^{-1})

It is advisable to schedule some solvent injections after a standard mixture and after the positive control samples to eliminate the presence of peaks by carry-over (so-called memory effects).

For the proficiency test ANTH1014, 25 of the 38 laboratories which had carried out a confirmatory analysis, passed the proficiency test by using LC-MS/MS methods (4). The test passed when 10.0 out 15.0 points were obtained. We obtained a score of 13.0 out of 15.0 (87%). The percentages for the laboratories passing the test varied from 67 to 100% (4).

An overview of the results of our laboratory is shown in Table 4.

Table 4. Summary of results obtained in proficiency test ANTH1014.

Sample number	Compounds detected in screening	Compounds confirmed + quantification (µg L^{-1}) (n=2)	Z-score
0071	closantel	closantel (51.01; 49.06)	0.2
0169	fenbendazole	fenbendazole (1.40; 1.34)	0.0
	oxfendazole sulphone	oxfendazole sulphone (15.36; 16.76)	-0.4
	oxfendazole	oxfendazole (5.50; 5.49)	0.0
		fenbendazole sum (22.69; 24.01)	0.7
	febantel	febantel (5.56; 5.59)	no z-score calcultated
0226	-	-	Blank sample
0372	albendazole	albendazole (0.23, 0.20)	-1.3
	albendazole sulphone	albendazole sulphone (54.34; 54.59)	0.2
	albendazole sulphoxide	albendazole sulphoxide (143.23; 140.35)	1.8
	albendazole-2 aminosulphone	albendazole- 2 aminosulphone (0.24; 0.23)	-1.9
		albendazole sum (183.80; 181.30)	1.5
0402	ketotriclabendazole	ketotriclabendazole (0.87; 0.73)	-1.0
	triclabendazole	triclabendazole (12.14; 12.00)	1.3
	triclabendazole sulphone	triclabendazole sulphone (89.85; n.a.)	1.5
	triclabendazole sulphoxide	triclabendazole sulphoxide (27.80;n.a.)	-1.8
		triclabendazole sum (112.05; 111.78)	1.8
	levamisole	levamisole (11.04; 10.87)	-1.2
0403	rafoxanide	rafoxanide (20.00; 21.80)	-0.8
0580	ketotriclabendazole		
	triclabendazole	triclabendazole (3.90; 3.05)	1.3
	triclabendazole sulphone	triclabendazole sulphone (19.43; n.a.)	1.3
	triclabendazole sulfphoxide	triclabendazole sulphoxide (6.98 ; n.a.)	0.3
		triclabendazole sum (26.06; 25.28)	1.1
	levamisole	levamisole (9.05; 8.49)	-0.7

n.a.: not analysed

Conclusions

A fast, specific and easy to perform multi-residue LC-MS/MS method was developed and validated for the detection of anthelmintics in milk. The method can be used in practice as a screening tool and in case of a suspect sample a second injection on minimum two transitions can be performed. The applicability of the method was shown by obtaining good results in the proficiency test ANTH1014 organised by the EU-RL Berlin.

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B ZERO CAP TOTAL: A NEW MASTER-CURVE CALIBRATED IMMUNOASSAY FOR THE DETECTION OF CHLORAMPHENICOL IN MILK

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Abstract
Chloramphenicol (CAP) is an antibiotic whose use has been banned in food-producing animals due to serious health risks for consumers. In order to verify the presence of CAP residues in animal matrices, a number of immunoassays have been available on market for decades, having suitable limits of detections for many matrices in compliancy to EU MRPL of 0.3 ppb. They are all characterized by the presence of a calibration curve to be implemented every session.
Tecna's aim was to develop a new, reliable and cost-effective kit with no need to run any calibration apart the zero standard. The removal of calibrators allows analysts to save wells and to exploit the whole kit for the analysis of routine samples.
B ZERO CAP *total* is an enzyme immunoassay for the detection of CAP in animal matrices in 60 min. The sample quantification is granted by a batch-related master-curve in the range 0.025 – 2.00 ng mL^{-1}. The direct analysis of defatted milk is possible. CCβ was validated according to EU 657/2002 at 0.05 ppb with no false negative determinations.

Introduction
Over the last decades, safety and economical concerns have been the main driving forces behind the development of rapid test kits for drug residue screening in food and feedstuffs. Such screening methods are mainly based on binding reactions occurring in lateral flow devices or enzyme-linked immunosorbent assays (ELISAs). While the first assays are characterized by extreme ease of use and quickness, the latter lead to more reliable, robust and sensitive results. ELISA kits are therefore widespread among industries and control laboratories as routine analytical tools to verify the compliancy of goods to production requirements or regulations, thus reducing the number of samples to be submitted to expensive, time-consuming instrumental analysis.

For low-medium analytical throughput, anyway, ranging from a couple to a dozen samples per time, ELISA kits could not be so cost-effective. Since a calibration curve must be run every session in order to calculate sample concentration, a number of wells must be spent for calibrators all the times. Such cost becomes irrelevant only when the laboratory has the possibility to collect a high number of samples and exploit the whole kit in one single session.

Tecna's aim was to provide small laboratories and industries with ELISA test kits with no need to run any calibrator apart from the "zero" standard. Thanks to the reduction of the wells that is required for the assay calibration, the cost per sample drops irrespective of the throughput. Besides, the removal of calibrators shows the additional advantage to reduce operative mistakes that can occur when manipulating the standards (*i.e*. calibrators mismatch, contamination) and solves the issue related to standards stability.

Chloramphenicol (CAP) is a broad-spectrum antibiotic whose use has been banned in food-producing animals in the European Union due to its severe side effects on both animal and human health. In the present work, Tecna first developed a novel classical ELISA kit for the quantitative detection of CAP in animal matrices, I'screen CAP total. Once all matrices were validated, the feasibility of a master-curve calibrated version of the assay (batch mean standard curve) was verified by comparison of results between the two ELISA assays. The master-curve calibrated kit, named *B ZERO* CAP *total*, was validated in terms of specificity, sensitivity, accuracy and precision.

Materials and Methods

ELISA reagents
Plastic microtiter plate was coated with anti-CAP antibodies. CAP standard solutions were prepared in amber plastic vials, containing 0, 0.025, 0.075, 0.25, 0.8 and 2 ng mL^{-1} CAP in assay buffer. A proper 0 standard was developed for milk analysis only. CAP-HRP enzyme conjugate (horseradish peroxidase) was prepared as 100x concentrated, to be diluted in its buffer every session. Sample dilution buffer was prepared 5x concentrated. ELISA washing buffer was prepared 10x concentrated. Both developing and stop solution were ready-to-use.

ELISA procedure and result calculation
Reagents were brought at room temperature. 50 µl of each standard or sample were added in the corresponding well. A volume of 50 µl enzyme conjugate was added. Each standard/sample was run in duplicate. The reaction was incubated 30 min at room temperature. The plate was washed with the assay washing buffer by means of a squeeze bottle for a total amount

of four washing cycles. All the wells were filled with 100 μl developing solution and the reaction was incubated 30 min at room temperature. A volume of 50 μl stop solution was then added. The absorbance of each well was measured within 30 min at 450 nm (Tecan Infinite F50 ELISA plate reader).

The mean absorbance (OD) of standards and samples were obtained. Mean OD values were related to that of 0 standard to get the B/Bo (%) result. The calibration curve was obtained by drawing standard B/Bo values of CAP standard solutions versus known concentrations onto a semi-logarithmic system of coordinates. Similarly, sample OD values were related to the mean absorbance of the milk 0 standard and the CAP concentration was obtained by interpolating their B/Bo onto the calibration curve.

Sample preparation

20 blank raw *bovine* milk samples were collected in the region Friuli Venezia Giulia (Italy) and analysed within 48 h after milking. Milk samples were centrifuged at 3,000 *g* for 10 min at 4°C. The fat was discarded and the samples were directly tested in the assay. Since the dilution factor is 1, the measuring range in the sample is 0.025 – 2.00 ppb.

I'screen CAP total milk validation

The assay was validated according to EU regulation 657/2002. Blank milk samples were analysed in order to determine the limit of decision (CCα) of the assay. The detection limit (CCβ) was obtained by spiking samples with different amounts of CAP. Accuracy of the assay was investigated in the range of 0.05-0.3 ppb. Precision was evaluated under repeatability (intra-assay coefficient of variation, CV%) and reproducibility (inter-assay CV%) conditions.

B ZERO CAP total development and validation

A batch master-curve was established as the mean value of *n* = 6 curves of *I'screen CAP total*. The validation data collected were recalculated by interpolation of samples B/Bo value onto the master-curve, in order to evaluate specificity, sensitivity, accuracy and precision of *B ZERO CAP total*.

Results and Discussion

I'screen CAP total milk validation

Blank samples were prepared according to the procedure described in the "Materials and Method" chapter within two independent sessions. The mean B/Bo (%) was 93 ± 5% (*n* = 40 determinations). Blank samples were measured as < 0.025 ppb CAP with 90% specificity. It was therefore not necessary to set any limit of decision.

The same materials were spiked at 0.05 and 0.1 ppb of CAP. Both levels were measured by the assay with no false negative determination, obtaining 100% sensitivity. Assay CCβ was therefore set at 0.05 ppb CAP. The mean B/Bo of 0.05 and 0.1 ppb spiked samples were 72 ± 4% and 64 ± 3% (*n* = 40), respectively, thus guaranteeing 21 and 29 points of average difference in the B/B0 ratio from blanks respectively. The mean recovery at 0.05 ppb spiking level was 124 ± 30%. The mean recovery at 0.1 ppb spiking level was 118 ± 20%. The comparison of B/Bo data between blank and spiked materials is shown in Figure 1.

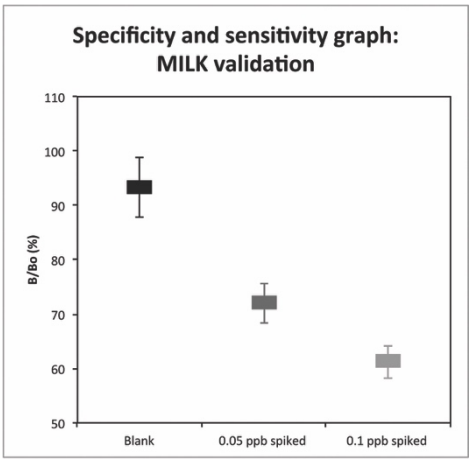

Figure 1. Comparison between blank and spiked milk samples mean B/Bo (%). Mean B/Bo value (n=40) of blank, 0.05 ppb and 0.1 ppb spiked milk samples are represented as rectangles. Bars show the standard deviations.

Three randomly selected samples were spiked at MRPL (0.3 ppb) and run in five replicates each. The mean recovery turned to be 109 ± 11% (*n* = 15 determination). The mean intra-assay coefficient of variation (CV) was 7%, the mean inter-assay CV was 8%.

I'screen CAP *total* turned to be a reliable test kit for the quantitative detection of CAP in milk. Thanks to the establishment of a proper milk 0 standard, no sample preparation was necessary. The lower is the sample handling, the higher is the reproducibility (Biancardi *et al*., 2012).

B ZERO CAP total set up

Six curves obtained with *I'screen* CAP *total* in six analytical sessions were used to set the *B ZERO kit* master-curve. The mean CV of B/Bo values of CAP standard solutions was 6%. Such a high reproducibility of standard curves is the absolute condition to have a master-curve calibrated ELISA kit, in order to assure accuracy of results. B/Bo values and curves are shown in Table 1 and Figure 2.

Table 1. Standards B/Bo values obtained in six sessions (Roman numbers). For each CAP standard solution mean B/Bo, SD and CV value was obtained.

Concentration (ng mL^{-1})	I.	II.	III.	IV.	V.	VI.	Mean	SD	CV (%)
0.025	87	81	84	79	81	85	83	3	4
0.075	71	66	66	68	64	72	68	3	5
0.25	49	43	44	46	42	44	45	3	6
0.8	29	25	26	26	25	30	27	2	8
2.0	18	16	15	17	15	18	16	1	7

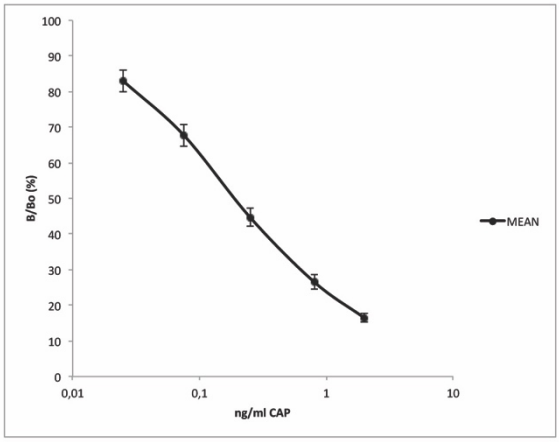

Figure 2. Mean curve obtained as the master-curve for B ZERO CAP total.

B ZERO CAP total milk validation

B/Bo values obtained for blank and spiked milk samples with *I'screen* CAP *total* were interpolated onto the master-curve. No differences were noticed in terms of α and β error, since assay specificity was 90% and CCβ was confirmed at 0.05 ppb, with 100% sensitivity and a mean recovery of 114 ± 14% (*n* = 40 determinations). The mean recovery at 0.1 ppb spiking level was 91 ± 16% (*n* = 40 determinations).

Mean recovery for 0.3 ppb spiked samples was 90 ± 10% (*n* = 15 determinations). The mean intra-assay CV remained 7%, the mean inter-assay CV 8%.

By correlating together all the data collected during the kits validation, a linear correlation was obtained between *I'screen CAP total* and *B ZERO CAP total*, having R^2 value 0.995 (Figure 3).

Student's t-test showed no differences between values at 0.05 ppb (CCβ spiked milk sample), while both at 0.1 and 0.3 ppb data were found to be statistically different. The mean dosage of 0.1 ppb spiked milk samples was found 0.12 ± 0.02 ppb (*n* = 40) with *I'screen* CAP *total* and 0.10 ± 0.02 ppb (*n* = 40) with *B ZERO* CAP *total* indeed. The mean dosage of 0.3 ppb spiked milk samples showed some more difference, since *I'screen* CAP *total* led to 0.33 ± 0.03 ppb (*n* = 15) while *B ZERO* CAP *total* to 0.27 ± 0.03 ppb (*n* = 15). This lower correspondence of results at higher CAP levels leads to a correlation with 0.83 slope, but it does not affect the overall reliability of *B ZERO* CAP *total*. Considering that CAP is a banned substance, it was assumed that the achievement of same specificity and sensitivity was the most important aspect.

Moreover, *B ZERO* CAP TOTAL is a cost-effective and easy-to-use ELISA test kit for the quantitative detection of CAP. The comparison of well consumes between the classical assay format and the novel *B ZERO* one is shown in the example in Figure 4. For the analysis of three milk samples, the classical ELISA requires an overall expense of twelve wells for calibrators, two

wells for the milk 0 standard and six wells for samples, for a total of 20 wells. By means of B ZERO CAP total the wells needed are only eight, corresponding to 60% wells saving.

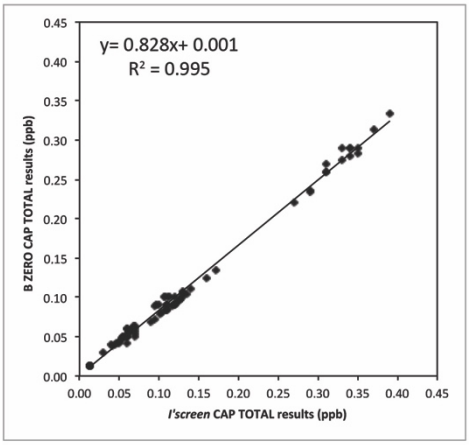

Figure 3. Correlation between results obtained with I'screen CAP total and master-curve calibrated B ZERO CAP total (n = 105 determinations per kit). Data included all the spiking experiments.

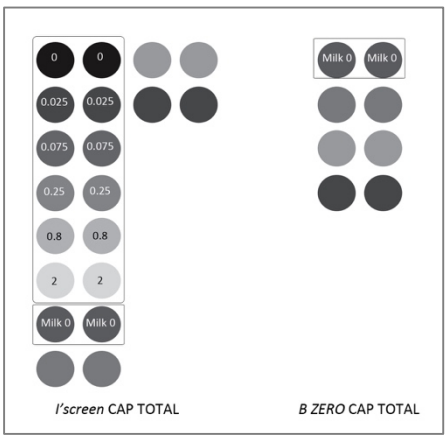

Figure 4. Comparison of the test lay out for analysis of three milk samples between I'screen CAP total and B ZERO CAP total.

Other matrices validation and ruggedness assessment

Both *I'screen* and *B ZERO* CAP *total* were validated for urine, muscle, seafood and honey analysis as well. In direct analysis of swine urine with no digestion, a CCβ was set at 1 ppb for both CAP and its metabolite CAP glucuronide. A solvent extraction procedure was applied for tissue and honey. The CCβ at 0.1 ppb for swine and *bovine* muscle and shrimp and 0.15 ppb for honey.

Since the design of *B ZERO* CAP *total* is based on the assumption of reagents ruggedness, a final investigation was arranged in order to verify the effect of temperature onto *I'screen* and *B ZERO* performances. ELISA-binding reactions are sensitive to temperature changes indeed. The effect of using cold reagents (just taken out of the fridge) and incubating at 35°C (thus simulating a possible summer laboratory environment) was studied when analysing a 1 ppb CAP and 1 ppb CAP glucuronide spiked urine sample. No significant differences were found when both thermic stresses were applied, thus demonstrating strong robustness of both test kits.

Conclusion

Two new ELISA kits for CAP determination in samples of animal origin were developed. The first one was a classical ELISA kit, while the second was a master-curve calibrated assay derived from the first. Thanks to reagents robustness that led to comparable accurate and reproducible validation results between the two assays, it was demonstrated that it is not necessary to run a calibration curve every analytical session. The master-curve calibrated kit, named *B ZERO* CAP *total*, had same specificity, sensitivity and precision as the classical ELISA kit.

Both test kits are therefore suitable for official control bodies and industries for analysing a number of animal matrices at level below or equal to EU MRPL. The advantage of *B ZERO CAP total* is its easy-to-use and the low cost required for analysis in case of a small number of samples.

Acknowledgements
Gratitude to Barbara Moretti (Latteria sociale di Cividale e Valli del Natisone), Paola Marangoni and Silvia Ambrosoli (G.B. Ambrosoli S.p.A.) for kindly providing the samples for test kit validation.

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RESIDUE DEPLETION OF IVERMECTIN IN CHICKENS

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Abstract

Helminth infections are widely spread in the poultry industry. This may stimulate extralabel use of some drugs, such as ivermectin (IVM). Pharmacokinetic and residual studies of IVM in poultry, however, are scarce. Our aim was to determine time restrictions for broiler chickens fed with balanced feed mixed with IVM for 21 days with respect to residue levels in edible tissues.

Sixty chickens of one-day old were fed with feed supplemented with IVM at 5 mg kg^{-1} feed for 21 days. Groups of six treated animals were sacrificed at 0, 1, 2, 4, 8, 10, 15, 20 and 28 days after the end of the treatment. Liver, skin/fat, kidney and muscle samples were obtained. IVM was determined by liquid-chromatography with fluorescence detection after automatic solid phase extraction with SPE C18 cartridges.

The highest concentrations were measured in the liver, which is logical given that IVM is a molecule that undergoes extensive hepatic metabolism. The optimal withdrawal time for edible tissues of these animals are within the permitted residual levels were: liver 12 days to 8 days for skin/fat and 10 days for kidney.

Introduction

Domestic birds are frequently affected by internal parasites (*Capillaria* spp., *Ascardia* spp, *Heterakis gallinarum*, *Syngamus trachea)* and external parasites (*Dermanyssus gallinae*, scabies mites -*Cnemidocoptes mutans*-, fleas -*Ceratophyllus gallinae*- and some ticks -*Argas persicus* (Sainsbury, 1987; Sharma & Bhat, 1990; Bennett & Cheng, 2012). Another ectoparasite of great economic impact on the poultry farm is the beetle poultry litter, *Alphitobius diaperinus*, with a significant negative impact on performance and production of birds. Currently, formulations based on ivermectin (IVM) for the treatment of parasitic infections of birds are available in the market, but still there are no studies on the profile of tissue depletion of these molecules in chickens destined to human consumption.

Ivermectin (IVM) is a macrocyclic lactone, endectocide of wide spectrum, widely used for treatment and prevention of internal and external parasites in food-producing animals. IVM efficacy has been demonstrated in the treatment of *Ascardia* spp. and *Capillaria* spp. in pigeons (Schepkins *et al*., 1985). It is a very lipophilic molecule, so its residues remain for long periods in the treated animal tissues, especially those with a high fat content (Baynes *et al*., 2000).

Residue studies are of fundamental importance in public health. Consumer safety is based on a series of measures including maximum residue limits (MRLs) and acceptable daily intakes as the most important. There are no established MRLs for edible tissues of chicken, but the European Union set the MRLs in edible tissues of food-producing mammals as follows: 100 ng g^{-1} in liver and fat, and 30 ng g^{-1} in kidney. Extrapolation of MRLs to the relevant minor species has been considered.

The aim of this study was to determine restriction periods for broiler chickens fed with balanced feed mixed with premix containing IVM for 21 days, in order to establish acceptable residue levels for human consumption according to the European Union regulations.

Materials and Methods

Study Design Treatment and Administration

Sixty one-day old BB chickens were fed with a prestart and initiator feed supplemented with IVM at 5 mg kg^{-1} for 21 days. This means that if in 21 days, a chicken consumes 1 kg of feed during the same time, it also consumes 5 mg of IVM (238 µg day^{-1}). The chickens treated with IVM were euthanized in groups of 6 animals at the following times post-treatment: 0, 1, 2, 4, 8, 10, 15, 20 and 28 days. The last time point corresponds with the endpoint for commercials purposes. Six chickens used as control (free from IVM) were euthanized before experiment. Immediately, after slaughter liver, skin-fat, muscle and kidney samples were collected. Each sample was properly conditioned, placed in plastic bags, heat sealed, labelled and stored at -20°C until assay.

The animal experiment protocol was in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS).

Reagents

Ivermectin (IVM) standard was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC grade and purchased from JT Baker (Phillipsburg, NJ, USA). *N*-methylimidazole and trifluoroacetic anhydride were from

Sigma-Aldrich Chemical Company (USA). Solid phase extraction (SPE) columns (Strata, C_{18}, 100 mg, 1 mL) and analytical column (Kinetex C_{18}) were obtained from Phenomenex (USA).

Ivermectin analysis

Analysis of Ivermectin in tissues was performed by high-performance liquid-chromatography (HPLC) with automated solid phase extraction and fluorescence detection. Tissue samples (muscle, liver, kidney and skin plus fat) were thinly sliced and 2 g were homogenized in 1 mL of acetonitrile (Ultra Turrax T_{25} basic, IKA). The homogenate was mixed 20 min, sonicated 10 min (Ultrasound Bath) and centrifuged at 2,000 *g* for 10 min at 4°C. The clear supernatant was transferred to a new tube and the extraction procedure was repeated once again. The total supernatant obtained was placed on the appropriate rack of Aspec XL automatic solid phase extraction apparatus (Gilson, France). Automatic sample preparation was performed using SPE C_{18} cartridges (Strata C_{18}, 100 mg, 1 mL, Phenomenex), which were conditioned with 2 mL of methanol, and followed by 2 mL of water HPLC quality. All samples were applied to cartridges, washed with 1 mL of water and 1 mL of methanol/water (25%), dried with air during 2 min, and finally eluted with 3 mL of methanol HPLC grade. The eluted volume was evaporated at 60°C to dryness in a vacuum concentrator (AVC 2-25CD Christ, Germany). Fluorescent derivative was obtained by dissolving the dry residue obtained in 100 µL of *N*-methylimidazole / acetonitrile (1:1, v/v) and 150 µL of trifluoroacetic anhydride / acetonitrile (1:2, v/v).

Standard curve

Standards were prepared by adding 0.5, 1, 2.5, 5, 10, 20 and 30 ng of IVM to test-tubes, evaporating to dryness at 60°C and dissolving and derivatising as described above.

Apparatus

The chromatographic system consisted of an isocratic pump (Gilson Inc. 307), an automatic injector (Gilson Inc. 234), a FluoroMonitor IM III Detector (Sp Thermo Separation products) and Eppendorf CH-30 Column Heater (set at a 30°C). The system is controlled through the Unipoint ® Gilson system. An C_{18} column (Kinetex, 2.6 µm, 4.6 mm x 100 mm; Phenomenex, Torrance, CA, USA) was eluted with a mixture of acetic acid 2%, methanol, acetonitrile (4:32:64) at a flow rate of 1.5 mL min^{-1}. Identification of IVM in bird tissues was accomplished by comparison with the retention times of the reference standards. The precision of the extraction procedure and chromatography technique was evaluated by processing as replicates in six different occasions, aliquots of pooled different tissue samples containing known amounts of IVM.

Method validation

The following parameters were evaluated for the analysis of each matrix: linearity (concentrations of ivermectin ranging between 0.5 and 30 ng g^{-1}), precision and accuracy, limit of quantitation (LOQ), limit of detection (LOD) and selectivity. The mean accuracy (% recovery) should be within the range 85-115% and the variation in precision should be ≤ 20%. The LOD was estimated through the analysis of 10 aliquots of control tissue (free of IVM). The noise of the base-line was measured; the average and the standard deviation were calculated, the LOD corresponds to three of those SD (signal-to-noise ≥ 3/1). The LOQ is defined as the level where the reproducibility of the replicate analysis does not exceed a variation coefficient of 20% and the accuracy is from 85-115% after the analysis of 12 replicates of fortified sample matrix with the lowest concentration.

Withdrawal time

The withdrawal periods for edible tissues of chickens (muscle, liver, kidney and skin plus fat) were estimated by linear regression analysis of the log transformed tissue concentrations and determined at the time when the upper one-sided 95% tolerance limit for the residue was below the MRLs, with a confidence of 95% (EMEA, 2002). Ivermectin concentrations in function of time found in muscle, kidney, liver and skin/fat were plotted and analysed with the program WT version 1.4 in order to recommend a period of withdrawal time for this experimental formulation.

Results

This method performed accurately and reproducibly over a range of 0.5 - 30 ng g^{-1} for IVM. The linearity was between r = 0.9913 to 0.9975 values in all tissues assayed. The chromatographic analysis time was short and IVM was presented in 2 min as a sharp and symmetrical peak with no interfering peaks (Figure 1).

The specificity of the method was demonstrated by the absence of interferences and the adequate symmetry of chromatograms. The extraction efficiency of the analyte was determined by comparison of the peak areas from fortified blank samples with the peak areas from direct injections of equivalent quantities of standards.

Precision and accuracy (intra- and inter assay) of the method were determined by evaluation of replicates of drug-free samples (n = 6) fortified with IVM at different concentrations (0.5, 5.0, 20 ng g^{-1}). The percentage of recovery (accuracy) was

87.4%, 81.3%, 85.2% and 83.7% in liver, kidney, muscle and fat, respectively, with a CV of 3.0, 1.9, 0.64 and 2.3%, respectively. The limit of detection (LOD) was established in 0.65, 0.84, 1.0 and 0.60 ng g^{-1} for skin + fat, liver, kidney and muscle sample, respectively. The limit of quantitation (LOQ) was 1 ng g^{-1} for muscle, liver and skin + fat, and 2 ng g^{-1} for kidney.

The validated analytical methodology showed satisfactory results of sensitivity, precision and accuracy that allow its use for the detection and quantification of tissue residues of IVM in chickens (Table 1).

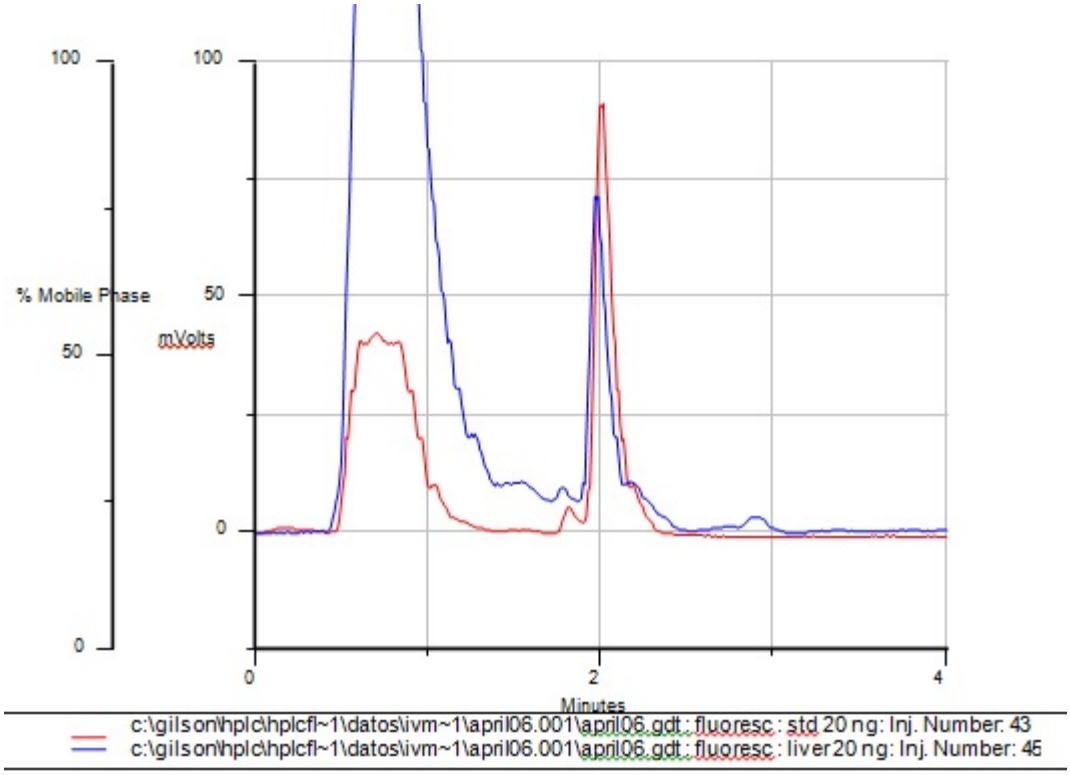

Figure 1. HPLC Chromatograms of IVM standard solution and liver spiked with 20 ng g^{-1} of IVM.

Table 1. Ivermectin recovery from tissue samples fortified with 20 ng of IVM.

	Liver			Kidney			Muscle			Fat		
ng g^{-1}	ng g^{-1} calculated	Accuracy (%)	RSD %	ng g^{-1} calculated	Accuracy (%)	RSD %	ng g^{-1} calculated	Accuracy (%)	RSD %	ng g^{-1} calculated	Accuracy (%)	RSD %
20	18.25	91.25	-8.75	16.85	84.25	-15.75	17.15	85.75	-14.25	16.85	84.25	-15.75
20	17.65	88.25	-11.75	16.24	81.2	-18.8	16.86	84.3	-15.7	16.32	81.6	-18.4
20	16.85	84.25	-15.75	16.09	80.45	-19.55	16.98	84.9	-15.1	17.01	85.05	-14.95
20	17.86	89.3	-10.7	16.21	81.05	-18.95	17.12	85.6	-14.4	16.25	81.25	-18.75
20	17.24	86.2	-13.8	16	80	-20	17.09	85.45	-14.55	16.77	83.85	-16.15
20	17.09	85.45	-14.55	16.13	80.65	-19.35	16.98	84.9	-15.1	17.22	86.1	-13.9
Mean	**17.49**	**87.45**	**-12.55**	**16.25**	**81.27**	**-18.73**	**17.03**	**85.15**	**-14.85**	**16.74**	**83.68**	**-16.32**
SD	**0.52**	**2.62**	**2.62**	**0.30**	**1.52**	**1.52**	**0.11**	**0.55**	**0.55**	**0.38**	**1.91**	**1.91**
CV	**3.00**	**3.00**	**-20.87**	**1.87**	**1.87**	**-8.13**	**0.64**	**0.64**	**-3.69**	**2.29**	**2.29**	**-11.73**

IVM tissue concentrations

In Figure 2 the mean tissue concentrations of IVM in muscle, kidney, liver and skin + fat at different days after the end of treatment are presented. The residue levels were low, with the highest concentration measured between the first and second day post-treatment in all tissues. The tissue with the highest concentration was liver, followed by skin + fat, kidney, and muscle. The higher concentrations were found in liver tissue (Figure 2), which is expected since IVM is a molecule that undergoes extensive hepatic metabolism, mainly by hydroxylation processes.

Linear regression analysis of the logarithmic transformed data can be considered for the calculation of the withdrawal periods. Using this approach, the withdrawal time is determined as the time when the one-sided, 95% upper tolerance limit of

the regression line with a 95% confidence level is below the MRL. According to the residue concentrations found, optimal withdrawal times for edible tissues were 12 days for liver, 8 days for skin / fat and 10 days for kidney.

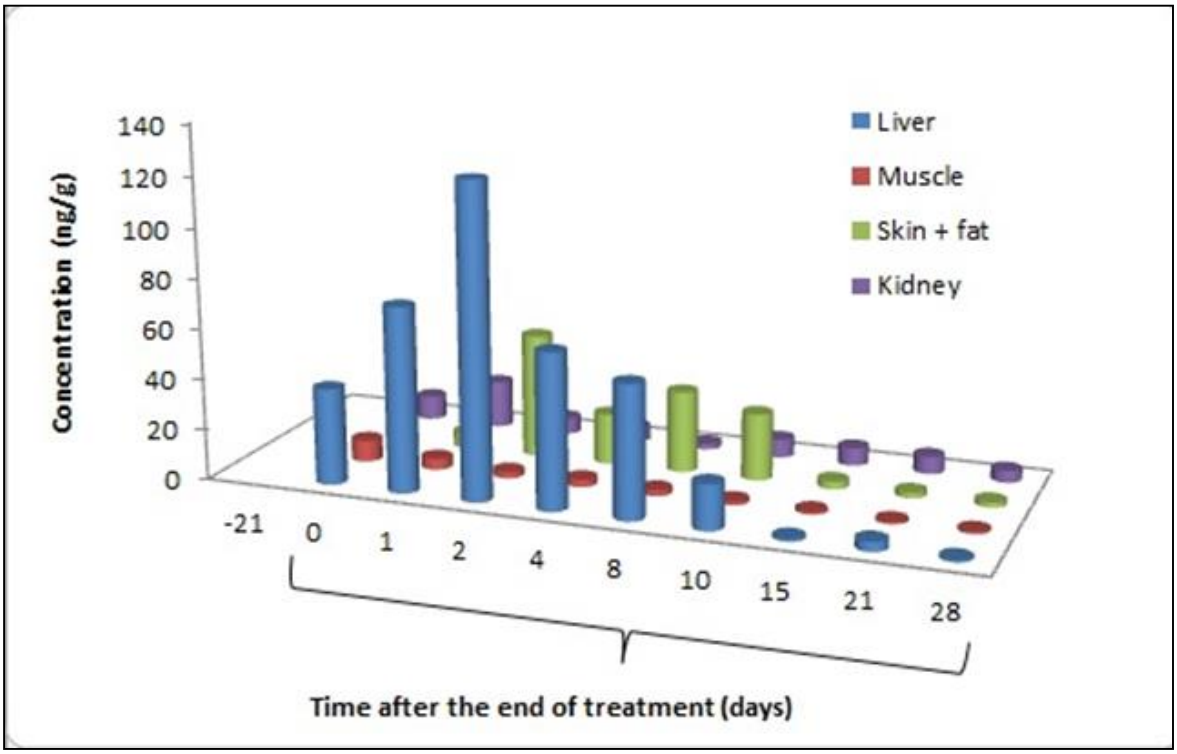

Figure 2. Mean tissue concentrations of ivermectin in chickens after oral administration of IVM during 21 days with the food

Discussion and conclusions

The available information reporting tissue residue profiles after IVM treatment is scarce in poultry compared to other species. Macrolide endectocides, as ivermectin, are lipophilic compounds, so high concentrations are expected in edible tissues, particularly in those with a high fat content. Our results do not match with Miller (1990), who administrated IVM to chickens with a diet fortified at 2 μg g^{-1} of feed for 5 weeks and who found no residues of IVM in the livers. So, this author was not able to establish any withdrawal period. The study performed by Miller (1990) was mainly on the IVM efficacy against *Alphitobius diaperinus*, not on the tissue profile depletion of IVM. In Miller's study, experimental animals were sacrificed at the end of the treatment, *i.e*. week 5, and therefore determined IVM residues in liver only at this sampling time point.

According to the regulations of the European Union, chickens fed for 21 days with this type of supplement containing ivermectin at 5 mg kg^{-1} are suitable for human consumption at 12 days post-treatment, *i.e*. at 33 days of age in this case. Considering that broiler chickens are 45-54 days old when destined for the market, it can be stated that with the dosage scheme proposed in our study, the chickens would indeed be suitable for human consumption. These results suggest that the IVM withdrawal period of growing chickens should be shorter than that of mammals, but pharmacokinetic studies need to be performed to test this hypothesis.

Acknowledgements

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NOVEL DESIGN AND SYNTHESIS OF CHLORAMPHENICOL INMUNOREAGENTS FOR THE ANALYSIS OF BIOLOGICAL SAMPLES

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Abstract
Chloramphenicol is a broad-spectrum antibiotic used in the food industry to treat multiple infections in cattle. The use of chloramphenicol is completely banned according to the European commission legislation establishing a minimum required performance limit of 0.3 ppb. Although several techniques have been described for its monitoring, an accurate and reliable method is necessary as a screening tool in order to select the samples that have to be analysed further. Immunochemical methods have proved themselves valuable as screening tools for the monitoring of different pollutants. In this work, an indirect competitive ELISA for the detection of chloramphenicol (CAP) in milk samples is described. Two immunizing haptens were synthesized, which thus maximise different specific epitopes on CAP. With generated antisera and using the prepared competitor, several combinations with good detectability were obtained. One combination giving an LOD of 0.004 ± 0.003 µg L^{-1} in buffer was chosen for to design an ELISA, which was validated according to the Commission Decision 2002/657/EC criteria established for qualitative screening methods. The ELISA developed showed a CCα and CCβ of 0.079 ± 0.049 µg·kg^{-1} and 0.148 ± 0.102 µg·kg^{-1} in milk, respectively. No relevant interferences from structurally related compounds, such as florfenicol or thiamphenicol, were observed.

Introduction
Overuse of antibiotics in the food industry to prevent diseases and improve productivity has led to increase of bacterial resistance (1). Non-desirable effects of antibiotics on human health, along with the antibiotic-resistant bacteria that could infect humans through the food-chain or by contact with animals, together with the harmful impact of antibiotics on the environment, are serious reasons to consider strict control and continued revisions of the use of antibiotics in the food-industry.

The case of chloramphenicol is not least because of its serious effects in the environment, especially in aquatic ecosystems (2), and in human health. Chloramphenicol is a broad-spectrum antibiotic used to treat multiple infections such as urinary and digestive infections, being effective against many bacteria, both gram-negative and gram-positive, as well as a large number of anaerobic organisms. However, the abuse of this antibiotic can cause many problems for human health. One of the most serious adverse effects in human health associated with the use of chloramphenicol is bone marrow toxicity. This process may occur in two distinct forms: i) bone marrow suppression, which is usually reversible, and ii) aplastic anaemia, which is generally fatal.

As a response, EU government Council Regulation 2377/90 was implemented to establish a maximum residue level of veterinary drugs in food of animal origin. Chloramphenicol was included in this regulatory law and nowadays it is illegal in the EU and banned according to Council Directive 96/22/EC and Council Regulation (EEC) No 2377/90 establishing a minimum required performance limit of 0.3 ppb (4). In 2001, CAP was detected in shrimp imported into Europe from Asia, containing between 1 and 10 ppb of this antibiotic (3). A variety of methods for the detection of chloramphenicol in food matrices has been described, such as chromatography methods (5), SPR (6) and immunoassay techniques (7,8).

In this work we have produced different immuno-reagents for sensitive and specific determination of chloramphenicol. In addition, we developed and validated an ELISA assay for the detection of chloramphenicol residues in food samples according to the Commission Decision 2002/657/EC criteria established for qualitative screening methods.

Materials and Methods

Preparation of Chloramphenicol Haptens
Immunizing and competitor haptens CA1 and CA6 were synthesized from chloramphenicol base obtained from Sigma-Aldrich (St. Louis, MO, USA). Finally, the synthesis of hapten CA7 was only tested as a competitor and the preparation of hapten CA2 (chloramphenicol succinate) was also tested as a competitor and purchased directly from Sigma-Aldrich. The experimental details on the synthesis, purification and characterizationof the haptens will be published elsewhere.

Bioconjugates
Protein-hapten conjugates were prepared following the active ester procedure in which carboxylic acid groups of the haptens (2 µmol) were activated with *N*-hydroxysuccinimide (NHS, 12.5 µmol) and dicyclohexylcarbodiimide (DCC, 25 µmol) in anhy-

drous DMF (200 µL), and finally coupled to different proteins, HCH and BSA (10 mg), in 0.2 M borate buffer (1.8 mL). The protein-hapten conjugates were purified by dialysis against 0.5 mM PBS (4 x 5 L) and Mili-Q water (1 x 5 L) and stored freeze-dried at -40°C. Working aliquots were prepared in PBS at 1 mg mL^{-1} and stored at 4°C. Bioconjugates were characterized by MALDI-TOF-MS estimating the corresponding hapten density, and is described elsewhere (9).

Immunization and Polyclonal Antisera Production

As224-226 was obtained by the immunization of female white New Zealand rabbits with CA1-HCH, following the described protocol, well established in the laboratory. In the same way, polyclonal antisera As227-229 were obtained by immunization with CA6-HCH. The process of immunization was evaluated by the assessment of the binding affinity of different antisera against various coating antigens by indirect ELISA assay. Once an acceptable antibody titre was observed, animals were exsanguinated and the blood was collected. Finally, the antisera were obtained by centrifugation and stored at -80°C in the presence of 0.02% NaN_3.

Indirect CA6-BSA/As226. General protocol.

Initially, the capability to detect CAP and specificity of the antisera obtained were assessed by competitive ELISA assay following a general protocol. Previously, the optimal concentrations of immuno-reagents had been adjusted by means of two-dimensional checkerboard titration assays considering the avidity of the antisera for the better competitor antigen. Many combinations with similar analytical performances were obtained, but the best combinations are shown in Table 2. Finally, microtiter plates were coated with CA6-BSA (0.125 µg mL^{-1} in coating buffer, 100 µL/well) overnight at 4°C. The next day, after four washes with PBST (300 µL/well), chloramphenicol standards (from 1,000 nM to 0.0025 nM in assay buffer) were added to the ELISA plate (50 µL/well) followed by the antiserum As226 (1/32,000 diluted in assay buffer, 50 µL/well), and incubated for 30 min at room temperature while shaking. After this time, plates were washed as before and an anti IgG-HRP solution was added to the wells (100 µL/well), and incubated for 30 more min at room temperature and without shaking.

The plates were washed again and the substrate solution was added to the wells (100 µL/well). The plates were incubated at room temperature, without shaking and protected from light. Colour enhancement was stopped after this time by adding 4 N H_2SO_4 (50 µL/well) and the absorbance was read at 450 nm. The standard curves were fitted to a four-parameter equation according to the following equation: y=((A-B)/(1-(x/C)^D))+B, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve.

Specificity studies

The assay specificity was evaluated against a set of different antibiotics (Table 1). Standard curves were prepared in PBST and run in the ELISA assay following the protocol described before. The cross-reactivity values were calculated according to the following equation: (IC_{50} [nM] chloramphenicol / IC_{50} [nM] compound) x 100.

The cross-reactivity was negligible for all the tested compounds (<0.01%). In the case of thiamphenicol and florfenicol, the assay shows more selectivity, but this is not important due to the sensitivity of the assay toward chloramphenicol.

Milk

Milk samples were taken from a pool of different whole milks. This pool was freeze-dried and stored at -40°C in aliquots. Milk samples were reconstituted in Milli-Q water before use.

Decision limit (CCα) and detection capability (CCβ)

In order to determine the decision limit and detection capability, a method of validation, according to Commission Decision 2002/657/EC as proposed by dr. Companyó *et al*. (10), was followed. To check that all blank absorbances were higher than the CCα value, thus ensuring a 0% of false non-compliant results, different milk samples (20 blanks) were repetitively analysed on three different days by ELISA, as described above. The blanks were known to be free from chloramphenicol. The average signal (A) and the standard deviation (SD) of the signal of the 20 blank milk samples were determined. The CCα was then calculated as CCα (abs.) = A - 2.33×SD, while CCβ was calculated as CCβ (abs.) = CCα (abs.) – (1.64×SD).

To determine the β-error, which was lower than or equal to 5% (percentage of false complaints), assays were performed on three days using 20 blank milk samples spiked at the CCβ level with chloramphenicol. In these cases, a minus sign is required in the equations, because in a competitive immunoassays the correlation between analyte concentration and light absorbance is reciprocal. Both decision limit and detection capability were obtained in terms of absorbance. These values were later interpolated in the appropriate calibration curves.

Results and discussion

Different antibodies and immuno-reagents were produced to detect, in a selective and sensitive way, the chloramphenicol antibiotic in milk. The synthesis of the proposed haptens was performed successfully, and they were purified and characterized properly. Haptens CA1 and CA6 were covalently coupled through their carboxylic groups to the lysine amino acid residues of HCH and used to obtain antibodies. In the same way, these haptens were coupled to BSA, OVA and CONA in order to prepare competitors and develop the corresponding competitive ELISA. Following the same procedure, haptens CA2 and CA7 were coupled to BSA, OVA and CONA proteins to develop the ELISA assay.

Table 1. Combinations with the best analytical performances in buffer conditions after corresponding screening assays.

As	AT	[AT] (µg mL^{-1})	As Dilution	Amax	IC_{50} (µg mL^{-1})	Hillslope	R^2
As226	CA1-BSA	0.0156	1/64,000	0.9656	3,003	-0.7674	0.9984
As226	CA2-BSA	0.03125	1/32,000	1.224	1,202	-0.7554	0.9925
As226	CA6-BSA	0.0625	1/64,000	0.8285	0,3283	-0.6256	0.9916
As226	CA7-BSA	0.0625	1/64,000	1.035	0,5504	-0.5313	0.9967

After the corresponding screening of all the immuno-reactants, using CAP as analyte and following the ELISA procedure previously described, systems with CA6-BSA and CA7-BSA as a coating antigen and antisera raised against CA1-HCH showed best analytical performance. Table 1 shows the features of the best competitive ELISA assays and reveals that the best antibody/coating antigen combinations are heterologous. Immunoassay using As226 as the antiserum and CA6-BSA as the coating antigen was selected for further studies.

Using this indirect ELISA (CA6-BSA/As226), chloramphenicol can be detected in buffer with a limit of detection (LOD) of 1.99 ± 2.09 ng L^{-1} (90% response of the zero dose) and with a working range between 7.4 ± 4.5 ng L^{-1} to 356 ± 81 ng L^{-1} corresponding to 80% to 20% of the assay responses at zero doses (Table 2). Figure 1 shows a chloramphenicol standard curve for the developed assay according to data recorded in three assays performed in buffer at different days.

*Table 2. Analytical performances for the developed assay (**CA6-BSA/As226**) for CAP detection in buffer (N=4) and in raw milk (N=3).*

	Buffer	**Raw milk**
A(min)	0.066 ± 0.021	0.001 ± 0.008
A(max)	0.675 ± 0.024	1.289 ± 0.160
Slope	-0.746 ± 0.124	0.801 ± 0.083
IC_{50} (µg L^{-1})	0.063 ± 0.005	0.304 ± 0.058
R2	0.993 ± 0.006	0.993 ± 0.004
Working range(µg L^{-1})		
80%	0.011 ± 0.005	0.049 ± 0.017
20%	0.395 ± 0.070	1.610 ± 0.186
LOD (µg L^{-1}) at 90%	0.004 ± 0.003	0.016 ± 0.004

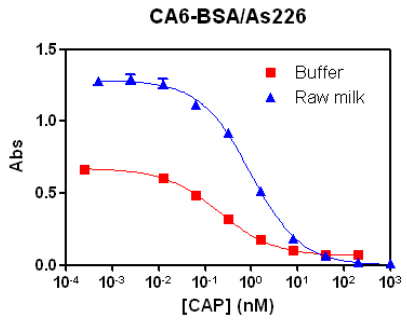

Figure 1. Chloramphenicol standard curve for the immuno developed using CA6-BSA as a competitor and As226 as the antiserum in buffer and in raw milk

Specificity studies of both immunoassays were performed preparing calibrating curves of different antibiotic families such as sulfonamides, β-lactams, quinolones and amphenicols. Table 3 shows that cross-reactivity values are negligible in all cases except for thiamphenicol and florfenicol. However, the IC_{50} values observed for both analytes, compared to CAP, are not significant. Consequently, it can be easily seen that the immunoassays developed are highly specific for CAP.

Table 3. Cross-reactivity values for CA6-BSA/As226

Analyte	CA6-BSA/As226	
	IC50 (µg L-1)	CR (%)
CAP	0,070	100
Enrofloxacin	> 200	< 0.01
Danofloxacin	> 200	< 0.01
Flumequine	> 200	< 0.01
Marbofloxacin	> 200	< 0.01
Acenocoumarol	> 200	< 0.01
Sulfamethoxazole	> 200	< 0.01
Sulfapyridine	> 200	< 0.01
Sulfamerazine	> 200	< 0.01
Sulfathiazole	> 200	< 0.01
Tilosine A	> 200	< 0.01
Tilosine B	> 200	< 0.01
Amoxicillin	> 200	< 0.01
Ampicillin	> 200	< 0.01
Penicillin	> 200	< 0.01
Neomycin B	> 200	< 0.01
Streptomycin	> 200	< 0.01
Thiamphenicol	13.9	< 0.5
Florfenicol	> 200	< 0.01
Tetracycline	> 200	< 0.01
Doxycycline	> 200	< 0.01

Matrix effect studies were evaluated preparing CAP standard curves in commercial whole milk at different dilutions and preparing an antiserum dilution in optimization buffer. According to the high matrix effect observed initially with raw milk, it was decided to adjust the concentration of the immuno-reagents in order to find the most suitable ones. Also, different physico-chemical parameters were evaluated so as to determine the optimum level for the best detectability. Microtiter plates were coated at 0.125 µg L^{-1} CA6-BSA as coating antigen and a dilution of antiserum 1/12000 As226 was tested, using 10 mM of phosphate saline buffer at pH 6.5 and 0.10% of tween-20, which gave the best results. Chloramphenicol standard curves were prepared directly in milk. It is worth noting that, in the case of milk, it was necessary to shake the plates at 750 rpm during the competitive step in order to improve the analytical parameters of the assay, as can be seen in Table 2 and Figure 1. It can be observed that it is possible to work directly with milk, achieving values of detectability of 0.016 ± 0.004 µg L^{-1}. The concentration producing 80% of the maximum signal was considered the limit of quantification (LOQ) and was found at 0.049 ± 0.017 µg kg^{-1}.

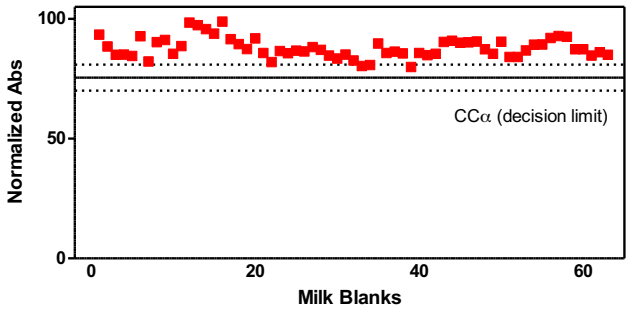

Figure 2. No false non-compliant verification for chloramphenicol. Blank milk samples were analysed for 3 days.

With the aim of validating our assay following the Commission Decision 2002/657/EC criteria, the limit of detectability (CCβ) and the decision limit (CCα) were established. Due to the different approaches suggested by the European Commission for screening methods for forbidden substances without a well-established permitted limit, a methodology proposed by dr. Companyó *et al*. was followed.

In this procedure, 20 blank milk samples were analysed in duplicate for 3 days to obtain a background value for non-contaminated samples (Figure 2). A CCα value of 0.079 ± 0.048 µg kg^{-1} was obtained. This value assures that this method can recognises a sample as a false compliant, thus enabling the screening of multiple number of samples simultaneously in order to simplify further chromatographic analyses.

Nevertheless, there might be a low risk of false non-compliant. For this reason, the detection capability, which reduces the probability of false non-compliant samples, was calculated. Calculated CCβ was 0.148 ± 0.102 µg kg^{-}.

In Figure 3, all the parameters needed to validate a screening assay, such ELISA, are represented. In this case, less than 5% of spiked samples were non-compliant, which is the permitted percentage of false compliant.

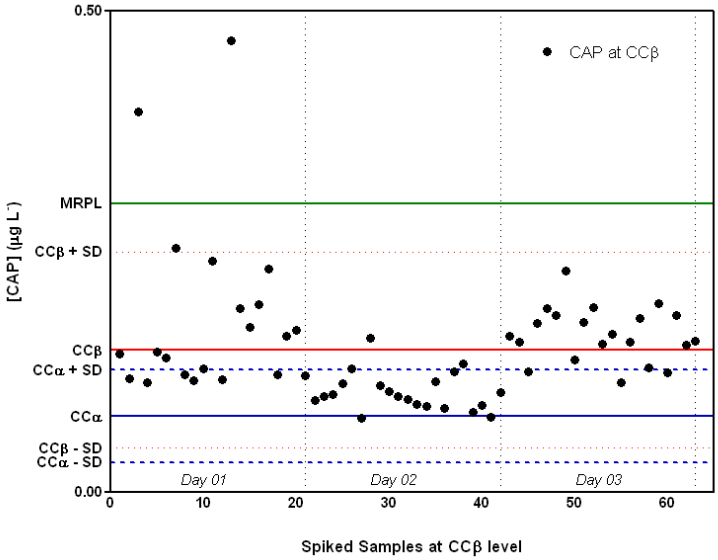

Figure 3. Summary of results obtained in the validation procedure, showing milk samples spiked at CCβ value. It is a false compliant verification study.

Conclusions

A selective ELISA assay for the determination of CAP in milk samples has been developed. Results showed that it is able to detect CAP in milk with good accuracy at concentrations lower than the MRPL level established by the EFSA, with the possibility of working directly with non-diluted milk and without sample pre-treatment. The ELISA was validated following the Commission Decision 2002/657/EC. The results reveal an ELISA the assay as a precise and quick screening method, *i.e*. a low percentage of false compliant samples was obtained.

Acknowledgements

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WIDE-RANGE SCREENING OF BANNED SUBSTANCES IN *BOVINE* URINE

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Abstract

European Union banned anabolic agents in food-producing animals, because their use could cause possible toxic effects on public health. These compounds and other banned substances included in the group A (Annex I of Directive 96/23/EEC) are determined in *bovine* urine within the National Residue Plans. In Italy, this official monitoring is performed with several different analytical methods to screen all the required classes/compounds. The aim of this work was the development and validation of an instrumental screening procedure for a wide range of banned compounds in *bovine* urine: dapsone, chloramphenicol, corticosteroids, lactones of the resorcylic acid (RALs), nitroimidazoles, sedatives, steroids and stilbenes. Urine is a very complex matrix, with several interfering substances, which can vary from animal to animal. Therefore, instrument conditions were developed for liquid chromatography coupled to hybrid high-resolution mass spectrometry (LC-Q Exactive, Thermo Scientific) with parallel reaction monitoring (PRM). Two chromatographic runs were carried out: ESI in negative mode for chloramphenicol, RALs and stilbenes and ESI in positive mode for all the other substances. The results demonstrated that the method was fit-for-purpose for the most of the 52 investigated analytes.

Introduction

Since 1988, monitoring plans to detect illicit use of growth-promoters in farm such as steroids, stilbenes or thyreostats play an important role to protect public health in the European Union. Annex I of Directive 96/23/EC classifies substances into two categories: A) substances having an anabolic effect or unauthorised; B) permitted veterinary drugs and environmental contaminants. Most controls targeting category A are carried out on urine samples which is the matrix of choice since it is relatively easy to collect and samples can be taken before slaughter avoiding contaminated meat to reach the market (Stolker *et al*., 2007). The commonly used strategy for their determination in the official control laboratories is the application, at first, of a screening test followed by a confirmatory method in case of suspect sample(s). The routine activities are generally carried out using several single-class methods to cover all the required classes with high times and costs. In addition, each applied method involves preliminary intensive work to develop, validate and obtain its accreditation. A way to improve the cost-effectiveness of these controls is to develop a wide-range multi-class screening method in urine. In fact, in the last years this strategy has been successfully realised for other groups of residues such as veterinary drugs, pesticides and mycotoxins, mainly thanks to the tremendous improvement of chromatographic and mass spectrometric equipment.

This work describes the development and validation of a targeted screening procedure for 52 banned compounds in *bovine* urine: lactones of the resorcylic acid (RALs), nitroimidazoles, steroids, stilbene and two banned veterinary drugs, dapsone and chloramphenicol. In addition, two classes belonging to group B, such as corticosteroids (B2f) and sedatives (B2d), were included, too. It is well known that corticosteroids could be associated to illegal growth-promoting treatments (Biancotto *et al*. 2013) and, on the other hand, sedatives can be used in cattle to prevent stress and death after steroid treatments. The applied technique has been the liquid chromatography coupled to hybrid high-resolution mass-spectrometry (LC-Q Exactive). Because of the several interfering substances, which vary from urine-to-urine, a parallel reaction monitoring (PRM) acquisition was necessary. Joining MS/MS experiments with high mass accuracy suitable detection capabilities were obtained for the most of investigated substances.

Materials and Methods

Reference standards

Individual stock standard solutions (1 or 0.1 mg mL^{-1}) were prepared in methanol (chloramphenicol, corticosteroids, dapson, nitroimidazoles and promazines) or in ethanol (β-agonists, RALs, steroids and stilbenes). Intermediate solution at 1 µg mL^{-1}) was prepared in methanol.

Chromatographic and MS conditions

Chromatography was performed on a Thermo Ultimate 3000 High Performance Liquid Chromatography system (Thermo Scientific, San Jose, CA, USA). Analytes were separated on a Kinetex XB-C18 column (100 x 3.0 mm; 2.6 µm – Phenomenex, Torrance, CA, USA). Two chromatographic runs were carried out: ESI in negative mode for chloramphenicol, RALs and stilbenes and ESI in positive mode for all the other substances. For the ESI(-) acquisition acetonitrile and water were used, whereas for the ESI(+) run the LC eluent A was acetonitrile containing 0.1% (v/v) acetic acid and eluent B was an aqueous solution con-

taining 0.1% (v/v) acetic acid. The gradient was the same for both runs and initiated with 100% eluent B for 0.5 min, continued with a linear decrease to 70% B in 10 min. This condition was maintained for 2.5 min and then linearly decreased to 5% B in 7 min. The system returned to 100% B in 0.5 min and it was re-equilibrated for 4 min (run time: 28 min). The column temperature was set at 40°C and the sample temperature was kept at 16°C. The flow rate was 0.4 mL min^{-1} and the injection volume 10 µL.

The mass spectrometer Q Exactive Plus (Thermo Scientific) was equipped with heated electrospray ionization source (HESI-II). The optimized HESI-II temperature was set at 350°C, the capillary temperature at 300°C, the electrospray voltage at 3.00 kV (positive mode) and 2.5 kV (negative mode). Sheath and auxiliary gas were 50 and 15 arbitrary units, respectively. The acquisition method was PRM (parallel reaction monitoring) for all analytes with 0.8 *m/z* as isolation window. All extract mass traces were based on a 5 ppm mass window (accuracy) and the resolution was set at 35,000 (FWHM at *m/z* 200).

Sample preparation

The deconjugation step was carried out adding five mL of acetate buffer 0.2 M (pH 5.2) to five millilitres of *bovine* urine and adjusting the pH at 5.2 with 1 M acetic acid. Then 100 µL of β-glucuronidase from *Helix pomatia* were added and the sample was placed at 42°C overnight or 55°C for two h. The de-conjugated sample was centrifuged and 5 mL of supernatant were transferred into a 15-mL tube and the pH was adjusted to about 7.0 with 1 M NaOH. The sample was then loaded into an OASIS HLB (60 mg/3 mL) cartridge pre-conditioned with 3 mL methanol and 3 mL 0.2 M acetate buffer (pH 5.2). The cartridge was washed with water and dried before elution. A NH_2 (500 mg/6 mL) cartridge conditioned with 5 mL methanol was placed under the previous one and both were then eluted with 4 mL of acetonitrile and 4 mL of methanol. After evaporation, the sample was reconstituted with 0.25 mL of a mixture of water/acetonitrile 90/10 (v/v) containing 0.1% of acetic acid. The final extract was filtered through a PTFE filter (pore size of 0.2 µm) and ultra-centrifuged (14,000 rpm) at 4°C. At the end it was transferred in vial for the injection into the chromatographic system.

Method validation

The validation study was carried out following Commission Decision 2002/657/EC (2002). For each analyte, the relevant MRPLs or recommended concentrations (RC) were taken into account (CRL Guidance Paper, 2007). Different *bovine* urines were spiked at three levels: 1, 2 and 5 µg L^{-1}. At the same time, the same urine samples from different animals were analysed without any spiking for a total of 126 analyses. The percentage of false negative (FN) results was assessed at each level to estimate the detection capability (CCβ) of each analyte.

Results and Discussion

The current trend in screening methods is to go beyond the ''multiresidual'' approach, where different analytes belonging to the same class and having similar structures are targeted, in favour of a ''multi-class'' approach, combining a large variety of target analytes with different structural features and physical and chemical properties. In the last years, several papers have been published about this kind of analytical methods determining dozens of substances belonging to important groups of residues such as veterinary drugs, pesticides and mycotoxins in food. In contrast, very few paper discussing the development of multi-class methods for the detection of banned substances in *bovine* urine are available (Leon *et al.* 2012; Leporati *et al.,* 2012). This means that the coupling "banned substances/*bovine* urine" is a more challenging goal probably because the required control levels are generally lower and the intrinsic variability of *bovine* urine is higher. In this sense, *bovine* urine can be considered a so-called "black matrix" (Varcarcel *et al*., 2001).

At the beginning of the method development, also the introduction of thyreostats (A2) was considered. However, for this class it was not possible to apply SPE purification, compromising the detection of other important compounds at the suitable levels (CRL Guidance Paper, 2007). The greater problem of such kind of procedures is to reach a sufficient selectivity. After preliminary experiments applying full-scan MS mode, it was concluded that, for the most of analytes, only the PRM (t-MS/MS) acquisition could give satisfactory results.

In PRM mode, the precursor ion is isolated by the quadrupole, transmitted via the C-trap to the HCD cell where corresponding fragment ions are generated and accumulated. Fragment ions are then transferred back into the C-trap and injected in the orbitrap mass analyser. The PRM experiment is managed by an inclusion list reporting the target precursor ions and their relevant RT windows. In Figures 1 and 2 the comparison between the initial full-scan MS acquisition method (Figure 1) and the final PRM one (Figure 2) is shown for the group of the ten steroids. The chromatograms were obtained for the same urine spiked at 1 µg L^{-1}. It is evident that the PRM mode improved the signal for most of the compounds, although for stanozolol (trace f) a false negative result was observed also with the optimised acquisition (see below). The same was noticed also for the other classes of analytes for which only the application of PRM allowed the detection at the levels of interest.

In Table 1 the preliminary validation data are listed. The evaluation of FN (false compliant) results was performed comparing the urine chromatograms with and without spiking. It is important to underline that the validation study has been performed analysing samples from different animals. As explained, there is a dramatic difference among each individual urine: certain

samples gave quite clean extracts whereas other ones were very rich of interfering peaks. Therefore, the validation study has to be performed also including these “worst urines”. To demonstrate the urine-to-urine differences (ion suppression), the chromatograms of “in-house dirty urine” (blank and spiked at 2 µg L^{-1} with all analytes) and those of the test material analysed within the FAPAS Proficiency Test PT 02246 “Synthetic Hormones in Bovine Urine” are presented in Figure 3. The final report of the PT organizer assigned the following values: 3.95 for methyltestosterone (MT), 2.65 for zeranol (ZER) and 7.58 µg L^{-1} for taleranol (TAL). In the chromatogram c) of the Figure, the MT signal is dramatically suppressed, whereas in the PT urine (Figure 3b) its peak is well detectable (3.95 µg L^{-1}). Analogously, for the chromatograms e) and f) with similar concentration of ZER (2.65 *vs* 2 µg L^{-1}, respectively), but with very different signal intensities: the ZER peak (16.43 min) in Figure 3f is very close to limit of detection, whereas in chromatogram e) is about fifty-fold higher.

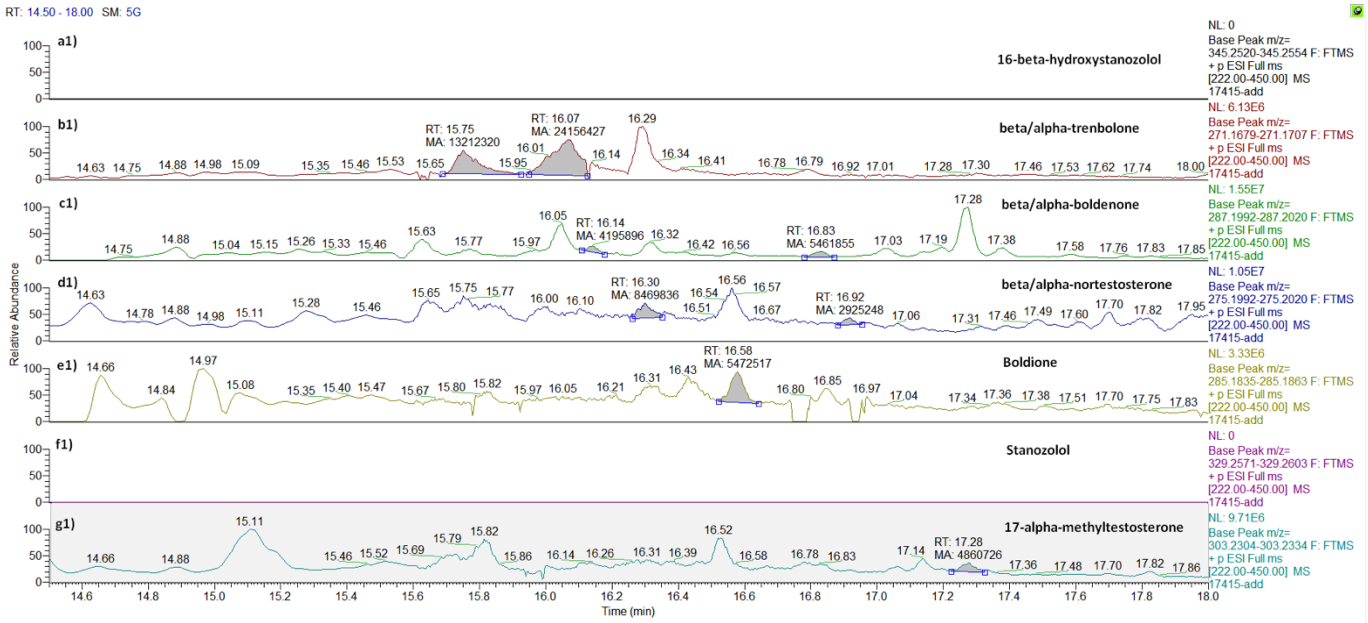

Figure 1. LC-HRMS/MS chromatograms of a urine sample spiked at 1 µg L^{-1} with the ten steroids (full scan MS acquisition).

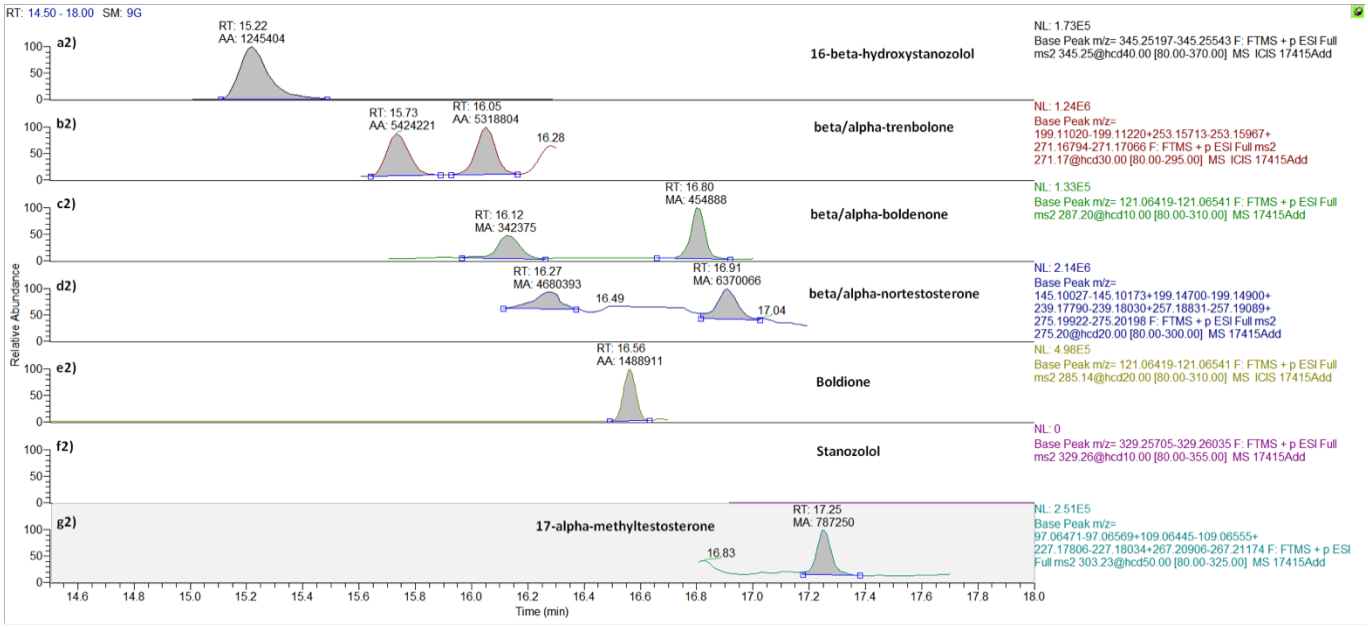

Figure 2. LC-HRMS/MS chromatograms of a urine sample spiked at 1 µg L^{-1} with the ten steroids (PRM acquisition).

The estimated detection capabilities were lower than or equal to 1 µg L^{-1} for all analytes, except for azaperol, azaperon, hexestrol (≤2 µg L^{-1}), chlorpromazine and β-nortestosterone (≤ 5 µg L^{-1}). Finally, for diethylstilbestrol (DES), propionylpromazine and stanozolol, the CCβ was higher than the higher concentration tested, *i.e.* 5 µg L^{-1}. As precautionary measure, for a certain analyte only when no FN results were detected (0 %), the correspondent level was considered the CCβ. Although Commission

Decision 2002/6757/EC allows up to 5% of FN, the number of analysed samples was not considered sufficient to have statistical certainty of the FN percentage (CRL Guideline, 2010). Therefore, further analyses are in progress to enrich the validation study and improve the knowledge about method performances.

In Table 1 also the recommended concentrations (RC) given by European Union Reference Laboratories (ex CRLs) are listed (CRL Guidance Paper, 2007). When these RCs were not available, the limits of detection listed within the Italian National Residue Plan were taken into the account. Evaluating the detection capabilities in the light of these levels, the developed screening method is not suitable for three compounds: diethylstilbestrol, stanozolol and β-nortestosterone. For some β-agonists very low limits have been fixed (0.2 µg L^{-1} for clenbuterol and mapenterol and 0.5 µg L^{-1} for isoxsuprine and clenpenterol) and there is some doubt that this screening method can reach these concentrations. On the other hand, it seems more important to control a wide range of substances at the same time than to reach limits under 1 µg L^{-1}. For chloramphenicol and propionylpromazine further experiments should be carried out to better investigate the achievable detection capabilities. At present, 43 out of 52 investigated analytes were detected at the European required levels.

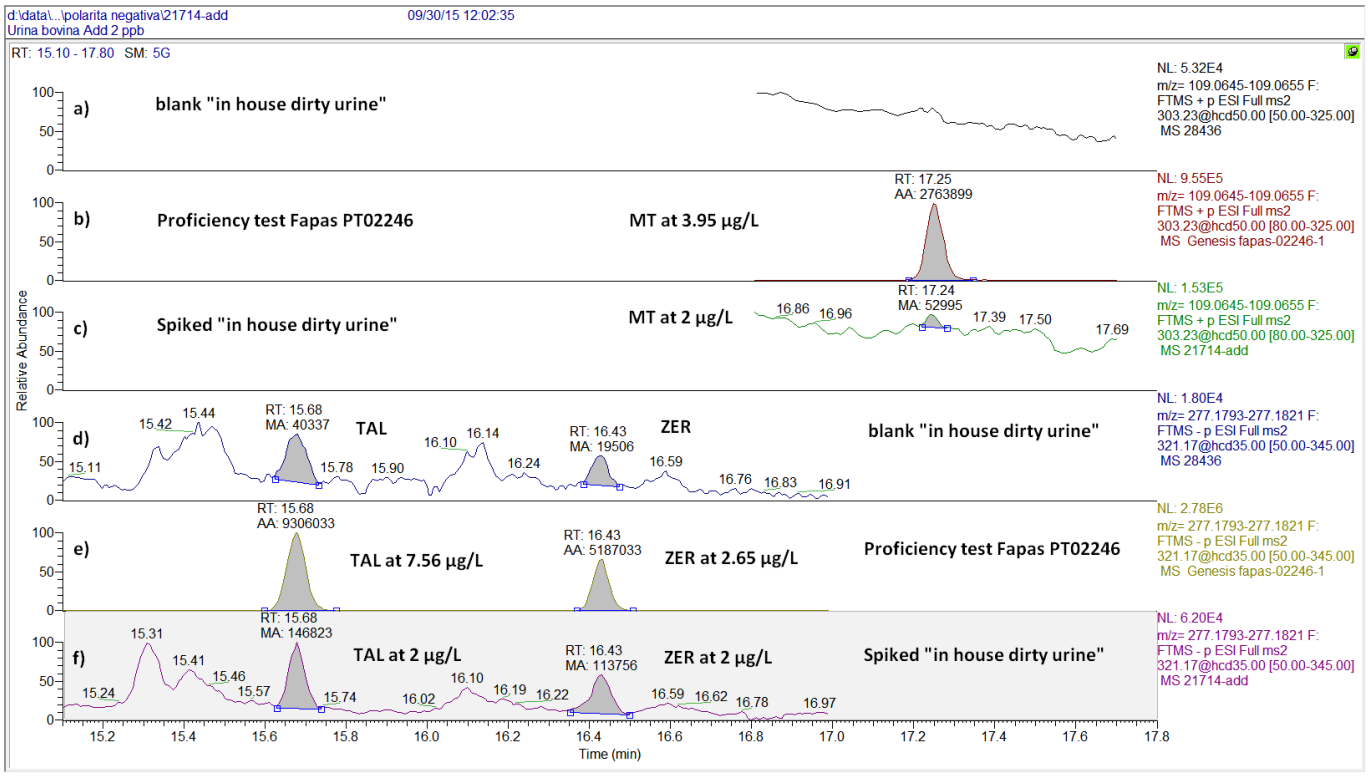

Figure 3. Chromatograms of an "in house dirty urine" without spiking (traces a) and d) for methyltestosterone and zeranol/taleranol, respectively) and with spiking at 2 µg kg^{-1} (traces c) and f) for methyltestosterone and zeranol/taleranol, respectively). The comparison with the sample analysed within the FAPAS PT 02246 (traces b) and e) demonstrated the dramatically different ion suppression effect for all the three analytes.

Conclusions

The developed method is suitable to detect 43 out of 52 investigated compounds. The chosen MS acquisition mode was PRM which provided high selectivity because the MS/MS data was acquired in high resolution mode that could separate co-isolated background ions from the target analyte ions. In SRM, only two-three transitions are generally monitored, whereas in PRM full MS/MS spectra are acquired that contains all the potential product ions allowing the selection of more suitable fragments depending on the analysed urine. The price to pay is the loss of performing untargeted analysis having chance to find unexpected contaminants, which is one of the most celebrated characteristics of modern HR analysers. The undoubtable gain is the high sample throughput. Increasing the number of simultaneously checked substances per sample and decreasing the applied screening methods to control the same set of compounds, a great improvement of the cost-effectiveness of the official controls is obtained.

Acknowledgements

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Table 1. Results of the validation study.

Analyte	Group	Spiking level (number of analyzed urines) 1 µg L^{-1} (24) FN Results	2 µg L^{-1} (22) FN Results	5 µg L^{-1} (17) FN Results	CCβ (µg L^{-1})	CRL RC (µg L^{-1})
β-zearalenol	RALs	0	0	0	≤1	2
α-zearalenol	(A4)	0	0	0	≤1	2
Zearalenone		0	0	0	≤1	2
Zeranol		0	0	0	≤1	2
β -zeranol (Taleranol)		0	0	0	≤1	2
Dienestrol	Stilbenes	0	0	0	≤1	2
Hexestrol	(A1)	2 (8%)	0	0	≤2	2
Diethylstilbestrol		6 (25%)	3 (14%)	2 (12%)	>5	1
β -trenbolone	Steroids	0	0	0	≤1	2
α -trenbolone	(A3)	0	0	0	≤1	2
β-boldenone		0	0	0	≤1	1
α-boldenone		0	0	0	≤1	1
β-nortestosterone		2 (8 %)	1 (5 %)	0	≤5	1
α-nortestosterone		0	0	0	≤1	1
16β-hydroxystanozolol		0	0	0	≤1	2
Boldione		0	0	0	≤1	2
17-α-methyltestosterone		0	0	0	≤1	2
Stanozolol		13 (54 %)	11 (50 %)	2 (12%)	>5	2
Terbutaline	β-agonists	0	0	0	≤1	3
Salbutamol	(A5)	0	0	0	≤1	1
Ractopamine		0	0	0	≤1	1
Clenbuterol		0	0	0	≤1	0.2
Isoxsuprine		0	0	0	≤1	0.5
Clenpenterol		0	0	0	≤1	0.5
Mapenterol		0	0	0	≤1	0.2
Azaperol	Sedatives	2 (8%)	0	0	≤2	-
Azaperon	(B2d)	2 (8%)	0	0	≤2	-
Carazolol		0	0	0	≤1	-
Acepromazine		0	0	0	≤1	30[a]
Promazine		0	0	0	≤1	30[a]
Propionylpromazine		3 (13%)	2 (9%)	1 (6%)	>5	30[a]
Chlorpromazine		5 (21%)	3 (14%)	0	≤5	30[a]
Metronidazole-OH	Nitroimidazoless	0	0	0	≤1	3 (plasma)
HMMNI	(A6)	0	0	0	≤1	3 (plasma)
Metronidazole		0	0	0	≤1	3 (plasma)
Ronidazole		0	0	0	≤1	3 (plasma)
Dimetridazole		0	0	0	≤1	3 (plasma)
Ipronidazole-OH		0	0	0	≤1	3 (plasma)
Ternidazole		0	0	0	≤1	3 (plasma)
Secnidazole		0	0	0	≤1	3 (plasma)
Ipronidazole		0	0	0	≤1	3 (plasma)
Prednisolone	Corticosteroids	0	0	0	≤1	2[a]
Beclomethasone	(B2f)	0	0	0	≤1	2[a]
Prednisone		0	0	0	≤1	2[a]
Betamethasone		0	0	0	≤1	2[a]
Dexamethasone		0	0	0	≤1	2[a]
Methylprednisolone		0	0	0	≤1	2[a]
Triamcinolone acetonide		0	0	0	≤1	2[a]
Triamcinolone		0	0	0	≤1	2[a]
Flumethasone		0	0	0	≤1	2[a]
Chloramphenicol	Veterinary drugs	0	0	0	≤1	0.3
Dapson	(A6)	0	0	0	≤1	5 (meat)

[a] *Italian National Residue Plan*

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NEW APPROACH BASED ON IMMUNOCHEMICAL TECHNIQUES TO MONITOR SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS) IN CLINICAL SAMPLES

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Abstract
Anti-estrogenic compounds such as tamoxifen, toremifen and chlomifen are used illegally by athletes to minimize physical impacts such as gynecomastia resulting from the secondary effects of anabolic androgenic steroids. The use of these compounds is banned by the World Antidoping Agency (WADA) and is controlled through analytical methods such as HPLC-MS/MS, which do not fulfil sample throughput requirements.
Compounds such as tamoxifen are also used to treat hormone receptor-positive breast cancer (ER+). Therapeutic drug monitoring (TDM) of tamoxifen may also be clinically useful for guiding treatment decisions. An accurate determination of these drugs requires a solid-phase extraction of patient serum followed by HPLC-MS/MS. In the context of an unmet need for high-throughput screening (HTS) and quantitative methods for anti-estrogenic substances, we have developed antibodies and an immunochemical assay for the determination of these anti-estrogenic compounds. The strategy applied took into consideration that these drugs are metabolized and excreted in urine as 4-hydroxylated compounds. An ELISA procedure was developed for the analysis of these metabolites in urine with an LOD of 0.15, 0.16 and 0.63 µg L^{-1} for 4OH-tamoxifen, 4OH-toremifen and 4OH-chlomifen, respectively, much lower than the MRPL established by WADA (20 µg L^{-1}).

Introduction
Selective estrogen receptor modulators (SERMs) are a family of compounds that interacts specifically with an estrogen receptor in order to block estrogenic compounds. This kind of compounds is commonly used to treat breast cancer, for which tamoxifen is most employed (1). Structurally similar compounds are toremifen and clomifen, in which instead of an ethyl group at the allyl core, a 2-chloroethyl group and a chlorine, respectively, is found.

In athletes, the use of anti-estrogenic compounds may compensate the effects of an extensive abuse of anabolic androgenic steroids which induce gynecomastia (2). For these reasons, the use of agents with anti-estrogenic activity has been banned in sports by the World Anti-doping Agency (WADA) (3), and anti-doping control laboratories must be able to detect the administration of the drug. To meet this requirement, several analytical methods have been developed for the control of anti-estrogenic agents, mainly based on chromatographic techniques coupled with mass-spectrometry (2,4-7). It is reported that SERMs are metabolized mainly as 4-hydroxylated variants which are excreted as glucuronide or sulphate conjugates.

Although several advances have been accomplished in order to obtain a highly sensitive technique minimizing sample pretreatment, it is necessary to proceed with a clean-up process of the sample so as to have a clean extract to inject in the chromatographic equipment. Alternatively, high-sample throughput capability of immunochemical methods could be a solution for the demands of the control for the illegal use of this drug by elite amateur and professional athletes.

It has often been shown that immunoassays can provide necessary reliability, low cost per analysis/sample, ease of use, selectivity, and detectability to analyse small organic molecules (8,9). Our research group has demonstrated the production of antibodies with tailored properties for anabolic steroid residues detection (10-12). Regarding SERMs detection by immunochemical techniques, antibodies for their determination have not been produced at the moment. Other strategies have been explored for the determination of tamoxifen, such as an amperometric biosensor method based on non-specific receptors (13) and the use of a biochemical assay based on an estrogen receptor to monitor tamoxifen (14). Thus, here we present the production of class-selective antibodies binding hydroxylated SERMs and the development of an immunoassay suitable for urine sample analysis according to the WADA requirements for anti-doping testing.

Materials and Methods

Chemicals and immuno-chemicals
The chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The standards 4-hydroxy-tamoxifen and 4-hydroxy-toremifen were synthesized following the procedure described by Gauthier *et al*. (15) and Yu *et al*. (16) with minor modifications. 4-Hydroxyclomifene was purchased from Synfine Research (Ontario, Canada). All other chemicals were of ana-

lytical grade. Biochemicals and immuno-chemicals were obtained from Sigma-Aldrich. The hapten (Z/E)1-[4-(2-Dimethylamino-ethoxy)-fenyl]-1-(4-hydroxy-phenil)-6-phenyl-hept-6-enoic acid (hTAM) used to immunise, was synthesized using the same experimental procedure as for 4-hydroxytamoxifen and 4-hydroxytoremifen, which will be published elsewhere.

Preparation of protein conjugates

Mixed anhydride method (hTAM-HCH (MA) and hTAM-BSA (MA)). The hapten (10 µmol) reacted with tributylamine (1.55 µL, 11 µmol) and *iso*-butylchloroformate (2.7 µL, 12 µmol) in 100 µL anhydrous dimethyformamide (DMF) and was added either to *bovine* serum albumin (BSA) or to horseshoe crab hemocyanin (HCH) (10 mg) as described (11).

Active ester method (hTAM-BSA (AE) and hTAM-HRP (AE)). The hapten (10 µmol) reacted with *N*-hydroxysuccinimide (5.75 mg, 50 µmol) and dicyclohexylcarbodiimide (20.63 mg, 100 µmol) in 200 µL anhydrous DMF and added to HRP (2 mg) and BSA (10 mg) as described (11). The conjugates were purified by dialysis and stored freeze-dried at -40°C, except the conjugates coupled to the hapten hTAM-BSA, which were purified by a Hi-trap desalting column. Work aliquots were prepared at 1 mg mL^{-1} in PBS and stored at 4°C. The characterization of the protein conjugates was performed by MALDI-TOF-MS by comparing the observed molecular weights of the prepared conjugates and the intact protein. The spectra were obtained by mixing 2 µL of matrix (10 mg mL^{-1} *trans*-3,5-dimethoxy-4-hydroxycinnamic acid in CH_3CN/H_2O 70:30, 0.1% trifluoroacetic acid) with 2 µL of a solution of the conjugates or proteins (5 mg mL^{-1} in MilliQ water). Because HRP has only one or two free accessible lysine constituents and the difference in molecular weight is so small, the molecular weights of the conjugates and the non-conjugated HRP was measured using the BSA as an internal standard.

Polyclonal antisera

Two female New Zealand white rabbits weighing 1 to 2 kg were immunized with hTAM-HCH (MA) according to the immunization protocol previously described (17) to obtain As211-213. The evolution of the antibody titer was assessed by measuring the binding of serial diluted antisera to microtiter plates coated with the hTAM-BSA (MA) conjugate. After an acceptable antibody titer was observed, the animals were exsanguinated and the blood was collected in vacutainer tubes provided with a serum separation gel. The antiserum was obtained by centrifugation and stored at -40°C in the presence of 0.02% NaN_3.

Hydroxyl-SERMs ELISA

Appropriate dilutions of the antisera and the enzyme tracer (hTAM-HRP (AE)) and/or coated antigen (hTAM-BSA (AE)) were established after two-dimensional checkerboard titration assays.

ELISA As212/hTAM-HRP. The microtiter plates were coated with the antiserum As212 (1/12,000 in a coating buffer, 100 µL/well) overnight at 4°C. The next day the plates were washed four times with PBST and the solutions of the different concentrations of standards, cross-reactants or samples were added (50 µL/well) followed by the solution of the enzyme tracer hTAM-HRP (0.125 µg mL^{-1} in PBST, 50µL/well). After 30 min at room temperature, the plates were washed again four times with PBST. The substrate solution was added (100 µL/well) and the enzymatic reaction stopped after 30 min at room temperature with 4 N H_2SO_4 (50µL/well). The absorbance was measured at 450 nm. The standard curve was fitted to a four-parameter logistic equation according to the following formula: y = (A–B/[1–(x/C)D]) + B, where *A* is the maximal absorbance, *B* is the minimum absorbance, *C i*s the concentration producing 50% of the difference between the maximal absorbance and the minimum absorbance, and *D* is the slope of the inflection point of the sigmoid curve.

Cross-reactivity determinations

Stock solutions of different compounds were prepared (10 mM in DMSO) and stored at 4°C. Standard curves were prepared in the same buffer for each ELISA format in the same range that the reference analyte and each IC_{50} determined in the competitive experiment described above. The cross-reactivity values were calculated according to the following equation: (IC_{50} 4OH-TAM/IC_{50} steroidal compounds)×100.

Urine sample and Excretion study samples

A pooled human urine sample was obtained by mixing samples from 10 individuals. Urine samples were obtained from excretion studies involving the administration of toremifene to healthy volunteers. The clinical protocol was approved by the Local Ethical committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). A single dose of 60 mg of toremifene (Fareston®) was administered to two healthy volunteers by oral route. In the first study, the urine samples were collected before administration and up to 82 h after administration. In the second study, urine samples were collected before administration and up to 10 days after administration. In addition, a spot sample was collected one month after toremifene administration. In order to compare toremifene metabolites with those of tamoxifen, urine was collected from 0 to 24 h after oral administration of tamoxifen (20 mg Tamoxifeno Ratiopharm EGF). Urine samples were stored at −20°C until analysis and they were analysed for all metabolites.

Urine sample pretreatment

Urine samples were treated as previously described with minor modifications (4). This method hydrolysed simultaneously the sulphate and glucuronide conjugates in order to obtain the free drug (10). First, an enzymatic hydrolysis was performed by mixing 1 mL urine sample with 1 mL 0.4 M PBS pH 7.0 and 5 µL β-glucoronidase. The mixture was incubated for 1 h at 55°C. After that, 500 µL carbonate buffer at 5% and, subsequently, 3 mL of *tert*-butyl methyl ether were added. The mixture was shaken and centrifuged at 4,000 rpm during 5 min. The organic layer was evaporated with a N_2 stream. The dried extract was reconstituted in 100 µL ethanol and 900 µL PBST.

Results and discussion

The aim of this work is to produce class-specific antibodies for the determination of SERM's main metabolites. The metabolites excreted for the most applied SERMs (7) are well known. Regarding tamoxifen, the main metabolites are Z/E-4-hydroxy-tamoxifen, desmethyltamoxifen and trans-metabolite E (18). Similar metabolism was also found later for toremifene (4) and clomiphene (2). In these articles, it was stated that the most relevant metabolites of SERMs are the 4-hydroxylated compounds and their corresponding sulphate and glucuronide conjugates. It is for this reason that we proposed the production of antibodies that would recognize all hydroxylated metabolites. The production of antibodies for the determination of small molecular weight molecules requires the design of a hapten that mimics the target analyte. Considering their chemical structure, the three targeted analytes, *i.e*. hydroxylated –SERMS, share three common epitopes. These epitopes are a phenolic group, an aryl group and a 4-(2-dimethylamino-ethoxy)-phenyl group (Figure 1).

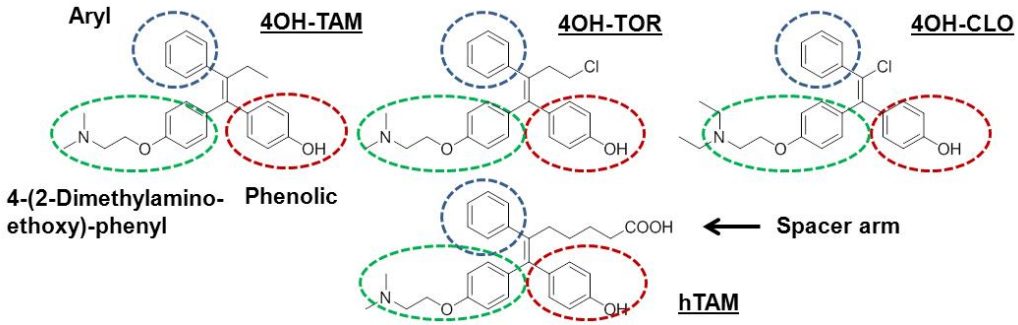

Figure 1. Chemical structures of 4-hydroxy-tamoxifen (4OH-TAM), 4-hydroxy-toremifen (4OH-TOR) and 4-hydroxy-Clomifene (4OH-CLO) and the proposed immunizing hapten (hTAM). The aryl group is highlighted in blue, the phenolic group in red, and the 4-(2-dimethylamino-ethoxy)-phenyl group in green.

Table 1. Recognition of structurally related substances, expressed by their IC_{50} and percentage of cross-reactivity with (E/Z)4OH-tamoxifen as the reference compound.

Compound	IC_{50} (nM)	CR (%)
(E/Z)4OH-Tamoxifen	1.19 (0.46)[a]	100
Tamoxifen	53.65 (19.9)	2.2
(Z)3-Methoxy-4OH-Tamoxifen	102.9 (42.9)	1.2
Toremifen	28.5 (11.5)	4.2
(E/Z)4OH-Toremifen	0.72 (0.30)	165
Clomifen	213.6 (87.5)	0.6
(E/Z)4OH-Clomifen	17.25 (7.26)	7.0
Testosterone	>1000 (>288)	<0.04
Estradiol	>1000 (>272)	<0.04

[a] *IC_{50} in µg·L^{-1} given between the brackets.*

The synthesis of the 4-hydroxylated metabolites of tamoxifen and toremifen was performed in our laboratory following the procedure described by Gauthier *et al*. (15) and Yu *et al*. (16) with a few modifications. According to the same procedure, the synthesis of the immunizing hapten hTAM was carried out successfully. The hTAM was coupled to HCH and BSA with a hapten density ratio of about 1 according to MALDI-TOF-MS analysis. The hapten density of the HCH conjugates could not be recorded due to its high molecular weight (> 2,000 kDa). However, because the BSA conjugate (hTAM-BSA (MA)) was prepared simultaneously from the same batch of the activated hapten, a suitable degree of conjugation to use the hTAM conjugate as immunogen was assumed. Thus, 211-213 were raised against hTAM-HCH (MA). The same hapten was coupled to BSA

and HRP in order to get the competitors for the development of the ELISA. In this case, the characterization by MALDI-TOF MS analysis revealed that the hapten densities for the hTAM-BSA (EA) and hTAM-HRP (EA) were 2 and 1, respectively.

Both formats, direct and indirect, were explored for the development of an ELISA for the detection of hydroxylated SERMs. From all the combinations tested with good detectability, the assay As212/hTAM-HRP was chosen as the best to follow the analytical characterization. The As212/hTAM-HRP assay achieved an IC_{50} of 2.4 nM in buffer expressed in immuno-reactive equivalents of 4OH-TAM.

Specificity studies were carried out in order to characterize the selectivity of the As212/hTAM-HRP assay (Table 1). The assay showed a high cross-reactivity with 4OH-TOR of 165%. However, the 4OH-CLO was recognized with only a 7% cross-reactivity, which was expected to be higher. But, if we consider the IC_{50} of 4OH-CLO, which is 17.2 nM (7.26 µg L^{-1}), the assay is also fit according to the WADA as it requires an MRPLs of 20 µg·L^{-1} for SERMs (3). Therefore, the parent drugs tamoxifen (IC_{50} of 19.9 µg·L^{-1}) and toremifen (IC_{50} of 11.5 µg·L^{-1}) can be detected with the assay following the requirements of the WADA. Other compounds tested, such as testosterone and estradiol, yielded negligible values.

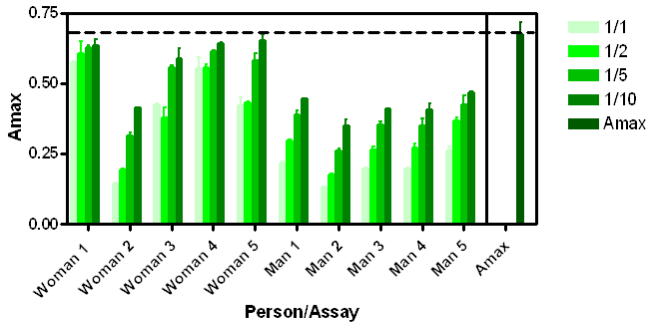

Figure 2. Matrix effect of urine samples from different healthy volunteers (Woman 1-5 and Man 1-5) compared with the buffer (Amax). The urines were not diluted (1/1), or 2, 5 or 10 times diluted.

Finally, we addressed the detection of SERMs metabolites in human urine. To evaluate the variability of the response, matrix effect was evaluated with urine from healthy volunteers. A high variability was found among the urines (Figure 2). In some cases, the value of the buffer (Amax) can be obtained by diluting the urine 10 times. In other cases, however, further dilutions would be necessary. When the urine is diluted more than 10 times though, the LOD becomes unacceptable for the detection of 4OH-CLO in this assay. For this reason, a clean-up step was proposed. Not only to minimize the variability caused by the different urines, but also to allow the hydrolysis of the glucuronide and sulphate conjugates excreted in urine.

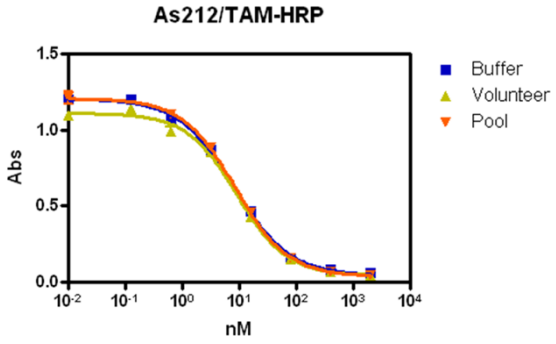

Figure 2. Matrix effect of a pooled urine sample and from one volunteer after a clean-up method compared with the analyte in buffer.

SERMs and their main metabolites are namely excreted as sulphate and glucuronide conjugates (7). With the aim to establish a simple sample treatment procedure compatible with the immunochemical method, urine was hydrolysed as previously described (12) and advantage was taken of an easy method for extracting SERMs metabolites from urine(4). Following this extraction protocol, the matrix-effect was minimized as exemplified in Figure 3 in which can be seen that the response of a pooled urine sample was almost the same as buffer. Like pooled urine, the matrix effect from a single person was also almost negligible.

An analytical characterization was performed in order to measure the hydroxylated SERMs applying the clean-up procedure. The results summarized in Table 2 revealed a great detectability for all the hydroxylated SERMs, reaching values of LOD of

0.04, 0.14 and 0.57 µg·L^{-1} for 4OH-TAM, 4OH-TOR and 4OH-CLO, respectively. All metabolites tested are under the required MRPL established by the WADA. Finally, recovery studies were performed obtaining values of 24 ± 8%, 40 ± 6% and 50 ± 15% for 4OH-TAM, 4OH-TOR and 4OH-CLO, respectively.

Table 2. Analytical parameters for each hydroxylated-SERMs in the As212/hTAM-HRP assay (n=3).

As212/hTAM-HRP	4OH-TAM	4OH-TOR	4OH-CLO
A(min)	0.05±0.006	0.054±0.016	0.05±0.01
A(max)	1.23±0.08	1.44±0.16	1.20±0.04
Slope	-0.75±0.02	-0.88±0.06	-0.69±0.04
IC_{50} (nM)	1.61±1.03	4.29±0.99	31.14±4.99
R^2	0.9991±0.0004	0.998±0.001	0.99±0.01
LOD (µg·L^{-1})	0.04±0.02	0.15±0.03	0.57±0.13
IC_{50} (µg·L^{-1})	0.61±0.36	1.76±0.36	12.38±1.56
WR (µg·L^{-1})	0.10±0.06/3.67±2.2	0.37±0.08/8.21±1.72	1.75±0.27/82.87±12.04

To summarize, the whole analytical protocol consisted, briefly, of hydrolysis of the urine, extraction, evaporation, reconstitution and ELISA measurements. Urine samples collected from patients treated with tamoxifen (patient T078u2), toremifen (M2-8 and M10-21) and clomifen (C066u3-5 and C066u6-10) were analysed by this procedure. The collection of these valuable samples made it possible to establish an excretion profile for each SERM (Figure 3), except for tamoxifen as only one sample was available. The results showed that all the samples tested, except for t=0, were positive, i.e. in excess of the MRPL established by the WADA. Hydroxylated tamoxifen was clearly found 24 h after administration. Hydroxylated toremifen can be traced several days in patient M10-21. Unfortunately, samples were not collected from patient M2-8 after 100 h, so that these data were confirmed. In any case, the metabolites can be found once the first sample is collected (t = 12 h). Hydroxylated clomifen was also found after the maximum time of sample collection (t=72h for patient C066u6-10).

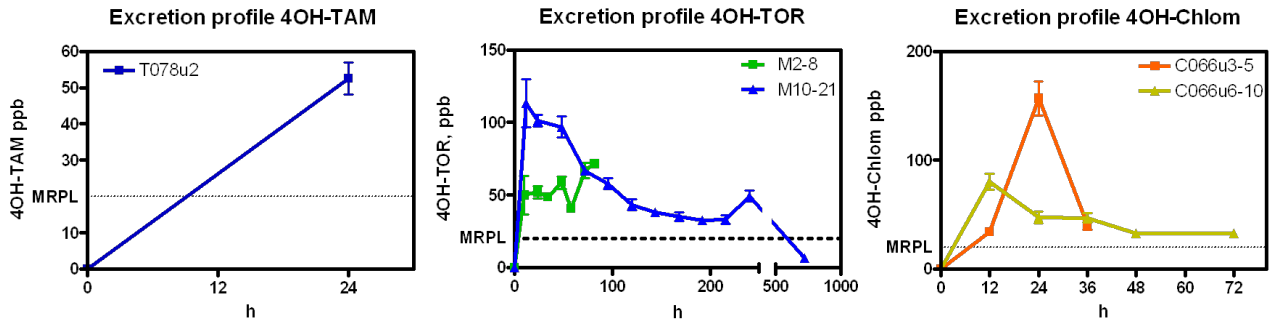

Figure 3. Different excretion profiles following SERM administration. The results are expressed in the corresponding immuno-reactive equivalents for its parent compound. The results are the mean and SD from n=3.

Conclusions

A new method has been established for the detection of the main metabolites of SERMs in human urine by an immunochemical method. The antibodies raised were produced after a rational design of the immunizing hapten that mimics the main metabolites of most employed SERMs. The whole process, consisting of an easy clean-up step of human urine and the subsequent immuno-chemical assay, reached very good limits of detection: 0.04, 0.14 and 0.57 µg·L-1 for 4OH-TAM, 4OH-TOR and 4OH-CLO, respectively. The LODs obtained are in compliance with the MRPL values recommended by the WADA regarding the illegal use of these steroid by athletes. The assay demonstrated its applicability for the analysis of urine samples to establish excretion profiles after tamoxifen, toremifen and clomifen uptake.

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A NEW SORBENT FOR CLEAN-UP OF SEAFOOD EXTRACTS PRIOR TO MULTI-RESIDUE VETERINARY DRUG LC-MS ANALYSIS

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Abstract
Much of the focus in liquid chromatography during the last decade has been on improving the speed and resolution of the separation taking full advantage of sub-2-µm particle columns and ultra performance liquid chromatography systems. One consequence of improving and/or shortening chromatographic analysis times in the analysis of veterinary drug residues is that the laboratory throughput bottleneck has now moved from analysis time to sample preparation time. This work presents the use of a novel sorbent for the effective clean-up of seafood for the analysis of multi class veterinary drug residues. The removal of excessive amounts of phospholipids can thus aid in extending LC column life and minimise ion-suppression and contamination in the mass-spectrometer.

Introduction
In order to ensure public health and safety, reliable analytical methods are necessary to determine veterinary drug residue levels in edible tissue samples such as fish and shellfish and infant formula powder. The compounds of interest range from polar water-soluble compounds to non-polar fat-soluble compounds. In order to maximise throughput and minimise costs it is desirable to determine the widest possible range of veterinary drug residues in tissue samples with a single analytical method.

The major constituents of a typical meat or seafood sample are water (up to 70%), protein (15-25%), fat (5-25%) and phospholipids (lecithin, 1-3%) [1]. Typically, during sample pre-treatment the protein is removed from the sample extract by precipitation and centrifugation. However, significant amounts of fat and phospholipid are co-extracted along with the target veterinary drugs. The presence of these co-extracted substances can lead to interference in the LC-MS analysis, contamination of the analytical column and other components of the LC system along with contamination of the mass spectrometer itself. Fats have traditionally been removed from tissue extracts using cumbersome hexane defatting steps or by the use of reversed-phase sorbents such as C_{18}-silica. Although these techniques may be effective for fat removal, neither of these procedures removes phospholipids.

In this study, sample preparation, clean-up and analysis protocols were developed for LC-MS/MS determination of a wide variety of veterinary drug residues in seafood tissue samples. This clean-up protocol was effective for the removal of both fats and phospholipids. Two types of tissue samples, shrimp (prawn) and salmon were chosen to demonstrate the suitability of the methodology. Samples were treated with an acidified acetonitrile/water solvent to precipitate proteins and to extract the veterinary drugs of interest. Then, a simple clean-up was performed using a novel SPE device, the Oasis PRiME HLB Cartridge. Representative compounds were chosen from major classes of veterinary drugs including tetracyclines, fluoroquinolones, sulfonamides, macrolides, β-lactams, NSAIDS, steroids and β-andrenergics. These compounds were spiked into the seafood samples prior to extraction and clean-up.

Materials and Methods

Sample clean up and analyte extraction
The procedure utilised in this study was developed from methods presented by Lehotay and refined by Tran [2-4]. Homogenised tissues were weighed (2.5 g) into a centrifuge tube (50 mL). Matrix-matched calibration standards and QC standards were generated by spiking the samples with appropriate amounts of desired analytes to generate a five-point curve and two QC samples at the concentrations outlined in Table 1. Precipitation and analyte extraction was carried out using 0.2% formic acid in 80:20 acetonitrile:water (10 mL). The sample was vortexed for 30 s and placed on mechanical shaker for 30 min. The sample was then centrifuged at 12,000 rpm for 5 min.

Solid-phase extraction clean-up was applied using Oasis PRiME HLB Cartridge (3 cc, 60 mg) and a pre-cleaned vacuum manifold. No cartridge conditioning was carried out. The vacuum was set to 1–2 psi. The supernatant (0.5 mL) was loaded and the pass through eluate was collected. An aliquot of each eluate (0.3 mL) was diluted three fold in aqueous 10 mM ammonium formate buffer (pH 4.5) and presented for UPLC-MS/MS analysis.

Chromatography and mass spectrometry

Chromatographic separation was achieved using an ACQUITY UPLC CSH C_{18} (100 x 2.1 mm, 1.7 µm) column on an ACQUITY UPLC IClass. A gradient was applied over 7 min, at a flow rate of 400 L min^{-1} using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), as follows: starting at 85% A with a linear decrease to 60% A over 2.5 min, drop to 5% over 1.4 min and hold for 1 min. Linear increase to 85% A in 0.1 min and allow to equilibrate for 2 min. The column temperature was maintained at 30°C and 5 µL injection volume was applied.

The UPLC inlet was coupled with a XEVO TQ-S tandem quadrupole mass spectrometer in electrospray ionisation mode, employing polarity switching. Time windowed MRM transitions were generated utilising QuanPedia, as detailed in Table 1. Various MS source conditions were applied to ensure ionisation of the analytes of interest; including source temperature (150°C), desolvation temperature (500°C), desolvation gas flow (1000 L hr^{-1}) and collision gas flow (0.15 mL min^{-1}).

Table 1. Summary of MS/MS transitions and matrix matched spiking for a selection of analytes.

Compounds	MRM	Cone (V)	Collision (eV)	Spike Level (low, high) µg/kg	Calibration Range µg/kg	Corr (R^2)	RT
Amoxicillin	366.2>349.1	30	8	12.5, 50	6.25–100	0.9978	0.70
	366.2>114.0	30	20				
Carbadox	263.0>231.0	25	15	25, 100	12.5–200	0.9978	1.43
	263.0>145.0	25	20				
Ceftiofur	524.3>241.1	30	16	250, 1000	125–2000	0.9975	2.84
	524.3>285.0	30	16				
Chloramphenicol	321.0>152.1	30	17	25, 100	12.5–200	0.9943	1.64
	321.0>257.1	30	15				
Chlortetracycline	479.3>444.2	30	21	25, 100	12.5–200	0.9955	0.97
	479.3>462.2	30	18				
Ciprofloxacin	332.1>288.1	30	18	25, 100	12.5–200	0.9918	2.99
	332.1>231.1	30	40				
Cortisol	363.2>121.0	42	52	50, 200	25–400	0.9989	3.45
	363.2>91.03	30	22				
Dexamethasone	393.2>373.2	30	10	25, 100	12.5–200	0.9980	1.09
	393.2>355.3	30	15				
Enrofloxacin	360.4>245.0	50	25	50, 200	25–400	0.9961	2.26
	360.4>316.1	50	25				
Erythromycin	734.4>158.1	30	32	2.5, 10	1.25–20	0.9982	0.61
	734.4>576.5	30	20				
Lincomycin	407.2>126.1	36	34	12.5, 50	6.25–100	0.9931	1.03
	407.2>359.3	36	20				
Lomefoxacin	352.1>265.1	31	22	50, 200	25–400	0.9960	3.79
	352.1>308.1	31	16				
Oxacillin	402.2>160.0	30	12	25, 100	12.5–200	0.9974	1.06
	402.2>243.1	30	15				
Oxytetracycline	461.2>426.2	30	21	25, 100	12.5–200	0.9952	1.06
	461.2>443.1	30					
Penicillin	335.2>160.1	20	30	12.5, 50	6.25–100	0.9903	3.46
	335.2>176.1	20	30				
Phenylbutazone	309.4>160.0	37	20	25, 100	12.5–200	0.9915	4.29
	309.4>103.9	37	20				
Ractopamine	302.2>164.1	30	15	75, 300	37.5–600	0.9915	1.03
	302.2>107.0	30	27				
Salbutamol	240.2>148.1	30	20	25, 100	12.5–200	0.9907	0.61
	240.2>222.1	30	12				
Sulfamerazine	265.0>92.0	30	28	25, 100	12.5–200	0.9918	0.91
	265.0>156.0	30	15				
Sulfamethazine	279.1>186.0	30	16	25, 100	12.5–200	0.9971	1.56
	279.1>92.0	30	28				
Sulfanilamide	156.0>92.0	30	15	25, 100	12.5–200	0.9977	1.73
	156.0>65.0	30	25				
Tetracycline	445.3>154.0	30	26	25, 100	12.5–200	0.9970	1.15
	445.3>410.2	30	21				
Tylosin	916.5>174.1	57	40	5, 20	2.5–40	0.9938	2.48
	916.5>101.1	57	45				

Results and Discussion

Table 2 shows the recovery data obtained from replicate analysis of spiked tissue samples. The use of RADAR full scan data acquisition demonstrated the effectiveness of the Oasis PRiME HLB Cartridge for removal of ≥95% of phospholipids from the shrimp extracts. The cartridge also removes more than 90% of hexane extractable fat, all in a simple pass through step.

Matrix effects averaged about 40% for both shrimp and salmon. The overall method recoveries are generally above 70% but significantly lower recovery was observed for some of the more polar compound classes, such as tetracyclines. However, the Oasis PRiME HLB cartridge clean-up contributes very little to any method recovery losses.

Table 2. Summary of recovery data obtained from replicate analysis of spiked tissue samples, where low and high spiking concentrations refer to information in Table 1 above.

	Shrimp				Salmon			
	Low level		High Level		Low Level		High Level	
Compounds	Recovery %	RSD(%) n=6	Recovery %	RSD(%) n=6	Recovery %	RSD(%) n=6	Recovery %	RSD(%) n=6
Amoxicillin	BLOQ	–	67	18	BLOQ	–	59	17
Carbadox	113	9	75	10	85	5	84	7
Ceftiofur	111	7	84	6	64	4	67	4
Chloramphenicol	106	7	77	12	79	7	69	10
Chlortetracyclin	79	7	63	17	67	5	65	7
Ciprofloxacin	190	14	103	15	109	9	95	4
Cortisol	99	8	80	6	82	4	82	4
Dexamethasone	112	9	79	7	89	8	79	6
Enrofloxacin	90	12	71	12	86	4	84	8
Erythromycin	110	7	83	8	85	9	86	7
Lincomycin	104	6	99	6	90	4	92	3
Lomefloxacin	126	11	90	11	97	4	92	5
Oxacillin	115	5	86	2	71	2	74	5
Oxytetracyline	125	11	92	7	83	5	76	4
Penicillin	112	10	86	6	70	10	71	6
Phenylbultazone	78	10	51	8	51	7	51	3
Ractopamine	102	9	87	8	87	3	90	4
Salbutamol	115	7	89	4	92	12	93	3
Suflanilamide	BLOQ	–	82	17	BLOQ	–	95	12
Sulfamerazine	107	7	91	7	83	3	77	12
Sulfamethazine	102	8	85	9	82	3	78	8
Tetracyline	106	7	77	12	79	7	69	10
Tylosin	116	10	98	4	76	7	87	3

Conclusions

A simple one-step pass-through clean-up protocol using Oasis PRiME HLB Cartridges was employed to remove greater than 90% of fats and phospholipids from the initial extracts of seafood commodities, allowing for accurate and repeatable quantification of a variety of multi class veterinary drug residues by UPLC-MS/MS.

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A NOVEL MULTISPOT ELISA FOR ANTIBIOTIC SCREENING

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Abstract
This study describes the development and performance of a preliminary investigation using a novel multispot ELISA for antibiotic screening in honey. Using a multiplex approach, the metabolites of the four main nitrofuran antibiotics (AMOZ, AOZ, SEM and AHD) and chloramphenicol (CAP) were simultaneously detected. Antibodies specific to the five antibiotics were printed onto the wells of a microtitre plate and a competitive assay format was employed. The performance of this assay was evaluated for feasibility as a screening tool for antibiotic determination in honey in order to replace the traditional ELISA. Single and five-plex calibration curves were examined in PBS buffer to determine feasibility. Finally, a multiplex curve was assessed in honey with IC_{50} of 1 ng g^{-1} for CAP, 4 ng g^{-1} for AMOZ and AOZ, approximately 100 ng g^{-1} for SEM and > 1,000 ng g^{-1} for AHD. This work has demonstrated the potential of multiplex analysis for antibiotics with results available for 40 samples within a 90 min period for analytes sharing a common sample preparation. This method has the potential to be used as a fit for purpose screening technique for antibiotics within the area of residue determination and food safety. Further improvements in the performance of the multiplex microarray especially for SEM and AHD will show a reduction in cost and time as well as high throughput screening of samples.

Introduction
Antibiotics are a vital component for the treatment and elimination of disease in human, plants and animals. Antibiotic residues from agricultural use must be carefully monitored as they can adversely impact public health due to allergenic and carcinogenic factors and may contribute to bacterial resistance. Apiculture relies on antibiotics to prevent disease spreading through bee colonies, however, the overuse of these antibiotics can cause residues in honey products. Honey is one of the many foods that are monitored for antibiotic residues such as nitrofuran drugs and CAP worldwide.

The use of the nitrofuran drugs and CAP in food-production has been banned within the European Union since the 1990s. The four main nitrofuran antibiotics are furazolidone, furaltadone, nitrofurantoin and nitrofurazone. Nitrofurans are quickly metabolised and have *in vivo* half-lives of only a few hours, therefore, they must be identified by their metabolites. These metabolite residues are 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), 1-amino-hydantoin (AHD) and semicarbazide (SEM), respectively. Honey producers, importers, exporters and regulators, therefore, need simple, fast and effective ways to test honey for antibiotics from nitrofurans and CAP.

A key role in the development of sensitive methods for nitrofuran metabolites and monitoring was developed by the EU FoodBRAND project. Within this project the first assays for nitrofurans were developed with both screening and confirmatory tests to measure tissue-bound nitrofuran residues (Cooper *et al*., 2004a, 2004b, Cooper *et al*., 2005). Analytical procedures for nitrofuran analysis in various matrices and screening and confirmation methods with respect to EU regulations are reviewed in Vass *et al*. (2008).

Multiplex assays are tools enabling the detection of multiple targets in one single test. They involve multiple antibody and target partners, each one having its own optimum working conditions. Furthermore, there is a probability of cross-talking interferences with the other targets within the assay which must be reduced or eliminated for a successful multiplex assay. Multiplex assays for nitrofurans include both a screening (O'Mahony *et al*., 2011) and an analytical method (Kaufmann *et al*., 2015) for the detection of all four nitrofurans simultaneously. A multiplex test for the detection of both the nitrofuran drugs and CAP are also available based on LC-MS/MS for shrimp samples (An *et al*., 2015, El-Demerdash *et al*., 2015, Veach *et al*., 2015). The development of sensitive, multi residue, rapid and high throughput screening methods are therefore important in the area of food safety and residue determination. The aim of this research was to evaluate the performance of a multispot ELISA as a screening tool for detecting five key banned antibiotics (AMOZ, AOZ, SEM, AHD and CAP) in honey samples in order to replace the traditional ELISA test kits.

Materials and Methods

Instrumentation
A sciFLEXARRAYER S5 (Scienion, Berlin, Germany) was used for spotting microtitre plates and a sciReader CL colorimetric microarray array reader (Scienion, Berlin, Germany) was used for scanning and analysing spot intensities.

Reagents
Antibodies and HRP conjugates for AMOZ, AOZ, SEM, AHD and CAP were provided by Tecna (Trieste, Italy). sciCOLOR T2 TMB/E was purchased from Scienion (Berlin, Germany). CAP, 2-NP-AMOZ, 2-NP-AOZ, 2-NP-SEM and 2-NP-AHD were all purchased from Sigma-Aldrich (Dorset, UK). Nunc microtitre plates were purchased from Fisher Scientific (Loughborough, UK).

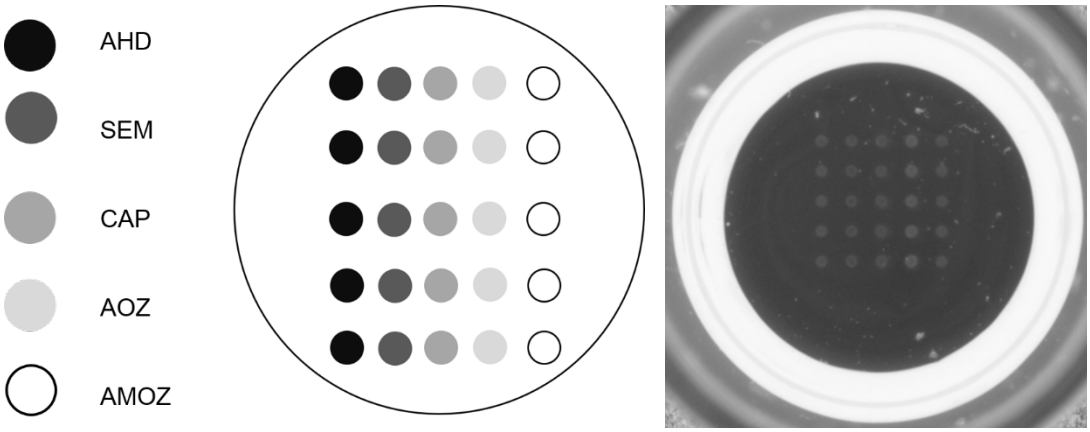

Figure 1. Layout of a well of a microtitre plate showing the printed antibiotics in a 5 x 5 multiplex microarray and a scanned image of a well using the Scienion reader.

Printing microarrays
Antibody solutions for each antibiotic were printed at a printing volume of 1 nL onto a microtitre plate using a sciFLEXARRAYER S5 spotter. For part one of the study (single spotting analysis) a 9 spot matrix format (3x3 array) was arrayed with a 1,000 µm spot to spot pitch composing of 9 replicates of each target in separate wells. For part two of the study (multi spotting analysis) a 25 spot matrix format (5x5 array) was arrayed with a 500 µm spot to spot pitch composing of 5 replicates of each target in the same well (Figure 1). After printing, the microtitre plates were incubated overnight at a humidity of 35%.

Assay Protocol
The printed microtitre plate was washed 4 times with ELISA wash solution and dried with lint free paper. Enzyme labelled HRP conjugate (50 µL) and sample/standard (50 µL) were applied to each well and incubated for 60 min at room temperature. The microtitre plate was washed 4 times with ELISA wash solution and dried with lint free paper. TMB/E (50 µL) was added to each well and incubated for 30 min at room temperature. The microtitre plate was washed twice with ELISA wash solution and dried with lint free paper. Finally, each well of the microtitre plate was scanned using the Scienion colorimetric reader (Figure 1).

Sample Preparation
Hexane (5 mL), 1 M HCl (0.5 mL) and distilled water (4 mL) was added to honey (1 g) and mixed with a vortex for 1 min. The sample was centrifuged at 3,000 *g* for 10 min and frozen at -80°C for 2 h in order to separate the phases. The upper phase was eliminated and the lower aqueous phase was allowed to defrost. 2-Nitrobenzaldyde (10 mM) in DMSO (200 µL) was added to the aqueous phase and the sample was incubated overnight at 37°C. Dipotassium phosphate (0.1 M, 5 mL), NaOH (1 M, 0.4 mL) and ethyl acetate (5 mL) were added to the sample and vortexed for 1 min. The sample was centrifuged at 3,000 *g* for 10 min. The upper organic phase (2.5 mL) was transferred to a glass test tube and evaporated at 50 – 60°C under a slow nitrogen stream. Finally, the residue was dissolved in 1 mL PBS (10 mM, pH 7.4).

Results and Discussion

Single and multi-calibration curves
An eight-point single calibration curve was prepared in PBS buffer (10 mM, pH 7.4) for each antibiotic (AMOZ, AOZ, SEM, AHD and CAP) and assessed as a single system (only one antibiotic printed) with no interaction from any other antibiotic. Single calibration curves were also assessed on microtitre plates printed as a five-plex system to see any interactions or cross reactivity with the other four printed antibiotics (Figure 2a). Finally, an eight point multi antibiotic (AMOZ, AOZ, SEM, AHD and CAP) calibration curve was prepared in PBS buffer (10 mM, pH 7.4) for an evaluation of each antibiotic as a five-plex multi system (Figure 2b). CAP showed similar IC_{50} concentrations between single and multi calibration curves while the IC_{50} increased approximately 3 times for both AMOZ and AOZ. Similar curve shapes were evident between singe and multiplex analysis.

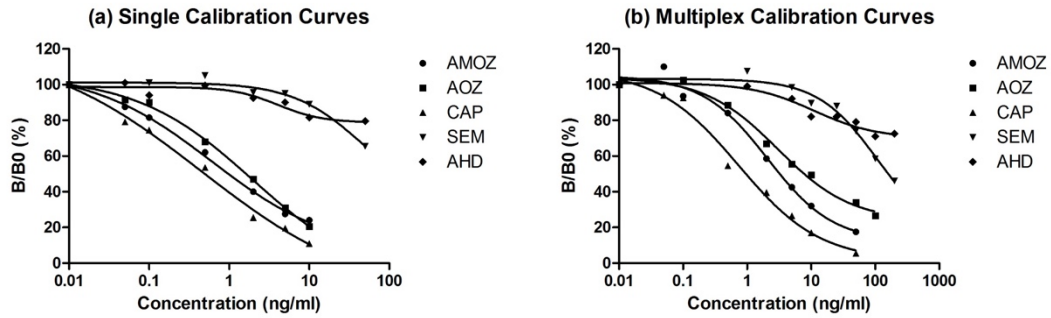

Figure 2. Single (a) and multiplex (b) calibration curves x 5 antibiotics (AMOZ, AOZ, CAP, SEM and AHD) in PBS buffer using a multiplex x 5 antibiotics printed microtitre plate (n=2 wells, 5 spots per well).

Matrix Effects

Three calibration curves were prepared for a full evaluation of matrix effects (Figure 3) including PBS buffer (10 mM, pH 7.4), extracted honey (spiked before extraction) and extracted honey (spiked after extraction to blank honey extract). A multiplex extraction was carried out with all five antibiotics (AMOZ, AOZ, SEM, AHD and CAP) spiked in the samples. Very little matrix effects were evident with SEM and AHD the only two antibiotics which showed an increase in the IC_{50} concentration.

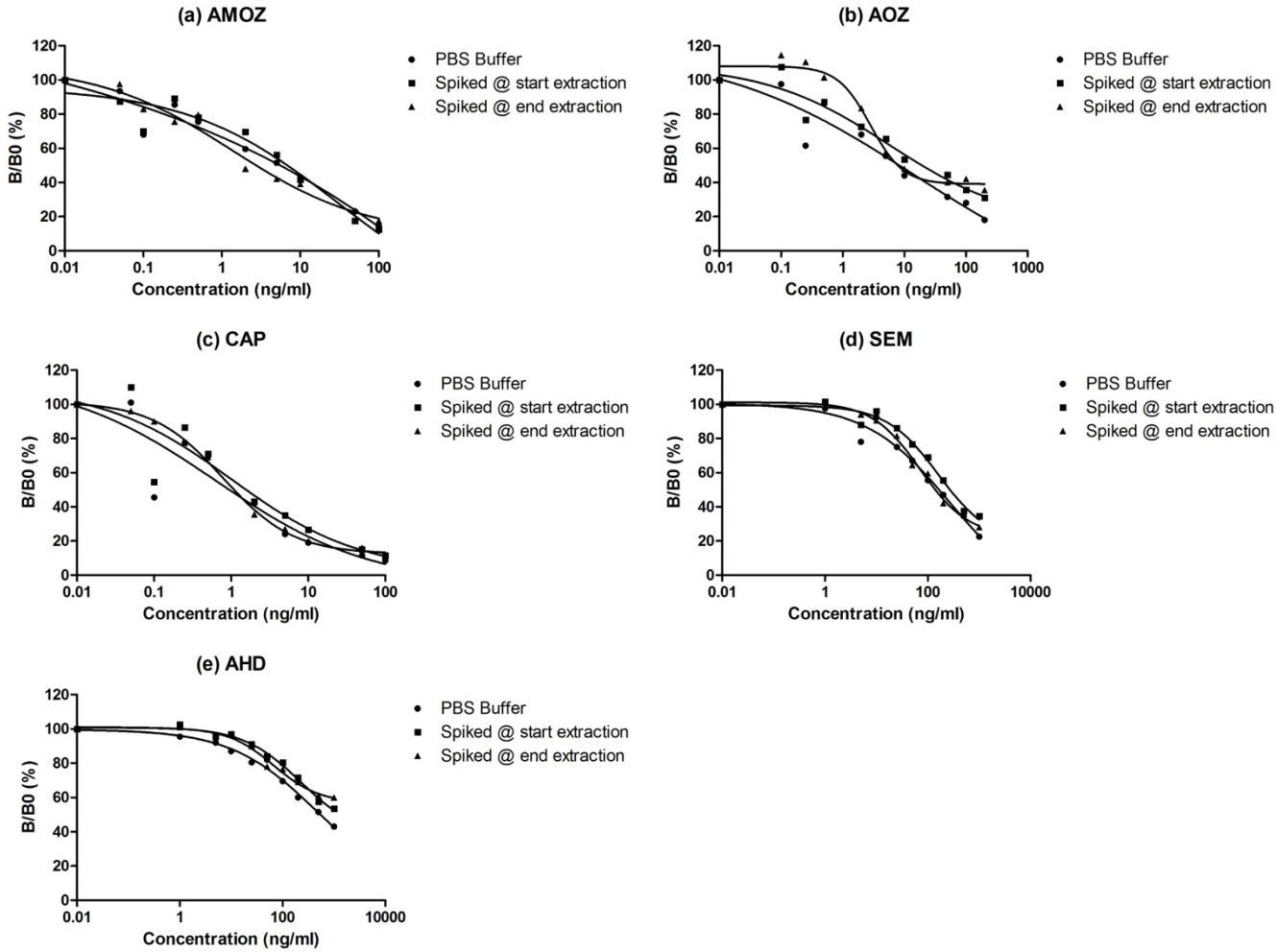

Figure 3. Matrix effects for multiplex x 5 antibiotics (AMOZ, AOZ, CAP, SEM and AHD) in PBS buffer, honey samples spiked at the start of the extraction and honey samples spiked at the end of the extraction to blank honey extract using a multiplex x 5 antibiotics printed microtitre plate (n=2 wells, 5 spots per well).

Sensitivity

Sensitivity was assessed by examining the IC_{50} of the assay and in cases where a full calibration curve was not achieved the 50 % inhibition of the assay (calculated from the 0 ng mL^{-1} standard) was used instead (Table 1). The IC_{50} was 0.7 ng mL^{-1} (AMOZ) and 1.1 ng mL^{-1} (AOZ) for single curve analysis (PBS). This increased to 3.0 ng mL^{-1} (AMOZ) and 3.5 ng mL^{-1} (AOZ) in a multiplex system (PBS) with a small increase to approximately 4 ng g^{-1} for both AMOZ and AOZ in a honey matrix. The IC_{50} was similar for CAP for both single and multi-analysis and when using both PBS and a honey matrix. The 50% inhibition level was used for both SEM and AHD because full calibration curves were not evident using the standards assessed. This was greater than 50 ng mL^{-1} for both SEM and AHD for single curve analysis in PBS (highest standard). For the multi calibration curve the calibration range was increased to 1,000 ng mL^{-1} for both SEM and AHD to obtain a better curve shape. The dynamic range for SEM was now much better and an IC_{50} of 79.2 ng mL^{-1} (multi analysis, PBS) was obtained, increasing to 100.5 ng g^{-1} in a honey matrix. For AHD the 50 % inhibition concentration was used as a full curve was still not evident. A concentration of 553.8 ng mL^{-1} (multi analysis, PBS) was obtained which increased to 1291.1 ng g^{-1} in a honey matrix.

Table 1. Mid-point (IC_{50}) for each antibiotic (AMOZ, AOZ, SEM, AHD and CAP) in both single and multiplex systems using both PBS buffer and a honey matrix (honey samples spiked at the start of the extraction and spiked at the end of the extraction to blank honey extract).

Antibiotic	Single Analysis PBS	Multi Analysis PBS	Multi Analysis Honey Spiked at start of extraction	Multi Analysis Honey Spiked at end of extraction
AMOZ	0.7	3.0	4.1	1.4
AOZ	1.1	3.5	4.0	3.5
CAP	0.7	0.8	1.1	0.9
SEM	> 50*	79.2	100.5	64.1
AHD	> 50*	553.8*	1291.1*	>1,000*

**A full calibration curve was not evident; therefore the 50 % inhibition was calculated from the 0 ng mL^{-1} standard.*

Conclusions

A sensitive multispot ELISA has been developed for the simultaneous screening of CAP, AOZ and AMOZ in honey. These antibiotics were successfully multiplexed into one assay. Antibodies for SEM and AHD were not as sensitive as for the other antibiotics and further improvements to improve the sensitivity to make this multi assay more applicable for regular testing for these antibiotics would be of benefit. Additionally, SEM and AHD showed cross reactivity with other printed antibodies. SEM was detected by the AMOZ, AOZ and AHD printed antibodies and AHD was detected by the AOZ printed antibody. This work has demonstrated the potential of multiplex analysis for antibiotics with results available for 40 samples within a 90 min period (after extraction). This technique has the potential to be used as a multi target screening technique for antibiotics within the area of residue determination and food safety and has the potential to be an advancement in the field of antibiotic detection.

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VALIDATION OF 4SENSOR® MILK KIT060 FOR THE TESTING OF RAW MILK ON THE PRESENCE OF B-LACTAMS, TETRACYCLINES, (DIHYDRO)STREPTOMYCIN AND CHLORAMPHENICOL

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Abstract
The 4SENSOR® Milk KIT060 (Unisensor s.a., Ougrée, B) is a fast and simple test for the control of residues of antibiotics in raw cows' milk on Russian Federation level (Customs Union, 2011). The 4SENSOR® Milk KIT060 was validated at ILVO-T&V on its applicability as a screening test according to Commission Decision 2002/657/EC and CRL Guidelines (*Anon*., 2010).
The kit demonstrated a detection capability profile totally in line with Russian Federation Maximum Permissible Levels (MPL, Customs Union Commission 2010), namely detection of penicillins at 4 µg kg^{-1}, tetracyclines at 10 µg kg^{-1}, streptomycin at 200 µg kg^{-1} and chloramphenicol at 0.3 µg kg^{-1}. The kit was efficient in different normal raw cows' milks and in specific extreme milks specially selected (or prepared) for this study (somatic cells, total bacterial count, fat, protein, pH). In some cases extreme milk caused false positive results (high somatic cell content or a low pH of 6.0) or a decreased detection capability (low protein content). In addition, the kit demonstrated an overall good performance on a wide panel of 300 milk samples from different Belgian farms as well as during two national ring test trials. However, it is recommended to confirm a positive result by repeating the test with a second dipstick.

Introduction

The 4SENSOR® Milk (KIT060) (Unisensor s.a., Ougrée, B) is a competitive antibody/receptor test for the rapid and simultaneous detection of residues of β-lactams, tetracyclines, dihydrostreptomycin, streptomycin and chloramphenicol (CAP) in raw milk. The time-to-result for the assay is approximately 10 min; the test procedure consists of two steps but the dipstick is introduced automatically using a HeatSensor (Unisensor s.a.) device, so in this way we can consider the test as a one step assay.
A validation study was performed at ILVO-T&V (Technology & Food Science Unit of the Institute for Agricultural and Fisheries Research of the Flemish Community) according to Commission Decision 2002/657/EC and to the CRL (Community Reference Laboratories) guidelines for the validation of screening methods for residues of veterinary medicines (*Anon.*, 2010).

Materials and methods

Test procedure
The appropriate number of cups with freeze-dried antibody/receptor is taken from the reagents' box and the cups are opened. To each microplate cup containing the dry reagents 200 µL of milk is added. The milk is mixed with the reagents by means of the pipettor. The cups are placed in a pre-heated HeatSensor, a specially designed block-heater and the timer activated for an incubation for 10 min at 40°C. The dipsticks are placed in a small device on top of the incubator. After some time, the dipsticks are automatically dropped in to the cups. At the end of the incubation the colour formation of the different test lines on the dipstick is interpreted. The ReadSensor (Unisensor s.a.) reader measures the colour formation at each test line and control line position and the LF Studio software calculates the ratio test line / control line. Calculation is obtained based on the measurement of the area of the colour formation. Ratio values ≤1.10 (≤1.25 for the CAP line) are considered as 'positive' for the antibiotic family/compound detected by the test line concerned; ratio values >1.10 (>1.25 for the CAP line) are considered as 'negative' or absence of the respective drug. The visual interpretation is as follows: when the colour of the test line is more intensive than the colour of the control line, the milk sample is negative ('no drug residue detected') for the group of antibiotics/substance concerned. In all other cases (test line is equal or less in intensity or missing) the milk is contaminated with drug residues of the group of antibiotics/substance concerned.

Detection capability
The detection capability of the 4SENSOR® Milk (KIT060) for all β-lactams and all tetracyclines listed as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010 and also for (dihydro)streptomycin and chloramphenicol was determined in this validation study. Each compound was individually spiked in blank raw milk at different concentrations. The increment between the different concentrations was depending on the concentration level in relation to the MRL, as indicated in Table 1. The blank raw milk was originating from four cows in mid-lactation that were not treated with antibiotics or chemotherapeutics during the last months.

Table 1. Increment between the concentrations tested.

Concentration (in µg kg^{-1})	Increment (in µg kg^{-1})
1-10	1
10-20	2
20-50	5
50-100	10
100-250	25
250-500	50
500-1,000	100
1,000-5,000	500

According to the CRL guidelines for the validation of screening methods (*Anon*., 2010), each concentration was tested 20, 40 or 60 times, respectively, in a time period of at least three days and by different technicians. The number of replicates is related to the closeness of the tested concentration to the MRL (Maximum Residue Limit, Commission Regulation 37/2010) or MRPL (Minimum Required Performance Limit, Commission Decision 2003/181/EC):

concentration tested is <0.5×MR(P)L: 20 replicates
concentration tested is 0.5 - 0.9×MR(P)L: 40 replicates
concentration tested is 0.9 - 1.0×MR(P)L: 60 replicates
concentration tested is >MR(P)L: 20 replicates

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Test robustness

In order to study the potential influence of the milk quality and composition on the performance of 4SENSOR® Milk (KIT060) results, individual normal and extreme raw cows' milk samples were collected and analysed in 10 replicates (blank and spiked (3 µg kg^{-1} benzylpenicillin, 10 µg kg^{-1} oxytetracycline, 200 µg kg^{-1} dihydrostreptomycin and 0.3 µg kg^{-1} chloramphenicol, respectively) for each test line of the dipstick. The milk parameters that were investigated are somatic cell count (>10^6 somatic cells per mL), total bacterial count (>$5 \cdot 10^5$ CFU per mL), fat content (<2 and >6 g per 100 mL), protein content (<3 and >4 g per 100 mL), and pH (6.0 and 7.5).

The false positive rate was determined by testing 300 different Belgian farm milk samples on the 4SENSOR® Milk. Finally, the test was used in two national ring trials.

Results and Discussion

Detection capability

Altogether, the 4SENSOR® Milk (KIT060) detects at least 23 compounds from the four different antibiotics groups at or below respective MRL levels or MRPL. The β-lactams nafcillin and cephalexin are missed at MRL but detected from a higher concentration, more precisely from 3× and 9× MRL on, respectively. Phenoxymethylpenicillin was detected at 3 µg kg^{-1}. No MRL for phenoxymethylpenicillin in milk has been established; phenoxymethylpenicillin is not registered for dairy cows.

The detection capability of the 4SENSOR® Milk (KIT060) for β-lactams, tetracyclines, (dihydro)streptomycin and chloramphenicol is shown in Figure 1. The detection capabilities for all four antibiotics families fit with Russian Federation requirements (tetracyclines: based on the parent drugs and not the 4-epimers). These detection capabilities fit also with European MRLs (except for nafcillin and cephalexin).

It is worth noting that the kit manufacturer has the intention to modify the detection capability of the kit for (dihydro)streptomycin to bring the CCβ closer to the MRL of 200 µg kg^{-1}.

Test robustness

Regarding the β-lactam test line, for the eight extreme different types of milks (×10 replicates) investigated in this study, all blank samples gave negative results except for some milks containing a very high number of somatic cells or a very low pH for which there is a risk of a false positive result. It is worth noting that most of these samples (and their mean value) gave correct negative results and that so extreme compositions/parameters are not likely to occur in bulk commingled milks from tanks and trucks, having a more normal composition/quality. Regarding the β-lactam spiked samples, milks containing an extreme low level of protein content showed significantly higher ratio results indicating a slightly decreased detection capability for β-lactam and led to false negative results for samples containing 3 µg kg^{-1} of benzylpenicillin (MRL and Customs Union MPL = 4 µg kg^{-1}). All the other types of milk spiked with β-lactams did show correct positive results in this experiment.

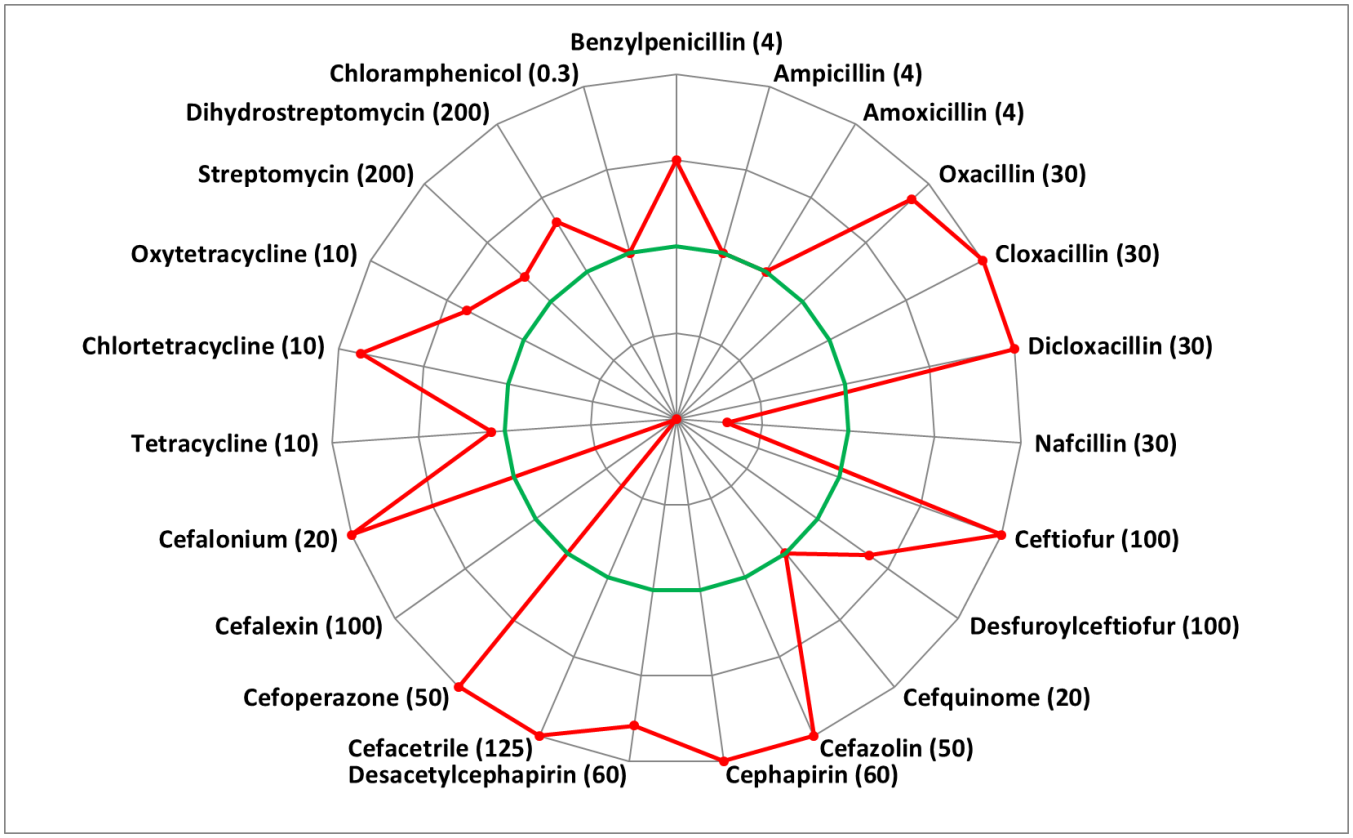

*Figure 1. Detection capability of 4SENSOR Milk (KIT060) for β-lactams, (dihydro)streptomycin, and chloramphenicol related to their respective MR(P)L (Commission Regulation (EU) N° 37/2010 and Commission Decision 2003/181/EC) or Customs Union MPL (Customs Union Commission 2010) and for tetracyclines related to their respective Customs Union MPL; legislation situation on 01/01/2016. Inner circle = 2 × MR(P)L or MPL; circle 2 = **MR(P)L or MPL**; circle 3 = 0.5 × MR(P)L or MPL; circle 4 = 0.25 × MR(P)L or MPL. MR(P)L or MPL (µg kg^{-1}) in cows' milk in between brackets after the name of each substance. Results obtained with Readsensor and cut-off ratio = 1.10 except for chloramphenicol (cut-off ratio = 1.25).*

Regarding the tetracycline test line, for the eight extreme different types of milks (×10 replicates) investigated in this study, no significant impact was observed on the performance of the test which gave correct negative and positive answers for all blank and spiked samples (but giving slightly more variable results in the samples containing extreme high level of somatic cells, compared to milk with a normal composition/quality and the other milk types).

Regarding the streptomycin test line, for the eight extreme different types of milks (×10 replicates) investigated in this study, all blank samples and spiked samples gave correct negative and positive results, respectively, except for two samples: one border line false positive result was observed for a milk sample with an extreme high number of somatic cells and one false negative result was observed for a milk sample containing an extreme low fat level and spiked with 200 µg kg^{-1} of dihydrostreptomycin.

Regarding the chloramphenicol test line, for the eight extreme different types of milks (×10 replicates) investigated in this study, all mean values obtained for the different populations of milks were as expected higher than the cut-off value (negative result) for blank samples and below the cut-off value (positive result) for spiked samples. However, false positive results were observed in some samples containing a very high number of somatic cells and a very high fat content. In the same way, a borderline false positive result was observed for a blank sample with a very high protein content.

In the opposite way, the chloramphenicol detection in some spiked samples was hampered for some extreme milks containing a low protein level, a low fat level or a high total bacterial count. In a lesser extent, a borderline false negative result was observed for one sample with a high fat content spiked with chloramphenicol at 0.3 µg kg^{-1}.

In summary, the three extreme parameters that affected the test the most were:

A) a high somatic cell content >10^6 per mL, usually giving a wider variability of results with a tendency of ratio decrease that can lead to some false positive results for β-lactams and chloramphenicol;
B) a low protein content <3 g per 100 mL, that gives a tendency of ratio increase showing less detection capability and some false negative results for β-lactams (benzylpenicillin at 3 µg kg^{-1}) and chloramphenicol (0.3 µg mL^{-1}) detection;
C) a low pH of 6.0, that can in a general way increase the detection capability of the test for β-lactam and tetracycline detection.

It is worth noting that such "extreme" milks that induced few cases of false positive results (somatic cells >10^6 per mL, pH 6), are normally not met in usual test situations and still less in bulk milk from large farm tanks or tanker silo. As an example a usual range of normal pH for milk is 6.6-6.9 and a normal mean somatic cell count for Belgian farm farms is 190,000 to 220,000 cells mL^{-1} with a limited percentage of samples showing more than 400,000 cells per mL. Extreme higher somatic cell counts (>10^6 per ml) could indeed impact on the performance of the dipstick with a risk of false positive results probably by influencing the flow or the dynamic of flow migration in these special milks.

During the screening of 300 different blank farm milk samples, no false positive result was observed for the chloramphenicol, β-lactam and streptomycin test lines. For 10 out of the 300 samples (3.3%); one strongly positive and nine weakly positive results were obtained on the tetracycline line, but when retested with another dipstick all gave a negative result. In the software program the correct positioning of the strips was checked, which was always the case.

So, it is advised to retest the sample with a second dipstick after a positive tetracycline result to confirm the positive test result.

Very good results in line with the detection capabilities and no false positive results on any of the four channels were obtained with 4SENSOR® Milk reagents in two national ring trials (Ooghe and Reybroeck, 2013 and 2014).

Conclusions

The 4SENSOR® Milk KIT060 demonstrated a detection capability profile for β-lactams, tetracyclines, streptomycin and chloramphenicol compounds totally in line with Russian Federation levels. This kit is therefore appropriate for the screening of raw cows' milk for milk industry inside Russia, Belarus and Kazakhstan as well as for European factories exporting milk to the Customs Union. Some interference was found caused by an abnormal high number of somatic cells (> 10^6 per mL). However, it is recommended to confirm a positive tetracycline result by repeating the test with a second dipstick.

Acknowledgements

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THE IMPACT OF ENZYMATIC HYDROLYSIS IN THE PROPER QUANTIFICATION OF RACTOPAMINE AS A DRUG RESIDUE IN SWINE MUSCLE MATRIX

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Abstract
Ractopamine is used as animal feed additive, but its adverse effects led to the necessity of the current monitoring programs of ractopamine residues in food products. In some countries, the maximum allowed limit is as low as 0.1 µg kg^{-1}, so its reliable quantification is indispensable. Ractopamine undergoes glucuronidation and the proportion of ractopamine conjugates is important in swine liver and kidney, but there is no information on muscle. Literature describes ractopamine assays using enzymatic hydrolysis to release its free form and assays without deglucuronidation, which could underestimate the drug content. This work aims to verify if there is statistical difference between ractopamine analysis with or without hydrolysis. Ten samples of swine muscle naturally contaminated with ractopamine were extracted in triplicate, with and without hydrolysis step, purified by solid phase extraction and quantified by UPLC/MS-MS. Based on paired t-test, the hydrolysis was considered an essential step. However, an excellent quadratic correlation can estimate the results of this hydrolysis through the test results without enzymatic release. Ractopamine analysis by different techniques can lead to different results and a proficiency test is required to reveal which method gives a proper quantification.

Introduction
Ractopamine (RAC; Figure 1) is a phenylethanolamine derivative that has been approved for use as a feed additive in some countries to enhance leanness in animal selected species. It is used with the purpose of increasing the rate of weight gain, improving feed efficiency and increasing carcass leanness. Although these benefits, the use of RAC has been related to adverse effects in cardiovascular and central nervous system (Yaeger *et al*., 2012; Zaitseva *et al*., 2014).

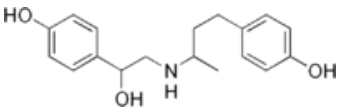

Figure 1. Structural formula of ractopamine

RAC is currently banned for use in several countries, therefore, there are monitoring programs of Brazilian meat intended for export. The limit of 10 µg kg^{-1} is used as the reference limit for regulatory action in analyses of the National Plan of Residues and Contaminants Control of the Ministry of Agriculture, Livestock and Supply. For other special monitoring programs, the reference limit is adopted necessary and appropriate for the purpose. In the case that Brazilian meat is intended for export to countries where the use of RAC is prohibited, the maximum residue limit (MRL) is 0.1 µg kg^{-1}.

Metabolism studies of RAC have been conducted in rats, dogs, pigs and cattle and showed that the drug undergoes glucuronidation in these animals. The studies showed that in swine liver and kidney matrices, the proportion of RAC conjugates has an important contribution (FAO FNP 41/16). Thus, it is necessary to realize to perform enzymatic hydrolysis in the analysis of RAC in those matrices, in order to release free RAC for extraction and determination. There is a lack of information on the ratios of parent drug to metabolites in other tissues, such as muscle. Despite this, RAC assays are available include enzymatic hydrolysis to release the free RAC form, and assays without this step, which could underestimate the real drug content if conjugates are present. Therefore, it would be interesting to know if enzymatic hydrolysis is relevant in muscle matrix. If it is proven to be relevant, the omission of this step would underestimate the true concentration of the analyte in the sample because only free RAC residues are quantified.

The goal of this work is to verify whether there is a difference between the analysis of of samples from naturally RAC-contaminated tissues, implementing or omitting enzymatic hydrolysis. Given the context of using RAC in animals in some countries and the need for its monitoring due to potential health risks, a reliable determination of RAC in swine matrices is of fundamental importance.

Materials and Methods

Reagents

All reagents were of analytical grade.

Procedure

The method used in the analysis was a validated adaptation of Liu *et al*. (2011). The method is based on the RAC extraction with acetate buffer, enzymatic hydrolysis with β-glucuronidase and a purification step that includes solid-phase extraction (SPE). The quantification is done by Ultra Performance Liquid Chromatography (UPLC) and Tandem Mass Spectrometry.

In the procedure of validation, two analytical curves were built. The first curve, lower concentrations, presented linearity in the range of 0.10 to 1.50 μg mL^{-1} (limit of quantification of 0.10 μg mL^{-1}) and second one, higher concentrations, in the range of 0.00 to 15.00 μg mL^{-1} (limit of quantification of 0.50 μg mL^{-1}).

Ten samples naturally contaminated with RAC were used in these experiments and one blank sample. Each sample was extracted and quantified in triplicate using enzymatic hydrolysis in the analysis and the same was done without using it. Based on the level of concentration, samples 1 to 5 were analysed in curve 1 and samples 6 to 10 in curve 2.

A second extraction experiment was done with five naturally contaminated samples using enzymatic hydrolysis with one difference: the duration of enzymatic hydrolysis was reduced to 2 h and the temperature of incubation was increased to 55°C. This experiment was done with the purpose to verify whether it is possible to reduce the time of enzymatic hydrolysis in order to get a faster time-to-result.

Chromatography separation was carried out on a 1200 Agilent LC System (Foster City, CA, USA). Mass spectrometric acquisition was carried out on an Applied Biosystems API 5000 triple quadrupole mass spectrometer (Foster City, CA, USA) operating in positive eletrospray (ESI) mode.

Paired t-test (Miller & Miller, 2010)

In situations when two different methods or conditions of analysis are compared by applying both of them to the same set of test materials, which contain different amounts of analyte, paired t-test is a powerful tool. Each batch is thus characterized by a pair of measurements, one value for each approach. Besides, to random measurement errors, differences between the analysis conditions may also contribute to the variation between the measurements. Here we wish to know if the different conditions produce significantly different results.

The test statistic calculates the difference, *d*, Equation (1), between each pair of results given by the two approaches. If there is no difference between the two approaches then these differences are drawn from a population with mean $\mu_d = 0$.$\mu_d = 0$. In order to test this null hypothesis, we test whether $\bar{d}$ $\bar{d}$ differs significantly from *0* using the statistic t_c.

$$t_c = \frac{\bar{d}}{\frac{s_d}{\sqrt{n}}} \quad (1)$$

$\bar{d}$ and s_d are the mean and standard deviation respectively of *d* values, the differences between the paired values. The number of degrees of freedom of *t* is $n-1$.

Correlation of determination (Massart et al*., 1997)*

The coefficient of determination, R^2, is very used often to show how much one variable can be predicted from the other variable. It is a measure that allows to determine the quality of a prediction from a certain model. The coefficient of determination is such that $0 \leq R^2 \leq 1$. This parameter represents the percent of the data that is the closest to the curve of best fit. For example, if the correlation coefficient *R* = 0.9500, then R^2 = 0.9025. This means that around 90% of the total variation in *y* can be explained by the relationship between *x* and *y* (as described by the fit). The other 10% of the total variation in *y* is not explained. The coefficient of determination can be calculated by Equation 2, based on ANOVA (Massart *et al*., 1997):

$$R^2 = \frac{SS_{Reg}}{SS_{Total}} \quad (2)$$

Considering *k* levels and *n* observations (Oliveira, 2012):

- $SS_R = \sum_i^k \sum_j^{n_i} \left(x_{ij} - \bar{x}_j\right)^2$;
- $SS_{Reg} = \sum_i^k n_i \left(\hat{x}_i - \bar{x}\right)^2$;

- $SS_{Total} = SS_R + SS_{Reg}$;
- $MS_{Reg} = SS_{Reg}/(k-1)$;
- $MS_R = SS_R/(n-k)$.

Results and Discussion

The results of the quantitation of RAC in naturally contaminated samples with or without using enzymatic hydrolysis (for a period of 16 h are summarized in Table 1, whereas those following 2 h or 16 h enzymatic hydrolysis are listed in Table 2.

Table 1. Results of quantitation of ractopamine according to extraction procedure - without or with enzymatic hydrolysis

Concentration level	Sample Identification	Procedure without hydrolysis	Procedure with hydrolysis
		Concentration (µg kg^{-1})	
1	0988A	0.21	0.43
	0988B	0.26	0.37
	0988C	0.27	0.44
2	2693A	0.29	0.64
	2693B	0.36	0.59
	2693C	0.41	0.58
3	0572A	0.36	0.66
	0572B	0.38	0.61
	0572C	0.40	0.67
4	1181A	0.80	1.24
	1181B	0.67	1.21
	1181C	0.70	1.14
5	0637A	1.10	1.46
	0637B	0.81	1.37
	0637C	1.14	1.45
6	1780A	0.99	1.93
	1780B	0.86	1.64
	1780C	1.01	1.67
7	1729A	1.33	2.38
	1729B	0.99	2.25
	1729C	1.25	2.29
8	2793A	2.12	3.07
	2793B	1.51	2.94
	2793C	1.76	3.13
9	2707A	1.88	3.10
	2707B	2.00	3.26
	2707C	2.04	3.46
10	0638A	3.00	6.72
	0638B	2.77	7.37
	0638C	2.56	6.37

The results of the statistical evaluations were as follows.
Samples 1 to 5: As $t_{calculated}$ = 8.993 > $t_{critical}$ = 2.144, there is a statistically significant difference between the approaches.
Samples 6 to 10: As $t_{calculated}$ = 5.285 > $t_{critical}$ = 2.144, there is a statistically significant difference between the approaches (without hydrolysis versus hydrolysis for 16 h).

Table 2. Results of the determination of ractopamine using either 2 h or 16 h enzymatic hydrolysis time.

Concentration level	Sample Identification	Procedure with hydrolysis	
		2 hours	16 hours
		Concentration (µg kg^{-1})	
1	1780A	1.50	1.93
	1780B	1.59	1.64
	1780C	1.57	1.67
2	1729A	2.02	2.38
	1729B	1.93	2.25
	1729C	2.52	2.29
3	2793A	2.46	3.07
	2793B	2.32	2.94
	2793C	2.51	3.13
4	2707A	2.46	3.10
	2707B	2.96	3.26
	2707C	2.55	3.46
5	0638A	5.69	6.72
	0638B	5.68	7.37
	0638C	5.84	6.37

As $t_{calculated}$ = 4.524 > $t_{critical}$ = 2.144, there is a statistically significant difference between the hydrolysis time approaches (2 hours versus 16 hours).

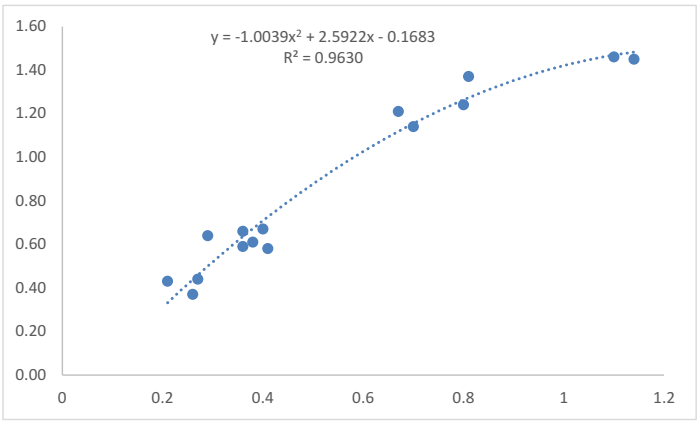

Figure 2. Results for the first analytical curve (range of lower concentrations)

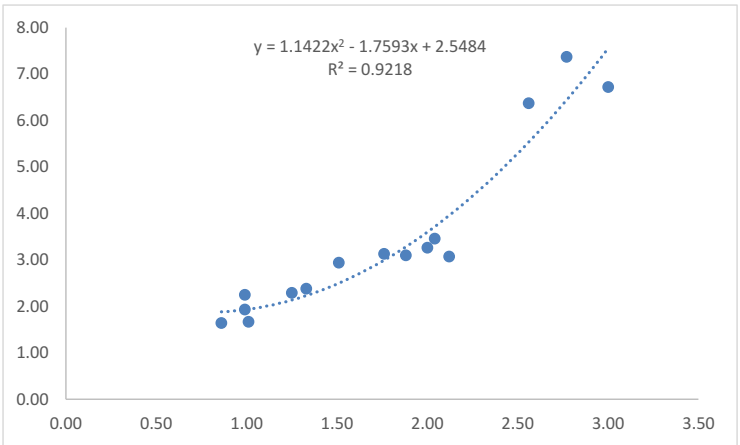

Figure 3. Results for the second analytical curve, range of higher concentrations (without hydrolysis versus hydrolysis of 16 hours)

Then, as there are systematic differences between the measurement procedures, this work calculates the coefficient of determination, Equation 2, in order to evaluate if one variable is able to predict strongly the other one (Figures 2 to 4).

Considering a quadratic fit, the R^2 = 0.9630 (Figure 2) means that more than 96% of the total variation in *y* can be explained by the relationship between *x* and *y*. The R^2 = 0.9218 in Figure 3 means that more than 92% of the total variation in *y* can be explained by the relationship between *x* and *y*, considering a quadratic fit as well. In a similar way, the R^2 = 0.9648 in Figure 4 says that more than 96% of the total variation in *y* can be explained by the relationship between *x* and *y* if a linear fit is considered.

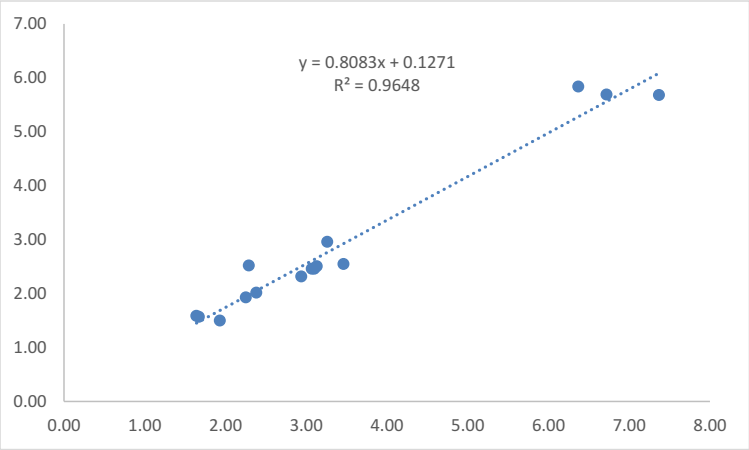

Figure 4. Results from the second analytical curve, higher concentrations (hydrolysis of 2 h versus hydrolysis of 16 h)

Conclusions

Statistically, results show that there is an expected impact of enzymatic hydrolysis on the determination of ractopamine in samples of naturally ractopamine-contaminated swine muscle. However, based on the high values of the coefficient of determinations, and thus strong correlation, the regression lines can be used to predict results derived from the enzymatic hydrolysis step without actually carrying it out or to reduce the incubation time for hydrolysis from 16 h to 2 h.

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PILOT PROJECT FOR A CHILEAN NATIONAL REFERENCE LABORATORY

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Abstract
Wageningen UR Chile, RIKILT Wageningen UR and the Chilean Food Safety Agency (ACHIPIA), developed a project sponsored by CORFO for the establishment of a National Reference Laboratories (NRL) System, considering the absence of NRLs in the country. Six priority areas were identified, inclusive veterinary Drugs, Microbiology, Marine Biotoxins and Pesticides. FARMAVET, an ISO 17025 accredited laboratory and responsible for the National Residue Control Program for the fisheries industry, was selected to take part of this project, particularly for marine biotoxins by LC-MS/MS. We proposed to organize a proficiency test (PT) to assess the quality of national laboratories. The method used in FARMAVET was validated according to the EU Harmonized Standard Operating Procedure. Professionals of FARMAVET were trained by RIKILT in the organization and statistical analysis of a PT. In this PT, there were four participants, and given the high variability of their results, which accounts for an uncertainty of the consensus value higher than 0.7 times the Horwitz-Thompson standard deviation, it was not possible to report their performance in z-scores, but a qualitative assessment was reported. In spite of the above, the experience was important for setting the basis for a NRL system and the reference tasks that implies to implement.

Introduction
Chile has an extensive network of laboratories of chemical analysis, ensuring the safety of animal food products. However, it does not have a network of National Reference Laboratories (NRL) as described in Directive 96/23/EC (Council of the European Union 1996). Considering this deficiency, Wageningen UR Chile together with the Chilean Agency for Food Safety (ACHIPIA) were granted a CORFO project with state resources for the establishment of a national network of reference laboratories. For this project, six priority areas were defined, which according to the risk assessment should have a NRL, namely, veterinary drugs, marine biotoxins, pesticides, food microbiology, dioxins and heavy metals. Considering the first stage of the project, three areas were prioritized, and the area of lipophilic marine biotoxins was assigned to the FARMAVET laboratory.

FARMAVET is a university laboratory specialized in chemical analysis, accredited under ISO regulation 17025. Currently, the laboratory has a fundamental role in the national network of food safety as the official laboratory of the National Fisheries Service (Sernapesca) performing residues analysis of veterinary drugs and contaminants in aquatic products for export. Within the scope of the laboratory, and according to the new requirements of the European Union regarding the analysis of lipophilic marine biotoxins, outlined in Commission Regulation (EU) N°15/2011 (European Commission 2011), the analytical method by LC-MS/MS was validated following the directions of the "EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS / MS" (European Union Reference Laboratory for Marine Biotoxins 2015). Currently this methodology is ISO 17025 accredited.

According to the Council Directive 96/23/EC, NRLs have several responsibilities, including: i) coordinating the work of other national laboratories responsible for residue analysis; ii) coordinating the standards and methods of analysis; iii) assisting the competent authority in organizing the plan for monitoring residues; iv) periodically organizing proficiency tests for each residue or residue group assigned to them; v) disseminating information supplied by Community reference laboratories, and vi) ensuring that their staff are able to take part in further training courses organized by the Commission or by Commission reference laboratories. While FARMAVET was not at the time of the project an NRL, its role as the official laboratory for Sernapesca demanded several responsibilities that included all items listed above, except for the organization of proficiency tests. Therefore, it was decided that the pilot should be aimed to conducting a proficiency test between national laboratories that had already validated the analytical method for lipophilic marine biotoxins by LC-MS/MS.

As for proficiency tests, as part of its quality assurance protocols, FARMAVET is constantly involved in such inter-comparisons, mainly with international laboratories. However, it does not have any experience organizing inter-laboratory rounds, therefore training by specialized professionals was required. At this point, the role of RIKILT Institute was critical in supplying materials for the test and providing the training required to carry out the pilot study, particularly on key aspects of the organization, materials preparation, the logistics of shipping the samples and, finally, the analysis the results.

Materials and Methods
The proficiency test was aimed to test the new methodology for the analysis of lipophilic marine biotoxins by liquid chromatography tandem mass-spectroscopy. The method validated in FARMAVET and used as reference for the analysis of samples,

follows the directives of the European Union Reference Laboratory belonging to the European Union for Marine Biotoxins (EU-RL-MB).

Briefly, the analytical method is based on the extraction of the lipophilic groups okadaic acid, pectenotoxins, yessotoxins and azaspiracids, from a sample of homogenized mussel tissue using methanol.

As previously noted, the methodology was already validated in FARMAVET at the onset of the pilot project, so the aim of this work, beyond the specific technical considerations of the method, was focused on the organization of a proficiency test for this new methodology in Chile. In order to achieve this, a five-days training was conducted at RIKILT Institute.

Sample preparation

For the test, three materials were prepared for each participant:

- Material A: 1.4 g homogenized *Mytilus chilensis* tissue, declared free from any of the lipophilic biotoxins by analysis.
- Material B: 1.4 g homogenized *Mytilus chilensis* tissue, naturally incurred with marine biotoxins at low concentrations.
- Material C: 1.4 g homogenized *Mytilus chilensis* tissue, naturally incurred with marine biotoxins at high concentrations.

As mentioned above, during the stay at RIKILT, the samples were prepared for testing. These samples were kept at RIKILT until they were shipped to FARMAVET.

On June 8th 2015, letters of invitation to participate in the proficiency test and registration forms were sent. To this call for participants, made using the information provided by Sernapesca and ACHIPIA, only 4 laboratories, located in Santiago and Puerto Montt, showed interest. This is due to the fact that to the date, they were the only ones who were using the methodology according to the European Union regulations.

Proficiency test samples were distributed on June 11th. Samples addressed to Santiago were distributed directly by ACHIPIA, while those going to Puerto Montt, at the south of Chile, were sent by a national courier system.

As stated in the letter of invitation, the results were sent by the four participating laboratories on July 10th.

Statistical evaluation

Given the low participation in this proficiency test, the results of this trial do not have statistical value. However, the results were analyzed for reference only and for internal evaluation of each participant.

For each biotoxin present in the test materials, the assigned value, that corresponds to the value determined by FARMAVET after analyzing 20 samples and during the homogeneity and stability test, was used to measure the performance of each participant. It was not possible to use the consensus value, because the high variability between participants resulted in an uncertainty of the consensus greater than $0.7\sigma_P$ (0.7 times the standard deviation of the assigned value).

The results of each laboratory, expressed in units of concentration, were evaluated based on the statistical parameter z-score. Each z-score is classified into the following scale:

$\|z\| \leq 2$	Satisfactory
$2 < \|z\| < 3$	Questionable
$\|z\| \geq 3$	Unsatisfactory

The z-score is calculated from: $z\text{-score} = (x-X)/\sigma_P$

Where, x is the average result of the laboratory, X is the assigned value, and σ_P is standard deviation of the assigned value.

Accordingly, the standard deviation of the assigned value (σ_P) was calculated using the Horwitz equation for analyte concentrations higher than 120 μg kg^{-1} and lower than 138 g kg^{-1}: $\sigma_P = 0.02c^{0,8495}$

For analytes in concentrations lower than 120 μg kg^{-1}, we used a complementary model: $\sigma_P = 0.22c$

For analytes in concentrations higher than 138 g kg^{-1}, we used a complementary model: $\sigma_P = 0.01c^{0,5}$

Where *c* is the analyte concentration expressed in g g^{-1}

Results

Considering that there were only four laboratories involved, according to the experts of RIKILT Laboratory, the results of this proficiency test have no statistical value; Nevertheless, it was proposed to do the analysis of results even with this low number of participants, in order to offer the participants a performance report, although in the form of an experimental trial. However, the results reported by the participants varied widely, resulting in an uncertainty of the consensus value higher than 0.7 times the Horwitz-Thompson standard deviation. This means that given the high uncertainty, it was not possible to do a statistical analysis on the results, or assign a z-score to each participating laboratory.

From the results, even despite the high variability, it is possible to conclude that no false positives were reported. As for false negatives, one of the participants did not detect yessotoxin in sample B, which is a false negative.

Table 1. Quantitative results for materials B and C.

Analyte	Assigned Value Material B	Material C
AO (μg kg^{-1})	73.2	344
DTX-1 (μg kg^{-1})	36.1	140
DTX-2 (μg kg^{-1})	20.7	54
DTX-3 (μg kg^{-1})	261	861
YTX (mg kg^{-1})	0.12	0.48
Homo-YTX (mg kg^{-1})	0.65	1.2
45 OH-YTX (mg kg^{-1})	0.20	0.77
45 OH-Homo-YTX (mg kg^{-1})	ND	0.09
PTX-1 (μg kg^{-1})	ND	ND
PTX-2 (μg kg^{-1})	ND	ND
AZA-1 (μg kg^{-1})	64.0	437
AZA-2 (μg kg^{-1})	38.2	99.0
AZA-3 (μg kg^{-1})	53.0	268

Discussion and Conclusions

The overall results of this proficiency test showed that while participating laboratories used the official method of the European Union, as well as FARMAVET, the lack of experience in the application of this methodology and in proficiency tests in general, may have caused the wide spreading of the data. Overall, when the assigned value was closer to the regulatory limit for lipophilic marine biotoxins, the participants showed less scattering, as was the case for material C results.

At the moment of the test, none of the participants had their method accredited by ISO 17025, as the test was only recently incorporated into their practice. This also implies that this was the first proficiency test for this group of analytes in which they took part. This was reflected also in the mistake made by one of the participants reporting YTX in μg kg^{-1} and not in mg kg^{-1}, as requested in the instruction letter.

Regarding the development of the project, while the overall objective of this specific pilot was the organization of a proficiency test for a specific analysis, the acquired experience is applicable to the entire scope of analysis tasks of the laboratory. Thus it goes beyond the analysis of marine biotoxins exclusively; test management is the same for any analysis. That is why FARMAVET plans to propose in the near future the organization of new laboratory tests for the Sernapesca laboratory network, for analytes used in national salmon farming, such as tetracyclines.

In this regard the support of RIKILT laboratory was critical, because they have vast experience in analysis of chemical residues and contaminants, as well as in the organization of proficiency tests, in which, as should be noted, FARMAVET constantly participates. Additionally, work in Wageningen UR allowed for an exchange of technical information regarding key points of lipophilic marine biotoxins analysis, based on which FARMAVET is constantly optimizing its analytical method.

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DEVELOPMENT AND VALIDATION OF A MULTI-CLASS METHOD FOR THE DETERMINATION OF ANTIBIOTIC RESIDUES IN HONEY USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A new, simple, and fast method has been developed for the determination of multi-class antibiotic residues in honey (sulfonamides, tetracyclines, macrolides, lincosamides, and aminoglycosides). The separation and the determination are carried out by liquid chromatography coupled to mass-spectrometry (LC-MS/MS). In the sample preparation, various parameters affecting the extraction efficiency including the type of solvent, the pH, the breaking efficiencies of *N*-glycosidic linkage by hydrochloric acid, the ultrasonic extraction and its duration compared to shaking technique, along with dispersive SPE clean-up were examined. Experiments with fortified samples demonstrated that 10 min ultrasonic treatment with acidified methanol (2 M HCl) followed by dispersive SPE clean-up with 50 mg PSA gave an effective sample preparation method for extracting several classes of antibiotics in honey. Anhydroerythromycin A, erythromycin A enol ether, and desmycosin were used as markers for the presence of both erythromycin A and tylosin A in honey samples.

The method was validated according to European Commission Decision (EC) No 2002/657. The recoveries of analytes ranged from 85 to 111%. Repeatability and intra-laboratory reproducibility were less than 20.6% and 26.8%, respectively. Decision limit (CCα) and detection capability (CCβ) ranged from 6 to 9 µg kg^{-1} and from 7 to 13 µg kg^{-1}, respectively, except for streptomycin and neomycin that showed slightly higher CCα at 25 µg kg^{-1} and CCβ at 34 µg kg^{-1}. Finally, the method was applied for the honey test material 02270 through an official FAPAS proficiency test (PT) for the determination of tetracyclines. PT results were found within a Z-score range of ±2, proving that the validated method is suitable to be used in routine analysis to ensure the quality of honey.

Introduction

Veterinary medicinal products (VMPs) are widely used in farm practices to prevent and control animal diseases. However, incorrect practices can lead to the potential presence of VMP residues in food-producing animals and may cause risks to human health (Coffman 1999; McEwen and Fedorka-Cray 2002; Landers, Cohen *et al.* 2012). Many studies indicate that VMPs can accumulate in edible tissues which can trigger allergic reactions in sensitive individuals. Furthermore, long-term exposure to low levels of VMPs could result in development of antibiotic-resistant bacteria, which would no more respond actively to drug treatment (Dayan 1993; Tollefson and Karp 2004; Graham, Paradis *et al.* 2014). Therefore, European Union (EU) (EU Reg No 37/2010 2009), U.S. Food and Drug Administration (CFR-21 Part-556, 2015), and other international regulatory authorities regulate VMPs intended to be used in food-producing animal production. In addition, they established maximum residue limits (MRLs) for VMPs to monitor their levels in food and to ensure that these residues impose no health risks to consumers.

In Apiculture, beekeepers treat their hives with antibacterial agents (Reybroeck, Daeseleire *et al.* 2012) against bacterial diseases such as American foulbrood (AFB) (Genersch 2010) and European foulbrood (EFB) (Forsgren 2010). But, in some countries, like United Kingdom (BeeBase 2015) and New Zealand (Biosecurity Order 1998) when bee colonies are infected with AFB, the hives must be destroyed by burning because spores are considered highly resistant and can remain infectious for more than 35 years (Haseman 1961; Genersch 2010).

So far, there are no MRLs for antibiotic residues in honey (EU Reg No 37/2010 2009), therefore, the presence of VMPs in honey is non authorized. The European Union Reference Laboratories (EU-RLs) (CRL Guidance 2007) provided recommended concentrations (RCs) for non-authorized substances like tetracyclines, sulfonamides, streptomycin, and macrolides (erythromycin and tylosin) in honey (Table 1) in order to improve and harmonize the performance of the monitoring analytical methods. While, several countries like Canada (FDR 2005), Belgium (AFSCA 2016), India (EIC 2015), Australia (DFI 1995; APVMA 2015), and Switzerland (DFI 1995) define their own tolerance levels for each class of veterinary drugs in honey (Table 1). For instance, in EU, a maximum required performance limit (MRPLs) of 0.3 µg kg^{-1} has been set for chloramphenicol in food (Commission Decision (EC) No 2003/181 2003). Furthermore, Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) drafted a guidance evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA 2013) for

the establishment of MRLs in honey based on the acceptable daily intake (ADI) of VMP residues and their depletion studies in honey.

Table 1. Tolerance levels in µg kg^{-1} for veterinary drugs in honey in several countries.

Classes	EU-RLs RCs	Belgium MRPLs	Canada MRLs	WRL	Australia MRLs	India MRLs	Switzerland MRLs
Streptomycin	40	20	125	37.5	-	10	10
Tetracyclines	20	20	-	-	-	-	-
Tetracycline	-	-	250	75	-	10	-
Chlortetracycline	-	-	100	30	-	10	-
Oxytetracycline	-	-	-	-	300	10	-
Sulfonamides	50	20	100	30	-	10	50
Macrolides	20	-	-	-	-	-	-
Erythromycin	-	-	100	30	-	-	-
Tylosin	-	-	200	60	-	10	-
Lincomycin	-	-	100	30	-	-	-
Penicillin	-	-	10	0.3	-	-	-

Honey is a complex biological matrix which contains a high concentrated aqueous solution of several sugars and other substances like vitamins, proteins, minerals, organic acids and enzymes (White Jr 1971; Ball 2007). The composition of these substances can vary depending on the nectar source and other external factors such as, seasonal and environmental conditions (White Jr and Chichester 1978; Anklam 1998). These variations pose analytical challenges regarding sample processing and analysis of trace contaminants in honey. One of these challenges is to remove interfering substances such as sugar, wax, and pigments from honey extract prior to VMP residue analysis to reduce matrix effect (Kujawski and Namieśnik 2008).

There are several other challenges for the analyst to overcome during the development of multi-class method for analysis of VMPs in honey (Kujawski and Namieśnik 2008). For example, sulfonamide residues in honey combine with reducing sugar to form *N*-glycoside bond, which lead to poor recoveries for almost all sulfonamides that could be found in the sample (Kaufmann, Roth *et al.* 2002). For that reason, it is necessary to include hydrolysis step to break sugar-sulfonamide bond in pretreatment process. Studies have demonstrated that methanol (Bernal, Nozal *et al.* 2009) and hydrochloric acid (Thompson and Noot 2005; Sajid, Na *et al.* 2013; Tölgyesi, Berky *et al.* 2013; Dubreil-Chéneau, Pirotais *et al.* 2014) were mainly used to hydrolyse *N*-glycoside bond in order to give better recovery. Whereas, macrolides are not stable at acidic conditions (Skinner, Taylor *et al.* 1993; Volmer and Hui 1998; Kim, Heinze *et al.* 2004); they are usually extracted under basic conditions to avoid their degradation (Wang 2004; Benetti, Piro *et al.* 2006; Bogialli, Di Corcia *et al.* 2007; Wang and Leung 2007). Erythromycin A degrades rapidly to anhydroerythromycin A in honey (Thompson and Van den Heever 2012) which is known to be an acidic matrix (pH ranges from 3.4 to 6 (Ball 2007). Other studies show that desmycosin (tylosin B), the degradation product of tylosin A, was identified in honey sample (Kochansky 2004; Adams, Heinrich *et al.* 2007; Thompson, Pernal *et al.* 2007).

Therefore, it is important to include not only the parent VMPs but also their metabolites or other transformation products when monitoring the use of their residues in honey. A similar phenomenon is observed for tetracyclines; these compounds can undergo structural epimerization in acidic conditions (pH 2-6) (Anderson, Rupp *et al.* 2005). Furthermore, they have a strong affinity to form a complex with divalent metal cations (Carlotti, Cesaretti *et al.* 2012), which lead to deficient recoveries during the sample extraction processing. To improve recoveries, the interaction can be disrupted by adding EDTA to the extraction solvent because it has greater affinity to chelate cations than tetracyclines (Anderson, Rupp *et al.* 2005).

Another issue associated to the development of multi-class analytical method is with aminoglycosidic antibiotics. These VMPs are highly polar organic basic compounds that show practically no retention in reversed phase liquid chromatography, unless an ion-pairing reagent such as perfluorocarboxylic acid is added into the mobile phase also considering suitable concentration to minimize the ionization suppression (Inchauspe and Samain 1984).

There have been several studies focusing on determination of VMPs in honey based on a single class residue methods (SRMs) in which they covered a limited number of analytes generally from the same family and similar chemical behaviour. However, there is an increasing effort in the development of multi-class analytical methods where those are capable of detecting a wide range of residues with different chemical and physical properties in the same sample (Kaufmann 2009; Kaufmann, Butcher *et al.* 2011). Several multi-class analytical methods in honey are described in the literature (Debayle, Dessalces *et al.* 2008; Hammel, Mohamed *et al.* 2008; Lopez, Pettis *et al.* 2008; Vidal, Aguilera-Luiz *et al.* 2009; Hou, Xie *et al.* 2011; Bohm, Stachel *et al.* 2012; Gómez-Pérez, Plaza-Bolaños *et al.* 2012; Wang and Leung 2012; Wang, Shi *et al.* 2013; Galarini, Saluti *et al.* 2015; Shendy, Al-Ghobashy *et al.* 2016). Most of these methods are based on using solid-phase extraction (SPE) for sample preparation and purification, with the aim to enhance the sensitivity of the detection and reduce interferences. However,

Hammel *et al.* (2008) describes four subsequent liquid-liquid extractions for the simultaneous determination of 42 antibiotics in honey prior to liquid chromatography electrospray-ionization tandem mass-spectrometry (LC-ESI-MS/MS) analysis. In addition, Wang J *et al.* (2012 and 2013), and Shendy *et al.* (2016) reported the use of QUECHERS method for quantification and confirmation of veterinary drugs in honey.

The aim of this study is to develop and validate a simple multi-class method for identification and quantification of 22 antimicrobial VMPs in honey by LC-ESI-MS/MS. These drugs belong to several classes of antibiotics that include sulfonamides (SAs), macrolides (MAs), tetracyclines (TCs), lincosamides (LCs), and aminoglycosides (AMGs). The extraction and clean-up steps were investigated and optimized by using ultrasonic-assisted extraction and dispersive solid phase extraction (d-SPE). Our objective is to determine the best conditions of extraction (pH and solvent) having in mind a practical routine use including reduced sample preparation and detection time but without affecting the multi-analytes recoveries. Furthermore, the matrix effect to which was subjected the mass-spectrometric signals was evaluated on multi-floral honey extracts during the validation procedure.

Experimental

Chemicals, reagents and solutions:

Analytical standards of sulfamethoxazole (SMXZ), sulfamerazine (SMRZ), sulfathiazole (STZL), sulfadimethoxine (SDMX), sulfadimerazine (SDMZ), sulfachloropyridazine (SCPD), oxytetracycline (OTC), doxycycline (DC), chlortetracycline (CTC), tetracycline (TC), anhydrotetracycline (AH-TC), anhydrochlortetracycline (AH-CTC), 6-epidoxycycline (EPI-DC), 4-epitetracycline (EPI-TC), 4-epioxytetracycline (EPI-OTC), demeclocycline (DMC), tylosin A (TYLA), erythromycin A (EMTC), anhydroerythromycin A (AH-ETMC), erythromycin A enol ether (ETMC enol), josamycine (JSMC), lincomycin (LNMC), dihydrostreptomycin (DH-STRP), streptomycin (STRP), neomycin (NEO), hygromycin (HGMC) deuterated sulfonamides sulfadoxine-D3 (SDX-d3), and sulfadimethoxine-D6 (SDMX-d6) were supplied by Sigma-Aldrich (Seelze, Germany). Desmycosine (TYLB) and carbon labelled ^{13}C erythromycin (ETMC-c13) were obtained from Toronto Research Chemicals (North York, Ontario, Canada). Tobramycin (TBMC) was purchased from Dr.Ehrenstorfer (Augsburg, Germany) and carbon labelled ^{13}C sulfathiazole (STZL-c13) from Witega (Berlin, Germany).

LC-MS grade acetonitrile (MeCN) and methanol (MeOH) were obtained from Honeywell Burdick & Jackson (Seelze, Germany). Ethylenediaminetetraacetic tetrasodium salt (Na_4EDTA) was purchased from Sigma-Aldrich (Madrid, Spain) and heptafluorobutyric acid (HFBA) for ion-pairing chromatography from Fluka (Saint Louis, USA). Hydrochloric acid solution (HCl) at 37% was obtained from VWR international (Fontenay-sous-Bois, France). Primary Secondary Amine sorbent (PSA) was purchased from Agilent technologies (Waldbronn, Germany). Formic acid (FA) analytical grade was obtained from BDH laboratory (Poole, England). Deionized water with resistivity 18.2 Ωm was prepared with Barnstead-Easy pure II from Thermo Fisher scientific (Hudson, USA). The pH values were measured on pH meter Thermo Orion 720A plus (Beverly, USA). Sonication process was carried out using Elma T 760DH ultrasonic bath with frequency of 40 kHz (Singen, Germany).

Individual stock standard solutions were prepared into appropriate organic solvents at a concentration of 500 µg mL^{-1}. Only AH-CTC was prepared at 120 µg mL^{-1}. MeOH was used to dissolve SAs, TCs, LCs, and MAs standards. However, AMGs stock standard solutions were dissolved in ultra-pure water with 1% FA using polypropylene volumetric flask. All stock solutions were kept at -20°C except for AMGs stored at +4°C. Multi-compound intermediate solutions for each antibiotic class (except for aminoglycosides) were prepared in MeOH at 20 µg mL^{-1} by appropriate dilution of the stock solution. The intermediate solution for AMG analytes was prepared in ultra-pure water at 20 µg mL^{-1} (only NEO and STRP were prepared at 100 µg mL^{-1}). For spiking purposes, three daily spiking solutions containing all analytes were prepared by diluting intermediate stock solutions in ultra-pure water to obtain the desired concentrations at 0.4, 0.6, and 0.8 µg mL^{-1} whereas, for NEO and STRP were prepared at concentrations of 2, 3, and 4 µg mL^{-1}. The internal standard (IS) mixture prepared as intermediate solution in ultra-pure water includes DMC, SDX-d3, and SDMX-d6 at 10 µg mL^{-1}; ETMC-c13, STZL-c13, and TBMC at 20 µg mL^{-1}; as well as HGMC at 40 µg mL^{-1}. Freshly prepared solutions were used by diluting 10 times the IS intermediate solution in water for spiking the samples.

Sample extraction

An amount of 2 ± 0.05 g of honey samples were weighed into a 50-mL disposable centrifuge tube. Samples were spiked with 100 µL of the internal standard working solution and kept overnight in the dark. Afterwards, 2.5 mL of ultra-pure water was added to dissolve the honey by shaking for 1 min. Then, 2.5 mL of acidified MeOH (HCl, 2 mol L^{-1}) was added; the sample was shaken by hand for 1 min and then sonicated for 10 min. The pH of honey extract was adjusted to 2.0 by adding 460 ± 5 mg of Na_4EDTA. The samples were shaken for 1 min and centrifuged for 10 min at room temperature at 4,000 rpm using Eppendorf 5810 (Hamburg, Germany). Following that, 1 mL of the extract was transferred to a centrifuge tube containing 50 ± 5 mg PSA for dispersive clean-up. The mixtures were shaken for 1 min and centrifuged for 10 min at room temperature at 4,000 rpm. Finally, 500 µL of the purified extract were transferred into LC plastic vials for chromatography.

Table 2. Ionization parameters for the analytes with chromatographic retention time.

Class	Names	Retention time (min)	Precur-sor ion	Product ion	Fragmen-tor (V)	CID (V)	(IS) used
Sulfonamides	SMXZ	10.413	253.8	253.8>108 *	120	25	SDX D3
				253.8>155.9	120	10	
	STZL	7.325	255.9	255.9>155.9*	80	10	STZL C13
				255.9>108	80	25	
	STZL C13 *(IS)*	7.503	261.9	261.9>161.8*	100	10	-
	SMRZ	6.847	265.1	265.1>108*	100	30	STZL C13
				265.1>155.9	100	10	
	SDMZ	8.268	279.1	279.1>186.1*	120	15	STZL C13
				279.1>108	120	30	
	SCPD	10.069	284.9	284.9>155.9*	120	10	SDX D3
				284.9>108	120	25	
	SDMX	11.704	311.1	311.1>155.9*	120	20	SDMX D6
				311.1>108	120	30	
	SDX D3 *(IS)*	10.3	314.1	314.1>155.9*	100	15	-
	SDMX D6 *(IS)*	12.06	317.1	317.1>161.8*	120	20	-
Lincosamides	LNMC	9.950	407.1	407.1>126.1*	80	35	ETMC C13
				407.1>359.2	80	15	
Tetracyclines	AH-TC	14.523	427	427>410*	100	15	DMC
				427>154	100	25	
	DC	13.596	445.1	445.1>428.1*	120	15	DMC
				445.1>154	120	30	
	EPI-DC	13.413	445.1	445.1>428.1*	100	15	DMC
				445.1>154	100	30	
	TC	11.610	445.1	445.1>409.8*	120	15	DMC
				445.1>154	120	30	
	EPI-TC	11.136	445.1	445.1>409.8*	100	15	DMC
				445.1>427	100	10	
	AH-CTC	15.400	461.1	461.1>444*	100	15	DMC
				461.1>154	100	30	
	OTC	11.071	461.1	461.1>425.8*	120	20	DMC
				461.1>443.1	120	10	
	EPI-OTC	10.960	461.1	461.1>425.8*	120	15	DMC
				461.1>443.1	120	10	
	CTC	13.249	479	479>443.8*	100	20	DMC
				479>461.8	100	15	
	DMC *(IS)*	12.58	464.9	464.9>447.9*	140	15	-
Aminoglycosides	TBMC *(IS)*	12.69	468.2	468.2>163.1*	100	20	-
	HGMC *(IS)*	10.07	528.2	528.2>177.1*	140	30	-
	STRP	10.213	582.2	582.2>263.1*	140	30	HGMC
				582.2>245.8	140	40	
	DH-STRP	10.166	584.2	584.2>263.1*	120	30	HGMC
				584.2>246	120	40	
	NEO	13.194	615.4	615.4>160.9*	140	30	TBMC
				615.4>292.9	140	20	

Table 2. (continued)

Class	Names	Retention time (min)	Precursor ion	Product ion	Fragmentor (V)	CID (V)	(IS) used
Macrolides	AH-ETMC	15.608	716.3	716.3>157.8*	120	30	ETMC C13
				716.3>557.9	120	10	
	ETMC	14.763	734.4	734.4>157.8*	160	25	ETMC C13
				734.4>578	160	15	
	ETMC enol	15.421	716.3	716.3>157.8*	140	30	ETMC C13
				716.3>557.9	140	15	
	ETMC C13 (IS)	15.73	718.4	718.4>160*	120	30	-
	TYLB	14.069	772.3	772.3>173.5*	180	30	ETMC C13
				772.3>131.6	180	35	
	TYLA	15.031	916.2	916.2>173.6*	200	40	ETMC C13
				916.2>771.9	200	30	
	JSMC	15.981	828.4	828.4>108.9*	200	45	ETMC C13
				828.4>173.9	200	35	

*IS: Internal standard; *: Quantification ion*

Liquid-chromatography mass-spectrometry

The chromatographic system consisting of HPLC Agilent technologies 1200 series (Morges, Switzerland) was coupled to an Agilent 6410 tandem mass-spectrometry (Wilmington, USA) set in positive mode electrospray-ionization (ESI). The analysis was conducted on a reversed-phase Zorbax SB C_{18} column (100x2.1 mm; 3.5µm) obtained from Agilent technologies (Wilmington, USA). Three mobile phase components A: ultra-pure deionized water with 100 mM HFBA, B: acetonitrile, and C: ultra-pure deionized water was used for chromatographic separation according to the following gradient program: the initial condition started from 10% of A, 5% of B, and 85% of C. The eluent A was kept unchanged at 10% during the analysis. The eluent C decreased from 85% to 50% over 10.5 min. After that, this percentage was linearly decreased to 25% within 1.5 min, then to 10% in 1 min. This composition was held for 1 min and increased to 85% in 0.5 min, followed by a re-equilibration time of 8.5 min. The flow rate was set at 0.5 mL min^{-1} and the total running time prior to re-injection was 23 min. The injection volume was 30 µL. To minimize the contamination of the system with sugar, a divert valve was used to divert the column elution directly to waste from 0 to 4 min, and plug again to MS/MS from 4 to 23 min in each sample run.

All antimicrobial VMPs were detected using ESI source in positive mode under the following working conditions: The source temperature was 350°C; desolvation nitrogen gas was at flow rate of 7 L h^{-1}; capillary voltage was +4.0 KV and the nebulizer pressure was 30 psi. The collision energy (CIV) as well as the fragmentor for each analyte was optimized through direct injection of 0.5 µg mL^{-1} standard solution into mass spectrometry. Table 2 summarizes the specific MS/MS parameters for the targeted analytes. From the MS/MS optimization, the two most intense transitions per analyte were selected for operation in MRM mode.

Validation procedure

The method was validated on blank multifloral honey material and according to Decision (EC) No 2002/657. For substances without MRL in honey but having MRL in other animal matrices such as meat and milk, the Commission recommended to assess the method performance in honey at concentration at least as low as recommended by the EU-RLs guidance of 2007 (CRL Guidance 2007). Therefore, adequate levels for spiking blank samples were chosen following this recommendation. Three levels of spiking were selected to evaluate the recoveries and corresponded to 1, 1.5, and 2 times the minimum required performance limit (Commission Decision (EC) 2002/657 2002). The first level was set at 20 µg kg^{-1} for all antibiotics except for NEO and STRP at 100 µg kg^{-1}. The method performance was evaluated for each analyte through the determination of the precision (Repeatability and intra-lab reproducibility parameters), the recoveries, the decision limit (CCα), the capacity of detection (CCβ), the specificity, the matrix effect, and the linear range.

An amount of 2 g blank honey samples were fortified with 100 µL of internal standard working solution and 100 µL of spiking solution containing all the analytes at three different concentrations (20, 30, and 40 µg kg^{-1}). However, for NEO and STRP the concentration levels were increased to 100, 150, and 200 µg kg^{-1}. The spiked samples were kept overnight in the dark. Six replicates for each level were analysed on three different days. For the quantification results, matrix fortified calibration curve was prepared by extracting five blank samples (2 g) fortified at level of 20, 40, 60, 80, and 100 µg kg^{-1} for all analytes except for NEO and STRP at 100, 150, 200, 250, and 300 µg kg^{-1}. For each run, the analysis was performed by injecting the

matrix-fortified calibration standards plus an additional blank sample twice at the start and at the end of the samples sequence, resulting in a total number of 30 analyses carried out within one day.

Results and Discussion

Optimization of sample preparation

Despite the fact that honey contains multiple compounds possibly responsible for matrix effect, antimicrobial VMPs also impose a problematic burden to overcome. SAs can form a very stable *N*-glycosidic bond with reducing sugar present in honey (Kaufmann, Roth *et al.* 2002). TCs can form chelating complex with metal ions and interact with silica group in a reversed phase analytical column. Therefore, they can elute with tailing peak (Stolker and Brinkman 2005). MAs are acid sensitive which results in the degradation of ETMC (Thompson and Van den Heever 2012), JSMC (Skinner, Taylor *et al.* 1993), and TYLA (Kochansky 2004; Thompson, Pernal *et al.* 2007). For that reason, Thompson *et al.* (2007) recommended to include both the parent ion and its major degradation products in LC-MS/MS analysis in order to verify the use of these antibiotics in apiculture. AMGs are highly polar compounds that cannot be satisfactorily retained on reversed phase unless ion-pairing agent was added to mobile phase at suitable concentration (Inchauspe and Samain 1984).

Our aim is to develop a simple extraction and clean up method that covers several types of antimicrobial residues with different polarity in honey without affecting recoveries. Therefore, choice of extraction solvent, effect of pH, acid hydrolysis pretreatment, and ultrasonic extraction versus traditional shaking were evaluated. Furthermore, dispersive SPE clean up with Primary Secondary Amine (PSA) was assessed through the determination of the remained amount of sugar content in honey. All experiments were performed in triplicate by spiking blank honey with all analytes at 100 µg kg^{-1}. These samples were kept overnight in the dark to permit sufficient absorption of the different standards prior extraction. In addition, recoveries for all analytes were estimated without correction for the losses during the sample preparation (thus showing their real recoveries). To assess the potential matrix effects, the peak area of the analytes in spiked samples was compared to the one obtained from analytes added to blank honey extract immediately prior to LC injection.

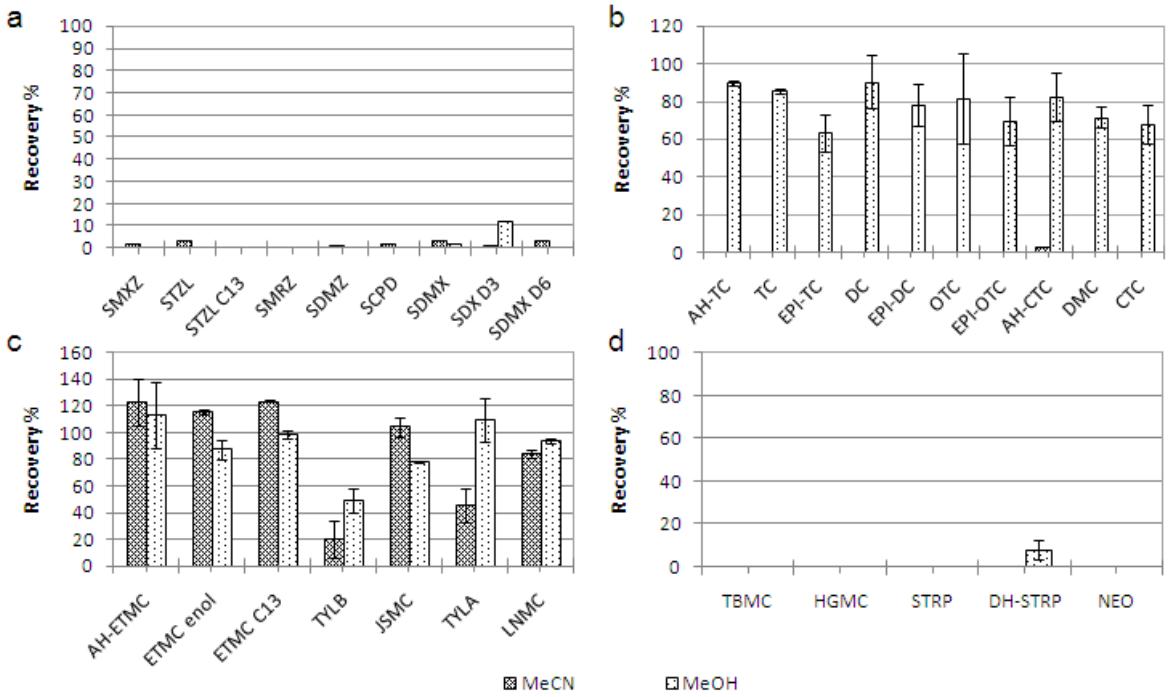

Figure 1. Evaluation of MeOH and MeCN organic solvents in sample preparation for the analysis of antibiotic residues in honey. A) SAs, b) TCs, c) MAs and LCs, d) AMGs.

Choice of extraction solvent in antimicrobial multi-residue method

The choice of the solvent for extraction is one of the most important parameters for the development of multi-class residue method. The solvent must be compatible with the analytes of interest and amenable to chromatographic separation system. Furthermore, another aspect to consider when selecting the extraction solvent is the safety for the laboratory personnel and the environment as well as its cost. In our study, MeOH and MeCN solvents were assessed for their ability to extract multi-class antibiotic residues. Only one parameter was changed during sample extraction which is the type of solvent. In the first

experiment, fortified honey samples (2 g) were dissolved in 2.5 mL of water then 2.5 mL of pure organic solvent was added into 50-mL centrifuge tube. The tubes were shaken for 1 min and ultra-sonicated for 30 min. Then, the extract was treated with 1 g Na_4EDTA followed by centrifugation at 4,000 rpm for 10 min. Finally, the extract was purified using 50 mg PSA then injected into LC-MS/MS.

Comparing MeOH to MeCN (Figure 1), MeOH was able to extract 17 of 32 compounds with recoveries higher than 60%. However, MeCN was suitable to extract macrolide residues from honey samples. Erythromycin A, aminoglycosides, and sulfonamides were not extracted with MeOH or MeCN. Acetonitrile is more effective in avoiding sugar co-extractive interferences from honey sample due to sugaring out phenomenon causing liquid-liquid partitioning between water and MeCN (Wang, Ezejias *et al.* 2008; Tsai, Chuang *et al.* 2010). However, it was found to be a non suitable solvent for sample preparation in analysis of multi-class of antimicrobials in honey. This could be due to high solubility of polar compounds (aminoglycosides and tetracyclines) in water fraction resulting in loss of these analytes during solvent partitioning in acetonitrile. Therefore, MeOH was assumed to be more suitable than MeCN for extracting more antimicrobial residues in honey. For sulfonamides, an acid hydrolysis step must be implemented prior to extraction to release *N*-glycosidic bond with the aim to avoid their underestimation. Chen *et al.* (2012) reported that a mixture of hydrochloric acid and methanol could even increase the breaking rate.

ETMC was not detected in our experiment, although the measured pH after adding Na_4EDTA during extraction was 10. The reason behind this observation is related to low pH (less than 3) due to the addition of HFBA ion-pairing reagent during the chromatographic separation. This yielded to ETMC degradation into ETMC enol and AH-ETMC A. Therefore, these two analytes were chosen as markers for analysis of ETMC in honey. It should be noted that the same degradation process for ETMC internal standard (EMTC-c13) occurred where an intense peak with a pseudo-molecular ion *m/z* at 718.4 giving daughter ion at *m/z* 160.

Effect of pH

The effect of pH on the extraction procedure was evaluated by adjusting its value through adding appropriate amount of Na_4EDTA to honey extract. In our study, three pH values 2, 5.1, and 10 were investigated. At pH 2 and 5.1, acidified MeOH with 2 M HCl was added to the extract after dissolving honey with water. After sonication for 30 min, 460 mg and 1g of Na_4EDTA were added to adjust the pH at 2 and 5.1 respectively. However, for basic extraction at pH 10, pure MeOH and 1g of Na_4EDTA were used. The optimal pH for each antibiotic class was assessed from the recovery values (Figure 2). For sulfonamides, best recoveries were found at pH 2 and 5.1; for tetracyclines and lincomycin, similar recoveries were obtained at all pHs. Acidification of honey sample is likely to disrupt the *N*-glycosidic linkage between sulfonamides and reduced sugar in honey. This increases the extraction efficiency of these analytes at pH 2 and 5.1 compared to 10, in which no acidic hydrolysis was applied. However, when analysing the sample at low pH values, the loss of macrolide residues occurs, except for TYLB correctly extracted. Figure 2 shows better recoveries for macrolides at basic pH; this observation was in line with results reported by Wang and Leung (2007). On the other hand, better extraction efficiency was observed for most studied aminoglycosides at pH 2 and decreased with increasing pH. Furthermore, better peak shape was obtained when the sample was extracted at pH 2 compared to those at pH 5.1 and 10 (Figure 3).

Our results showed that the extraction efficiency of most analytes was improved by acidifying the samples at pH 2. However, that pH has strong impact on macrolide antibiotics leading to their degradation except TYLB.

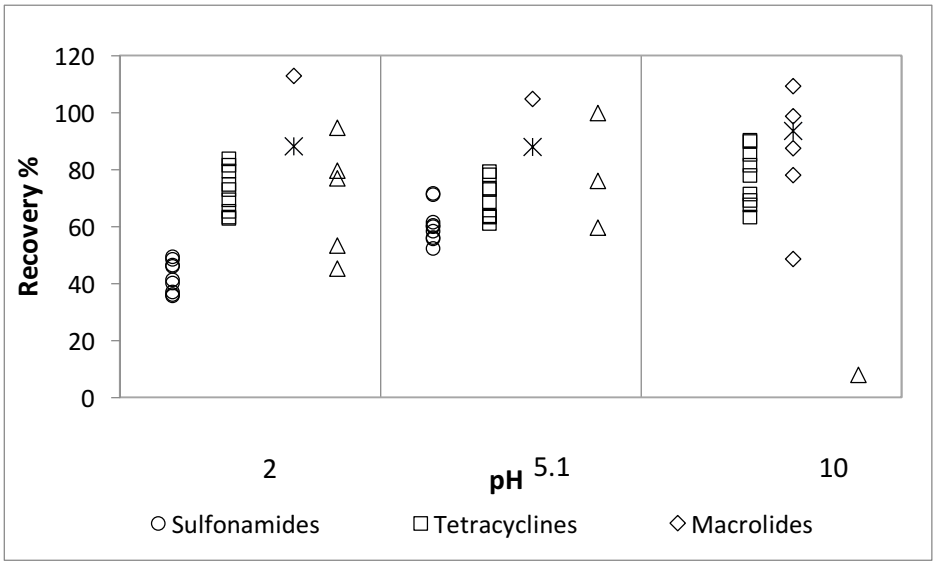

Figure 2. Influence of pH on extraction of antibiotic residues in honey

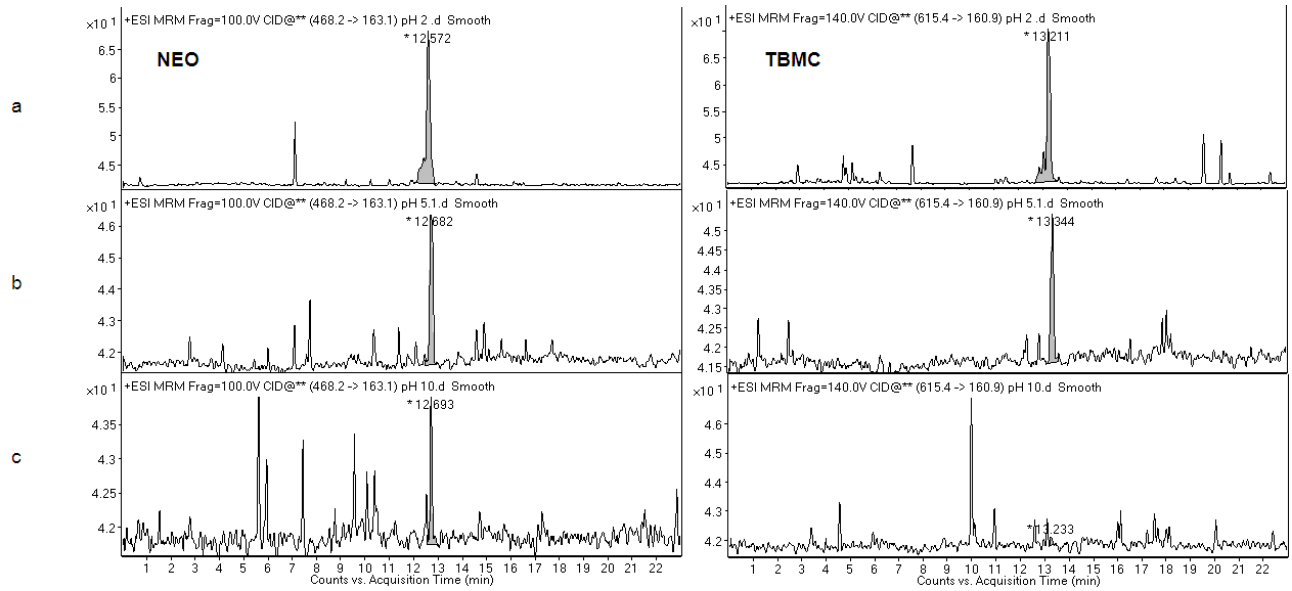

Figure 3. Improvement in peak shapes of NEO (left) and TBMC (right) at different pHs. a: pH=2, b: pH=5.1, c: pH=10

Effect of acidic hydrolysis

Several studies demonstrated that acidic hydrolysis is a crucial step to ensure the complete release of sulfonamides bonding sugar (Thompson and Noot 2005; Sajid *et al.* 2013; Tölgyesi *et al.* 2013; Dubreil-Chéneau *et al.* 2014). However, Bernal *et al.* (2009) reported the possibility of using pure MeOH to break the *N*-glycosidic bond. But Figure 1a, shows that pure MeOH was not able to disrupt the bonding. This observation was likely attributed to keeping our samples spiked for the whole night. Sajid *et al.* (2013) reported that methanol is adequate if the spiked honey was analysed within the same day immediately after spiking. But, when the spiked sample was kept for more than one day, no recoveries were found. Chen *et al.* (2012) and Tölgyesi *et al.* (2013) reported the use of acidified methanol to release sugar-bonded sulfonamides in honey and thus improving their recoveries.

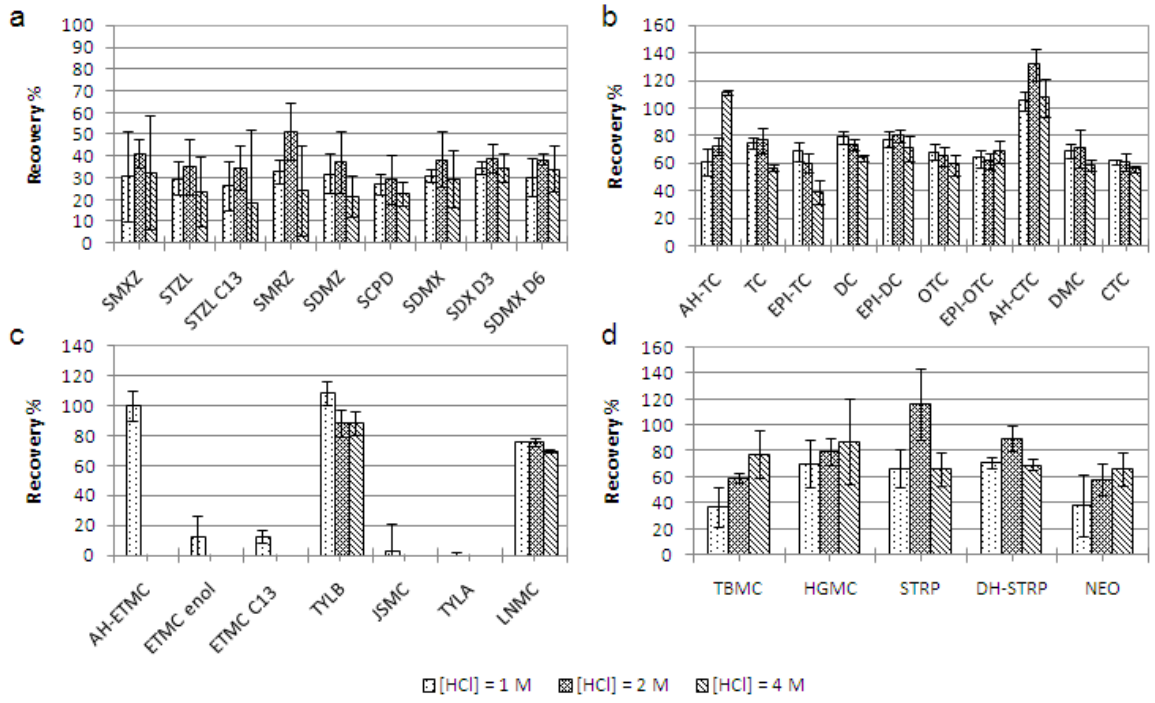

Figure 4. Analytes recoveries with different amount of HCl but equal pH value (pH 2). A) SAs, b) TCs, c) MAs and LCs, d) AMGs.

To avoid the possible underestimation of sulfonamides in honey sample, the breaking efficiencies of *N*-glycosidic bond were investigated with 1, 2, and 4 M hydrochloric acid prepared in MeOH. The pH of extraction was maintained at 2 by adding

proper amount of Na_4EDTA. The recoveries of analytes are displayed in Figure 4. It can be observed (Figure 4a) that there were approximately 5 to 10% higher recoveries for analysed sulfonamides with 2 M HCl than with 1 and 4 M. The breaking efficiencies were 27-35% for 1 M HCl, 29-51% for 2 M HCl, and 19-38% for 4 M HCl. We also observed that change in recoveries depended on the amount of used HCl.

For tetracylines, better recoveries (60-110%) were obtained at 1 and 2 M HCl. Among tetracyclines only AH-TC (Figure 4b) shows an increase in recovery with increasing hydrochloric acid concentration which lead to partial degradation of EPI-TC and TC into anhydro-tetracycline (AH-TC) (Pena *et al.* 1998). On the other hand, recoveries for aminoglycosides (Figure 4d) with 4 M HCl are higher than those with 1 and 2 M except for STRP and DH-STRP. Their maximum recoveries were found at 2 M HCl. Regarding macrolides, hydrochloric acid at 1 M generally provides better recoveries than 2 and 4 M. AH-ETMC and TYLB had recoveries of 100% and 108% respectively. However, poor recoveries (less than 20%) were obtained for ETMC enol, EMTC-C13, and JSMC. When the concentration of HCl increased, AH-ETMC, ETMC enol, EMTC-C13, and JSMC were not detectable. As a result, it is recommended to reach an optimized compromise among these different effects. To minimize the degradation of macrolides, extraction with 1 M HCl was the best choice but recoveries of aminoglycosides as well as the efficiency of *N*-glycosidic bond decreased at that concentration. Therefore, in our final method, hydrochloric acid at 2 M was chosen when performing extractions of multi-class antimicrobials.

Comparison of shaking versus sonication

In this experiment, we assessed the influence of mechanical shaking versus sonication on the extractability of antimicrobial residues in honey. The results were evaluated to study the efficiency of conventional shaking and ultrasonic-assisted extraction procedures through recovery experiments. Conventional orbital shaker IKA KS 501 (Staufen, Germany) was used to shake honey samples at 200 rpm. The duration of extraction was set to 30 min for both treatments. Figure 5 displays the comparison of different extraction approaches. It can be observed that the extraction efficiency for most antimicrobials in honey using the sonication method were higher than conventional shaking. Therefore, ultrasonic extraction was considered throughout this work. It is important to note that, the use of conventional shaking gave better results for AH-ETMC which was easily extracted while 30 min of ultrasonic extraction led to its degradation. We can conclude that all compounds give better recoveries using ultrasonic extraction except degradation products of erythromycin A.

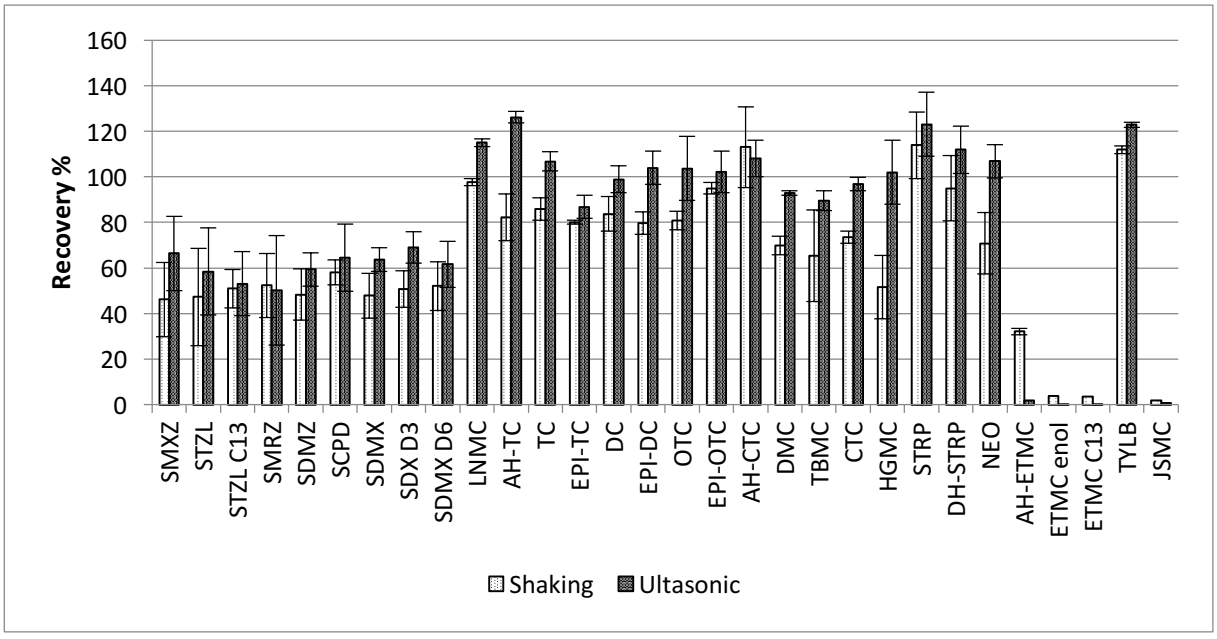

Figure 5. Comparison of extraction techniques (shaking and ultrasonic) to extract antibiotic residues in honey.

A second set of experiments was performed to investigate the efficiency of ultrasonic extraction at 10, 20, and 30 min. The effect of sonication time on the extraction of antibiotic residues in honey can be seen in Figure 6. It was found that the increase in ultrasonic extraction time from 10 min to 30 min caused a decrease in many recovered antimicrobials. Taking into consideration the possible degradation of some analytes such as AH-ETMC and ETMC enol at higher extraction time (Figure 6c), the optimum sonication time was selected at 10 min. Regarding JSMC, adopted acidic condition for sample preparation (pH 2) led to its degradation (Skinner, Taylor *et al.* 1993). It was shown also that neither sonication nor hydrolysis improved its recovery (less than 10%) during method development, thus, it was removed from the method.

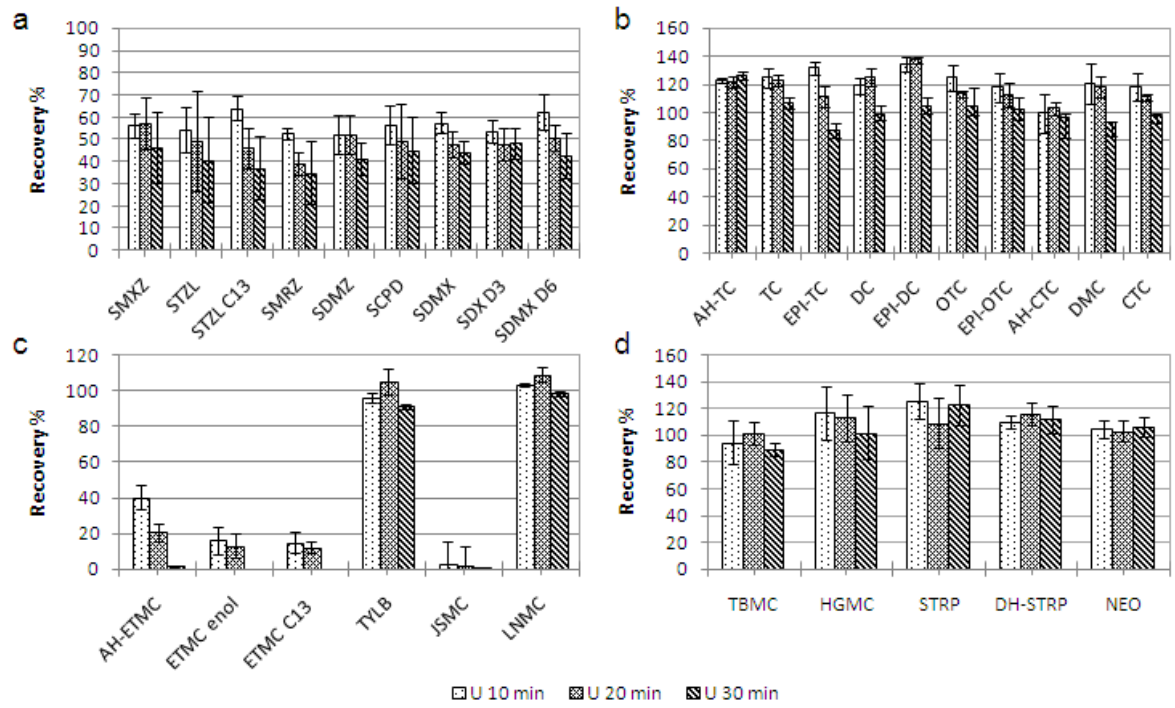

Figure 6. Influence of ultrasonication periods on extraction efficiency. A) SAs, b) TCs, c) MAs and LCs, d) AMGs.

Sample clean-up

Honey is a very complex matrix which contains interfering compounds (such as sugar, wax, and pigments) that can be co-extracted during the extraction process. This can reduce the lifetime of the analytical column and interfere with antimicrobials detection. Therefore, a clean-up step after extraction is mandatory to minimize co-extracted substances from honey extract. A number of multi-class methods use SPE for cleaning honey extract (Debayle *et al.* 2008; Lopez *et al.* 2008; Vidal *et al.* 2009; Hou *et al.* 2011; Bohm *et al.* 2012; Galarini *et al.* 2015). The most common SPE sorbents include HLB, polymeric reversed-phase, strong cation exchange, and mixed mode (reversed-phase/strong cation) exchange.

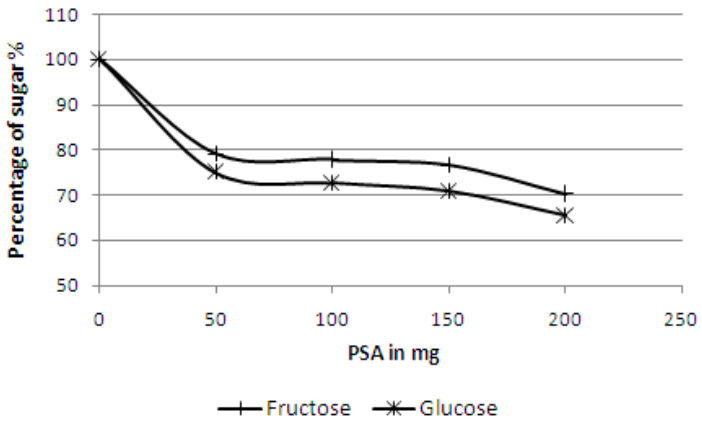

Figure 7. Clean-up capability of PSA sorbent to remove co-extracted hydrophilic component mainly sugar.

In our work, we evaluated a simple clean-up approach based on dispersive solid phase extraction (d-SPE). Primary secondary amine (PSA) was chosen for clean-up since both sugars and fatty acids can be removed from sample extract through hydrogen bonding (Anastassiades *et al.* 2003; Orso *et al.* 2014). Our experiment was performed to investigate the impact of PSA on the purification of 1 mL honey extract along with the recovery of analytes using different amounts of PSA sorbent 50, 100, 150, and 200 mg. An aliquot from each purified extract was taken for the analysis of sugar content (glucose and fructose) using LC with refractive index detector (RID). As it can be seen in Figure 7, 50 mg of PSA was found effective to remove about 20 to 30% of the tested sugar. However, increasing the amount of PSA >50 mg does not provide further decrease in sugar content. In addition, all target analytes achieved better recoveries when 50 mg of PSA was used (Figure 8). It is important to

note that a significant loss in recoveries of aminoglycoside residues was observed with increasing the amount of PSA. This might be attributed to its capacity to form hydrogen bonds with polar compounds. Therefore, 50 mg PSA was chosen as the most effective clean-up technique.

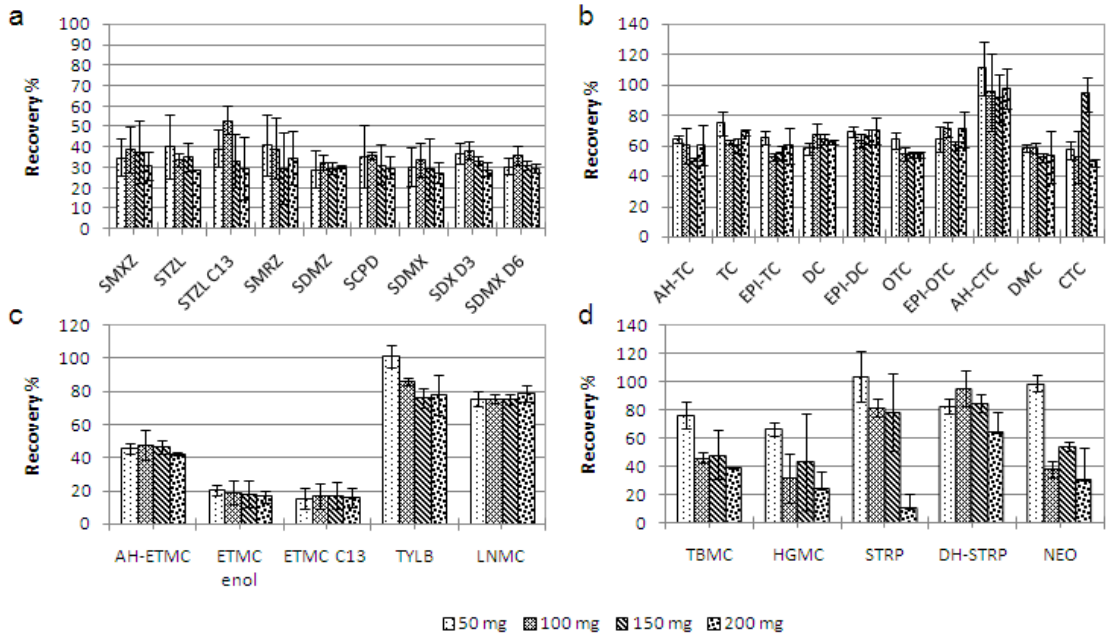

Figure 8. Effect of using PSA sorbent with different amounts on analytes recoveries.

Validation of the method

After method development, validation of the method was performed in accordance with European Commission Decision 2002/657. Identification of antibiotics is confirmed by presence of transition ions at the correct retention times (±2.5%) compared to the corresponding standards. Two MRM transitions are monitored for each analyte earning four identification points as described in Table 5 of Commission Decision (EC) No 2002/657. Furthermore, the measured ion ratio (least intense versus the most intense signal) was compared to those obtained from fortified honey samples and thus must fall within the criteria described in the Table 4 of Commission Decision 2002/657/EC.

To verify the absence of potential interfering substances at the retention time of the analysed antibiotic, blank honey samples, coming from different origins, were analysed more than 20 times. They were included over the 3 days throughout the sample queues on LC-MS/MS. These blanks showed no interfering peaks overlapping with our analytes in MRM mode, indicating adequate specificity for the analysis of the 22 antimicrobial residues in honey.

The linearity was determined by using matrix-fortified linear calibration curve. Blank honey samples were spiked with increment amount of analytes prior sample extraction at five concentration levels ranging 100 - 300 µg kg^{-1} for NEO and STRP and 20 - 100 µg kg^{-1} for the other compounds. Internal standards were spiked as well on blank honey samples at fixed amount for all levels (DMC, SDX-d3 and SDMX-d6 at 50 µg kg^{-1}; ETMC-c13, STZL-c13 and TBMC at 100 µg kg^{-1}; as well as HGMC at 200 µg kg^{-1}). The calibration curve showed good linearity with the coefficient of determination (r^2) higher than 0.988 (Table 3).

The CCα and CCβ were estimated according to ISO standard 11843-2 (2000) which allows the determination of both parameters using linear calibration curve. The decision limit CCα means the limit at and above which can be concluded with an error probability (α=1% for non-authorized substances) that a sample is non-compliant (Commission Decision No 2002/657/EC). The Commission Decision 2002/657 defines that CCα of a banned substance must be lower than the recommended limit and CCβ must be lower or equal to this recommended limit. As shown in Table 3, CCα and CCβ values for studied analytes range from 6 to 25 µg kg^{-1} and from 7 to 33 µg kg^{-1}, respectively. The highest CCα and CCβ values were obtained for NEO and STRP antibiotics, however, both decision limits and detection capabilities met the conditions of EU Decision.

Table 3. Results of CCα, CCβ and coefficient of determination r^2 for each analytes.

Analytes	Linear range (µg kg^{-1})	Coefficient of determination (r^2)	CCα (µg kg^{-1})	CCβ (µg kg^{-1})
SMXZ	20-100	0.992	8	11
SMRZ	20-100	0.994	7	9
STZL	20-100	0.993	7	10
SDMX	20-100	0.992	8	10
SDMZ	20-100	0.995	6	8
SCPD	20-100	0.991	9	11
OTC	20-100	0.996	5	7
DC	20-100	0.994	7	9
CTC	20-100	0.993	7	10
TC	20-100	0.992	8	11
AH-TC	20-100	0.993	8	10
AH-CTC	20-100	0.988	10	13
EPI-DC	20-100	0.993	8	10
EPI-TC	20-100	0.993	8	10
EPI-OTC	20-100	0.993	7	10
TYLB	20-100	0.991	9	11
AH-ETMC	20-100	0.992	8	11
ETMC enol	20-100	0.994	7	9
LNMC	20-100	0.991	9	12
DH-STRP	20-100	0.991	9	11
STRP	100-300	0.990	24	32
NEO	100-300	0.989	25	33

The recovery and precision of each antibiotic (except NEO and STRP) was determined on spiked blank honey samples at three different concentrations 20, 30, and 100 µg kg^{-1}. For NEO and STRP the spiked levels were 100, 150, and 200 µg kg^{-1}. The recoveries were estimated and corrected automatically from the matrix fortified calibration curve. Table 4 provides satisfactory results for all tested antimicrobials where the average recoveries are ranged between 85% - 111% at the three concentrations. These values fall within the range of acceptable bias (between -20% to +10%) when samples are spiked at concentration higher than 10 µg kg^{-1} (2002/657/EC).

In terms of precision, repeatability (CV_r) and intra-lab reproducibility (CV_R) experiments on three different days were investigated at three spiking levels and the results are shown in Table 4. We can observe that CV_r and CV_R values for all antimicrobials range from 5.8% to 20.6% and from 6% to 26.8%, respectively. Based on the Decision (EC) 2002/657, the CV_R for samples spiked at concentrations less than 100 µg kg^{-1} shall be as low as possible (targeting below 23%). Our CV_R values for all analytes were below 23% except for SMRZ (CV_R, 24.2%) and AH-EMTC (CV_R, 26.8%) at 20 µg kg^{-1}. Therefore, our method satisfactorily fulfils the requirements for intra-lab reproducibility.

Finally, matrix effect was evaluated by comparing calibration sets prepared one spiking analytes in solvent and the other spiking analytes in extracted blank honey but at the same concentration. Signal enhancement or signal suppression are generally due to in-source ionizing competition of the targeted analytes with co-extractive components in honey matrix samples from sugars primarily and also from carbohydrated fibers. Matrix effects (ME%) were calculated by comparing the slope obtained for each analyte in honey matrix (a_m) with that of the same analyte in solvent (a_s) and using the following formulae (Economou *et al.* 2012):

$$ME\% = \left(\frac{a_m}{a_s - 1}\right) \times 100$$

Figure 9 shows a significant matrix suppression ranging from -26 to -80% for sulfonamides, tetracyclines, lincosamides, STRP and DH-STRP. As for AH-ETMC and NEO, they showed tolerable matrix effects (less than +20%). On the other hand, only two analytes (ETMC enol and TYLB) showed matrix enhancement ranging from +35 to +55%. Matrix matched calibration was different from the one obtained in solvent, indicating a significant matrix effect for these analytes in honey. Therefore, matrix fortified standard calibration was used in quantification to avoid problems derived from suppression and enhancement effects.

Table 4. Method validation data of the developed method

	20 µg kg^{-1}			30 µg kg^{-1}			40 µg kg^{-1}		
Analyte	Rec %	CVr %	CVR %	Rec %	CVr %	CVR %	Rec %	CVr %	CVR %
SMXZ	94	15.0	16.9	98	14.0	16.3	90	7.3	11.4
SMRZ	98	19.8	24.2	91	14.5	16.1	96	12.6	13.2
STZL	100	19.2	17.6	104	16.4	17.2	107	12.3	11.8
SDMX	109	9.1	10.1	102	6.5	9.0	102	8.0	7.3
SDMZ	101	12.5	12.6	96	11.1	12.1	98	10.4	11.0
SCPD	106	17.3	18.2	96	9.2	10.0	93	12.5	11.8
OTC	100	10.9	12.3	103	7.8	14.8	103	10.9	12.0
DC	98	8.4	8.4	104	11.6	10.8	103	8.0	13.0
CTC	97	10.8	11.1	104	9.7	9.4	98	11.0	13.1
TC	97	9.3	13.4	100	10.3	13.1	101	9.0	9.1
AH-TC	102	14.2	16.1	100	7.3	7.2	102	7.8	9.7
AH-CTC	85	16.9	16.4	93	10.9	20.6	93	10.8	13.2
EPI-DC	100	8.8	8.2	104	6.0	6.0	103	7.2	11.5
EPI-TC	97	8.3	8.0	98	8.0	8.1	98	8.0	7.5
EPI-OTC	100	13.5	16.1	104	11.5	17.0	101	9.8	12.3
TYLB	95	17.1	20.0	92	8.4	10.7	111	10.2	13.2
AH-ETMC	100	20.6	26.8	103	9.5	20.1	102	8.7	18.3
ETMC enol	97	17.1	20.5	99	10.6	20.9	109	13.4	24.4
LNMC	103	13.5	15.7	102	9.5	11.0	100	10.7	12.2
DH-STRP	97	17.8	17.2	109	19.0	21.3	105	19.0	20.6
	100 µg kg^{-1}			150 µg kg^{-1}			200 µg kg^{-1}		
Analyte	Rec %	CVr %	CVR %	Rec %	CVr %	CVR %	Rec %	CVr %	CVR %
NEO	98.2	16.7	17.9	96.5	14.6	16.0	84.7	13.3	14.3
STRP	99.1	12.6	14.1	94.4	8.9	9.9	108.2	17.1	21.7

Quality control materials

To assess the quality of test results obtained through the validated method, an inter-laboratory proficiency test scheme was carried out for the determination of antibiotic residues in honey. FAPAS (Food Analysis Performance Assessment Scheme) honey test material 02270 containing a certified amount of unknown analytes of tetracyclines was used. The name of these analytes as their concentrations in honey were not provided before a final report was issued by FAPAS. These analytes were identified as chlortetracycline and doxycycline at concentration ranging from 3.3 to 8.5 µg kg^{-1} (assigned value 5.9 µg kg^{-1}) and from 41.6 to 106.9 µg kg^{-1} (assigned value 74.2 µg kg^{-1}), respectively. Our obtained concentrations were 58 µg kg^{-1} for doxycycline and 5 µg kg^{-1} for chlortetracycline with Z scores of -1 and -0.69 respectively. This was a good indicator that our developed and validated method provides reliable quantification results to determine tetracycline residues in honey product.

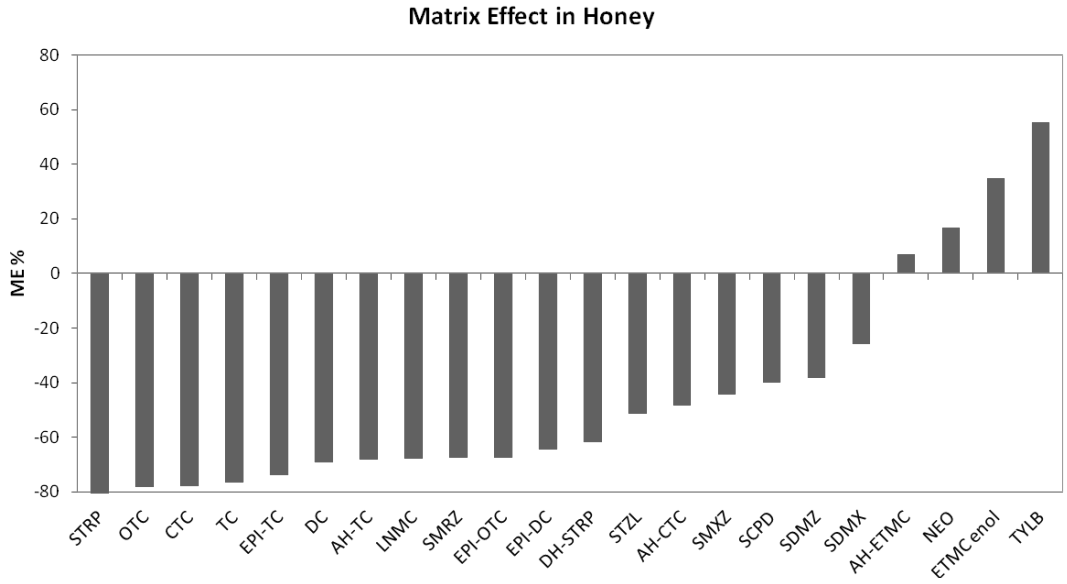

Figure 9. Matrix effects for the target compounds

Conclusion

A straightforward sample preparation has been developed for the determination of multi-class antimicrobial residues including sulfonamides, tetracyclines, lincosamides, and macrolides in honey using LC-esi-MS/MS. Up to 24 samples can be extracted within less than 2 h in one step, offering a high-throughput multi-residue analytical method in an acceptable cost and time for analysis.

In our study, methanol was found to be the most effective solvent for extraction of a wide range of antimicrobial residues in honey. The use of acid hydrolysis (2 M HCl) during sample treatment is essential to disrupt *N*-glycosidic sulfonamides bonding with the honey reducing sugars. Our experiment demonstrated the need to decrease the pH of honey samples to 2 in order to achieve good recoveries for most of the studied analytes. However, still the risk to lose acidic-sensitive antimicrobials such as macrolides occurs. As a result, degradation products of tylosine A and erythromycin A were selected as better marker residues to monitor the presence of these analytes in honey samples. Dispersive solid phase extraction with PSA 50 mg allows removing 20 to 30% of co-extractive sugars (20-30%) from honey extract; however further addition of PSA sorbent reduces the recovery of aminoglycoside residues.

The developed method was validated according to recommended criteria of Commission Decision (EC) No 2002/657 and satisfactory performance data were obtained for most studied analytes. Specificity, linearity, recovery, precision, CCα, and CCβ have been tested successfully. They were in compliance with EU legislation requirements demonstrating the fitness of this method for selected antimicrobials. Further work will be carried out by including this method in a surveillance monitoring program dedicated to determine antimicrobial residues potentially found in different types of honey in Lebanon.

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DETERMINATION OF GROWTH HORMONE-RELEASING HEXAPEPTIDE IN *BOVINE* URINE BY LC-MS/MS

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Abstract

The abuse of growth-promoters in animal husbandry changed over the years. To be ready for the future in terms of control of misuse of growth-promoters, method development for new classes of compounds is necessary. Besides by using well-known steroids and anabolic agents, muscle growth within cattle can also be altered by administration of growth hormone-releasing peptides (GHRP's). There is not much known about analysis and excretion of GHRP's in cattle urine. In this study, growth hormone-releasing hexapeptide (GHRP-6) was chosen as a model compound for this class of compounds. LC-MS/MS was used for compound detection and quantification. Detection and sample clean-up was challenging due to the tendency of the compound to adsorb to all kinds of surfaces. The compound was isolated from urine using 96-wells SPE Oasis HLB-plates. The method was fully validated according to Commission Decision 2002/657, the CCα was 0.37 ng mL^{-1} and the uncertainty of measurement 3.4%.

Introduction

Growth hormone-releasing peptides are secretagogues that act on a specific receptor on the hypothalamus. They are small peptides that induce the growth-hormone secretion. Many of the growth hormone-releasing peptides (GHRPs) available on the market are synthetic and used in human medicine to cure *e.g*. growth-hormone deficiency and acute ischemia (1,2). An overview of available peptides with growth hormone-releasing activity is given in Table 1.

The use of growth hormone-releasing peptides can be beneficial in animal husbandry due to the effect of growth-hormone secretion enhancement resulting in muscle growth. The main purpose of growth-hormone is to regulate the optimal statural growth in the body. A complex endocrine interaction with the pituitary somatotropins either induces or suppresses growth-hormone pulsatile release (3). The GHRPs however, take a different route in the body and do not undergo the suppression making the effects faster visible. "GHRPs and their analogues have no structural homology with growth hormone releasing hormones and act via specific receptors present at either the pituitary or the hypothalamic level." (4). GHRP-6 is a synthetic peptide that works as a growth-hormone secretagogue in both humans and animals. The growth hormone-releasing hexapeptide serves a clinical purpose of treating people with acute ischemia, as it maintains tissue viability in small intestine, liver and kidneys (2). It can be administered intravenously, subcutaneously, intranasally and orally. The GHRP-6 is small, stable, soluble and has a low toxicity, making it a good candidate to be used as a doping agent (5).

Table 1. Primary structure of peptides with growth hormone releasing activity.

Compound	*Sequence*
GHRP-1	Ala-His-D-bNal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-2	D-Ala-D-bNal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-4	D-Trp-Ala-Trp-D-Phe-NH_2
GHRP-5	Tyr-D-Trp-Ala-Trp-D-Phe-NH_2
GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2
Hexarelin	His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2

As a synthetic peptide, the structure of GHRP-6 is derived from an endogenous pentapeptide, met-enkephalin, an endorphin of the brain that binds to the opioid receptor (6). Like enkephalin, the growth hormone-releasing hexapeptide induces growth hormone release activity, and has a structure that was specifically selected to act on a particular receptor of the hypothalamus and pituitary (7). Table 1 shows the chemical sequence of GHRP-6. It can be concluded that all synthetic compounds contain at least a single artificial D-amino acid. The artificial amino acid was inclued to prolong the half-life of the peptides.

The metabolic pathway of the body is maintained by liver enzymes, exopeptidases and by kidneys. During metabolism, GHRP-6 is hydrolysed at the C-terminal end and the uncharged and natural amino acids are eliminated from the C/N-terminal end by exopeptidase (8). During the metabolic process, the GHRP-6 does not degrade into amino acids but into smaller peptides.

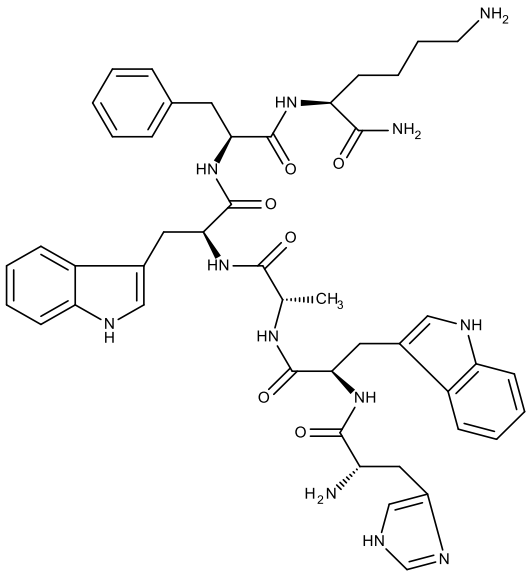

Figure 1. Chemical structure of growth hormone-releasing hexapeptide: His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2. The compound has a charged N-terminal histidine side chain, central aromatic residues and C-terminal lysine residue (3).

The enkephalin analogue, served as a basis to create structurally interrelated GHRPs to improve the GH-releasing activity. One of the peptides, growth hormone-releasing hexapeptide (GHRP-6), particularly has a direct effect on the pituitary (9).

Administration of growth hormone-releasing substances for production purposes is prohibited in the European Union to protect consumers from possible harmful effects (10). To determine illegal use, a highly sensitive method for regular screening of GHRP-6 needed to be developed. Bioanalytical method development for peptide quantification is complicated, due to the behaviour under various sample preparation and chromatographic conditions. Peptides have the tendency to stick to surfaces of materials used during sample preparation, such as pipette tips, test tubes and also to the stationary phase of liquid chromatography columns. There is very little information known about the exact behaviour of GHRPs in laboratory set up. The most investigated peptide up to this date is GHRP-2. The different solvents, stationary phases, eluents and sample clean up vary from peptide to peptide. The analyte of interest for this study was GHRP-6.

Materials and Methods

Materials and chemicals

Solvents, acids and standards were from Merck (Darmstadt, Germany), Sigma (Sigma-Aldrich, Steinhem, Germany) and ACTU-ALL Chemicals (Oss, The Netherlands). Pure water was purchased from ACTU-ALL Chemicals. SPE cartridges Oasis WCX 96-Well Plate 30 µm (30 mg) were bought from Waters (Milford, MA, USA). Isotope-labelled ^{13}C-GHRP-6 was purchased from Pepscan (Lelystad, The Netherlands). Each standard was dissolved in 5% formic acid and 10% dimethyl sulfoxide and stored at -20°C.

Sample preparation

A 100-µL portion of urine was taken and diluted with 100 µL 5% formic acid in water. The sample was then spiked with 100 ng mL^{-1} internal standard. The spiked samples were centrifuged at 14,000 rpm (Eppendorf, Germany) for 10 min. The supernatant was loaded onto pre-equilibrated (300 µL methanol, 300 µL water) SPE 96 Well-Plate Oasis HLB (30µm, 30 mg). For loading, positive pressure processor 96 (Waters, UK) was used. The sample was washed with several separate steps, 300 µL water, 300 µL 5% methanol in water. The third washing step involving 200 µL methanol/ water (30/70, v/v) was repeated twice to remove as much as matrix effects as possible. The sample was eluted onto a 96 Well-Plate with 2% formic acid in acetonitrile/ water (60/40, v/v). Test portion of 100 µL of the eluate was transferred to another 96 Well-Plate and the injection solvent was changed to 2% formic acid in acetonitrile/ water (30/70, v/v).

LC-MS/MS analysis

LC-MS/MS analysis was carried out on Xevo TQ-S (Waters, UK) with a Zspray ESI, positive ionisation. The following MS settings were used: capillary 2.50 kV, cone 50 V, source Offset 50 V, desolvation temperature 500°C, desolvation 950 L h^{-1}, cone 150 L h^{-1}, nebuliser 7.0 bar, LM Resolution1 2.50, HM Resolution1 14.50, LM Resolution2 3.00, HM Resolution2 15.00, Ion Energy2 0.8, collision Gas Flow 0.18 mL min^{-1}, collision 30/15 eV. For GHRP the MRM's 465.7>84.1 and 465.7>110.027 (CE 15) and for ^{13}C-GHRP 469.48>110.17 monitored.

LC Conditions

The chromatographic separation was obtained using a Acquity (Waters, Ireland) UPLC CSH C_{18}-column (100 mm × 2.1 mm id, 1.7 µm) (oven temperature 60°C) under gradient conditions (Table 2) with 0.1% formic acid in water as eluent A and 0.1% formic acid in acetonitrile as eluent B. The flow rate was set at 0.6 mL min^{-1}, and the samples were injected from Waters (UK) 96-Well Plate (1 mL) with injection volume set at 5 µL.

Table 2. Gradient profile for LC.

Time (min)	Flow rate (mL min^{-1})	%A	%B	Curve
Initial	0.600	100	0	Initial
0.5	0.600	100	0	6
7.00	0.600	60	40	6
8.50	0.600	60	40	6
8.60	0.600	100	0	6

Validation

Validation was performed according to Commission Decision 2002/657. Calculation was performed using ResVal V3.0. This data processing sheet uses ANOVA for calculating method performance characteristics. The calculations for CCα and CCβ are in accordance with ISO11843 (11).

The specificity of the method was determined by testing the blank sample against the CCα. The outlier test is based on the robust statistic approach. The ruggedness test is based on two tests, one of them tests if the results are within the repeatability limits of the method (n = 7) and the other is based on a dispersion test (R-chart).

The validation is performed by performing three consecutive experiments. For each experiment seven *bovine* urine samples are chosen. The validation was performed with 21 urine samples within one day. Three experiments were performed, seven urine samples were used for each experiment and spiked at four different concentration levels (0, 0.5, 1, 1.5 ng mL^{-1}) of the validation level (1 ng mL^{-1}). Linearity analysis was performed on eight different concentration levels. (0.25, 0.5, 0.8, 1, 1.5, 2, 4, 5 ng mL^{-1}). The samples are quantified against a matrix-matched calibration curve.

Results and Discussion

In Figure 2 an overview is given of one transition of GHRP-6 spiked to samples of urine. As can be seen from Figure 2, there is little matrix interference at low concentration levels of 0.25 ng mL^{-1}. To determine the linearity, a calibration curve (Figure 3) is constructed for the spikes in Figure 2 (0.25, 0.5, 0.8, 1, 1.5, 2, 4, 5 ng/mL).

Table 3. Performance characteristics of full validation.

Performance characteristic	Validation level	Validation level	Validation level
Level	0.5	1	1.5
Unit	ng mL^{-1}	ng mL^{-1}	ng mL^{-1}
Accuracy	96.9%	87.4%	94.4%
Covariation reproducibility	1.7%	2.2%	1.5%
Covariation Repeatability	1.7%	1.8%	1.4%

The method was fully validated; the performance characteristics are listed in Table 3. The CCα and CCß were determined according to ISO11843 and 2002/657/EC (12). Calculated CCα is 0.37 ng mL^{-1} and the CCβ is 0.74 ng mL^{-1}. Based on the validation it can be concluded that GHRP-6 can be reliable quantified with the method used. The standard deviation for the repeatability and reproducibility is very low. The method was considered specific and robust.

Conclusions

The aim of this project was to develop a confirmatory method for the detection of GHRP-6 in *bovine* urine. The sample clean-up steps involved spiking the sample with the isotopically labelled internal standard. The sample was centrifuged and loaded on to a SPE 96 Well-Plate Oasis HLB. The sample was clean with several washing steps and injected into the LC-MS/MS. The method was validated according to the directive 2002/657. The found decision limit (CCα) was 0.37 ng mL^{-1} with α-error of 5%. The detection capability (CCβ) of the developed method was found to be at 0.74 ng mL^{-1} with the β-error of 5%. Based on the validation it can be concluded that the developed method has specificity and sensitivity and can be used for confirming and quantifying GHRP-6 in *bovine* urine with 93% accuracy and 3.4% uncertainty.

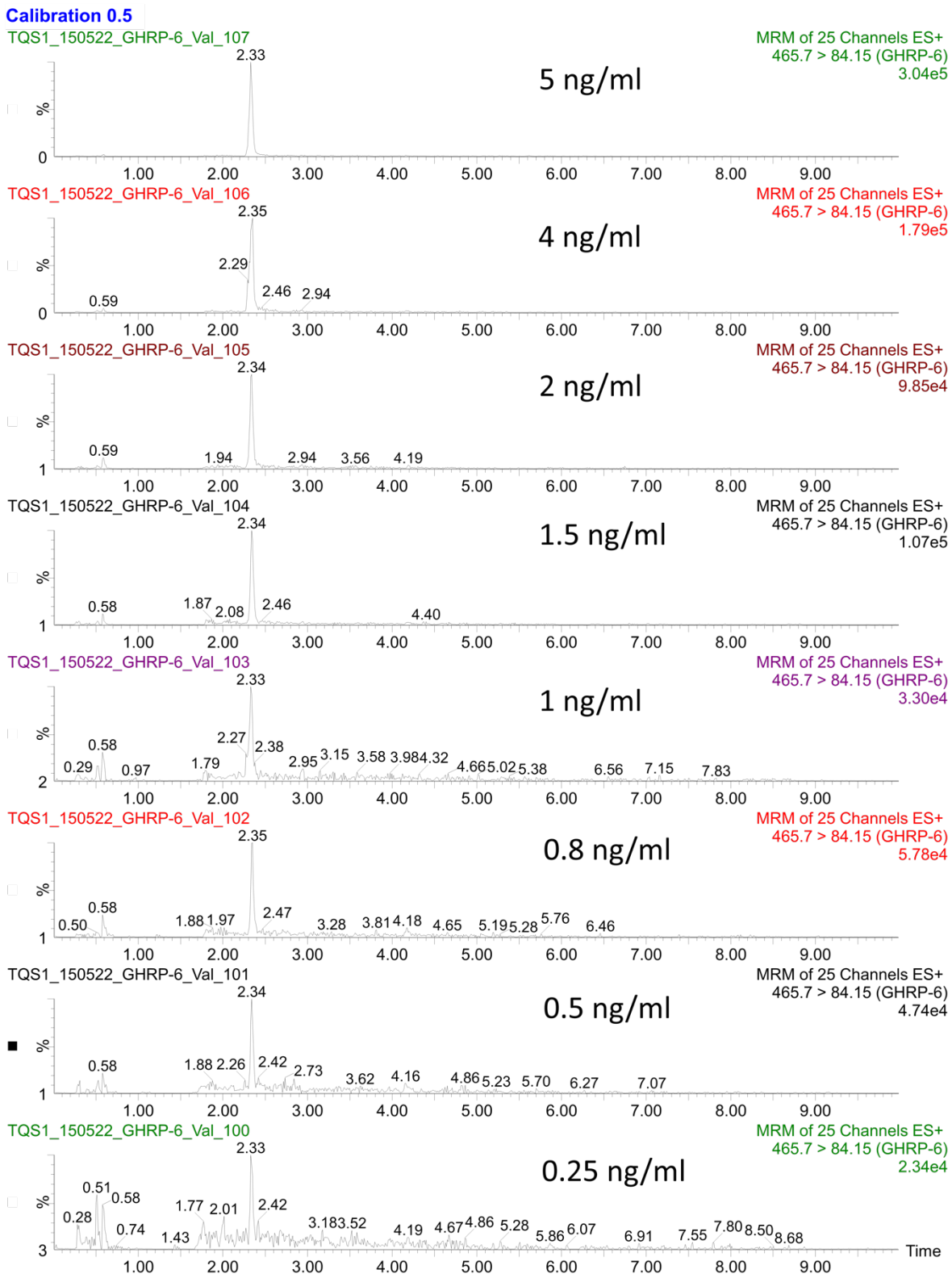

Figure 2. LC-MS/MS analyses of 0.25, 0.5, 0.8, 1, 1.5, 2, 4 and 5 ng mL^{-1} GHRP-6 spiked in urine. Chromatographic separation was carried out on a Waters CSH C_{18} column (2.1 × 100mm, 1.7 µm). Data processing was done for the mass transition 465.7> 84.151 m/z.

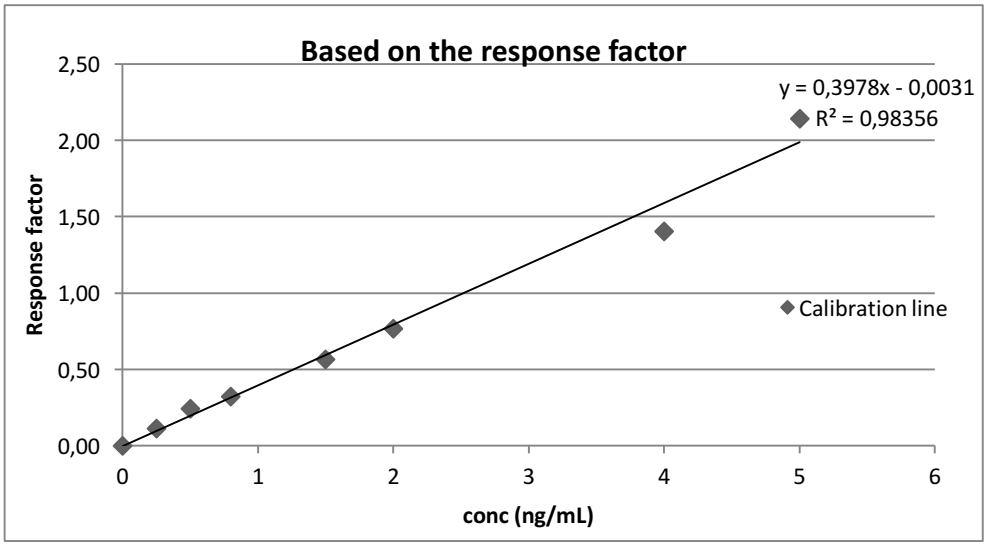

Figure 3. Calibration curve based on the relation between the concentration of the calibration standards and the response factor calculated from the isotope dilution mass ratio. Formula obtained from this graph was used to quantify GHRP-6 in samples spiked at validation levels, based on their response factors.

Acknowledgements

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DECONJUGATION OF STEROIDS IN URINE: IS THERE AN ULTIMATE METHOD FOR COMPLETE DECONJUGATION OF STEROIDS IN URINE?

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In memorial of Jeroen van den Brink who passed away in the summer of 2014. He performed this work as an intern at RIKILT.

Abstract

Analysis of steroids in urine contain a deconjugation step to remove glucuronide and/or sulphate moieties. This deconjugation step is performed with enzymes or chemically. For enzymatic deconjugation of glucuronide conjugates, β-glucuronidase from *Escherichia coli* is effective. Enzymatic deconjugation of sulphate conjugates is not possible without the risk of converting steroids. Chemical deconjugation is found to be very effective for sulphate conjugates, but also for glucuronide conjugates. However, the effectiveness of the deconjugation is influenced by the position of the substituent on the steroid. If the substituent is attached to the position three, chemical deconjugation is less effective than on the position seventeen. This effect is more pronounced for glucuronides than for sulphate conjugates, indicating that steric hindrance is causing this less effective deconjugation reaction. The optimal condition for chemical deconjugation is 60°C with methanolic HCl (1 M). The final protocol to deconjugate all phase II steroids is a combination of an enzymatic- and chemical-deconjugation. In case of a 17-conjugated steroid, only a chemical deconjugation step should be performed.

Introduction

Because steroids are relatively non-polar compounds, the steroid is substituted with either a sulphate or glucuronide group, to enhance solubility in an aqueous environment and thus to facilitate renal clearance. This process is called phase II metabolism. Steroids in urine are more than 90% present in a conjugated form. Examples of phase II conjugated steroids are depicted in Figure 1.

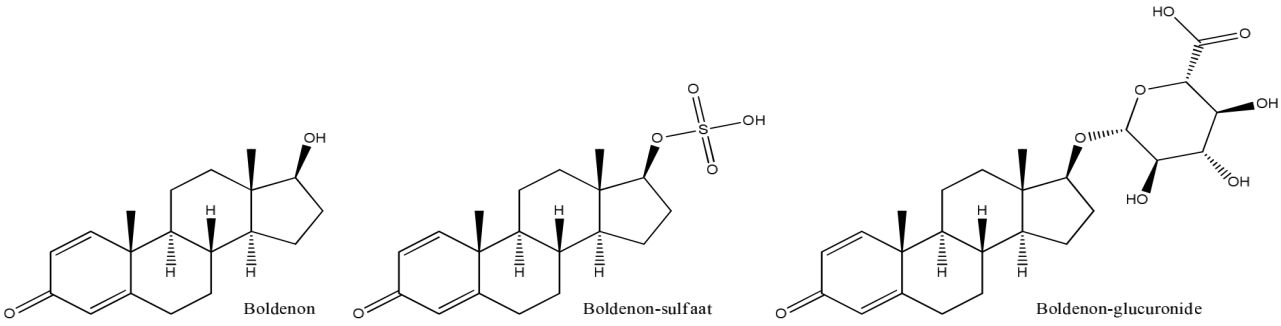

Figure 1. Example of free boldenone, boldenone-17-sulphate and boldenone-17-glucuronide.

To analyse steroids using GC-MS/MS, phase II substituents need to be removed. This deconjugation of steroids can be performed in two ways. Enzymatic hydrolysis removes the glucuronide or sulphate using specific enzymes. Deconjugation can also be performed by solvolysis, *i.e*. releasing the functional group in an organic solvent containing a strong acid or base [1].

Enzymatic deconjugation

With β-glucuronidase it is possible to remove glucuronic acid using enzymes extracted from bacteria (*E. coli*) or snails (*Helix aspersa*). Deconjugation of steroid sulphates can be performed with arylsulphatase extracted from bacteria (*Aerobacter aerogenes*), molluscs (*Patella vulgata*) or snails (*Helix aspersa*). There are different types of arylsulphatase enzymes, namely arylsulphatase A, arylsulphatase B and arylsulphatase C (also called steryl-sulphatase or steroid sulphatase). Arylsuphatase C is the most commonly used enzyme for steroid deconjugation. Arylsulphatase A and arylsulphatase B are not used because they are very inefficient in hydrolysing sulphate. The enzymes of *H. aspersa* is the most widely used because these contain both β-glucuronidase enzymes and arylsulphatase, and is therefore very effective for deconjugation of a large amount of steroids. The enzyme of *P. vulgata* has poor sulphatase effectiveness but is very specific to certain steroids (4).

It is important to choose the enzyme that fits best to the conjugated steroid. Results have shown that most enzyme sources are contaminated with substances responsible for the conversion of steroids to other compounds. These so called artefacts

are unwanted. The use of β-glucuronidase of *E. coli*, does not convert steroids (2) because it is a pure form. A well-known conversion of a steroid using enzymes extracts is shown in Figure 2.

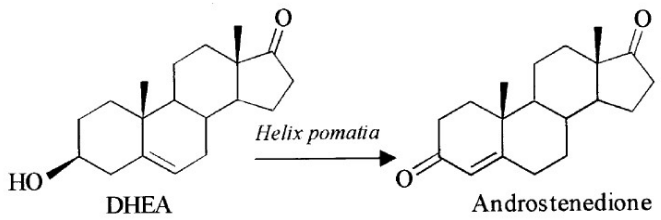

Figure 2. Conversion of dehydroepiandrosterone (DHEA) to androstenedione through conversion of enzymes of H. Pomatia *(2).*

When enzymatic deconjugation is applied to a large number of steroids that are present in urine, it is important to note that the effectiveness of this deconjugation may vary from steroid to steroid. It has been found that arylsulphatase only deconjugates specific steroids. This enzyme can deconjugate the 3-suphate of estradiol but cannot deconjugate the 17-sulphate (3). Important factors in the effectiveness of enzymatic deconjugation are hydrolysis time, pH, temperature and source of the enzyme.

Chemical deconjugation

Chemical deconjugation can also be used to remove phase II conjugation. To remove phase II substituents, a strong acid like hydrochloric acid or sulfuric acid in aqueous medium is used. Also ethyl acetate in combination with sulphuric acid is used for solvolysis of conjugates (4). A disadvantage of the use of chemical deconjugation is that relatively extreme conditions are being used at which degradation of steroids can take place. For this reason, it is important to avoid chemical deconjugation at high temperatures. This increases the chance of degradation effects during the solvolysis (5).

A form of solvolysis is methanolysis and is an effective method that is used in the detection of androgens in the urine of athletes in doping control (6). Methanolysis has been compared with protonic hydrolysis and enzymatic hydrolysis using enzymes from *H. aspersa*. The results of methanolysis compared to these methods vary with steroids but are at least as good. Methanolysis is an efficient and effective method and can be performed within 20 min (5). Parameters that are important for chemical deconjugation are the type of acid, temperature and solvolysis time. Using this approach, both sulphate and glucuronic acid conjugates can be effectively cleaved (4).

In this study, research was performed extensively to test enzymatic as well as chemical deconjugation with the goal to develop a full deconjugation method for steroids.

Materials and Methods

In general, standard chemicals and lab equipment were used. The conjugated steroids used in this study were 17β-androstenediol-disulphate (Steraloids), 17β-boldenone-glucuronide (German Sport University Cologne), 17β-boldenone-sulphate (Steraloids), dehydroepiandrosterone-sulphate (Steraloids), dehydroepiandrosterone-glucuronide (Sigma), 17β-estradiol-3-glucuronide (Sigma), 17β-estradiol-17-glucuronide; (Sigma), 17β-estradiol-3-sulphate (Sigma), 17β-estradiol-17-sulphate (Steraloids), 17β-estradiol-3-sulphate-17-glucuronide (Sigma), 17α-testosterone-sulphate (National Measurement Institute), 17β-testosterone-glucuronide (National Measurement Institute), 17β-testosterone-sulphate (National Measurement Institute).

Table 1. Variables optimised for the enzymatic deconjugation

Variable	1	2	3	4
Time (h)	0	1	4	24
T (˚C)	37	60		
Amount of enzyme (µL)	20	40		

Enzymatic deconjugation

From literature it is known that *Helix Pomatia* extracts can convert undesirably steroids. It is for this reason that enzyme sources were chosen for which such conversions have not been described, *i.e*. β-glucuronidase from *E. coli*. Arylsulfatase from *A. aerogenes* was also tested. To optimize the enzymatic deconjugation a factorial design is used. A total of 32 of all possible experiments were determined using GenStat ® (14th edition) and are summarised in Table 1.

Optimization of chemical deconjugation

Chemical deconjugation is performed with methanol as solvent (5), several different acids were used. The experimental design (32 experiments) is given in Table 2 using factorial design using GenStat ®.

Table 2. Variables optimised for the chemical deconjugation

Variable	1	2	3	4
Acid	Methanolic hydrochloric acid	Methanolic sulphuric acid	Methanolic hydrochloric acid from acetyl chloride	Methanolic hydrochloric acid from TMCS
Concentration (M)	1	2		
Time (h)	1	4		

Results and discussion

Enzymatic deconjugation

Optimization of the enzymatic deconjugation is carried out by using two enzymes: β-glucuronidase and arylsulfatase. From the experiments that have been conducted is was found that arylsulfatase, unlike the enzyme β-glucuronidase, is unable to deconjugate any of the used sulphate conjugates. For this reason, only the results of the β-glucuronidase deconjugation discussed.

The most important result is that for the component DHEA a significant difference is found between the effect of hydrolysis temperature at 37°C or at 60°C. The effectiveness at 37˚C is significantly better. Varying the hydrolysis time from 0 hour to 24 h revealed that there is a small difference between the results obtained at 0 h, 1 h, 4 h and 24 h. Also between the amounts of enzymes (20 and 40 µL) used, no significant differences in the results were found and was confirmed by an ANOVA analysis. It was concluded that a hydrolysis time of 15 min and 20 µL enzyme solution is sufficient (Figure 3).

The results of the entire experiment showed that the effectiveness of β-glucuronidase to deconjugate steroid conjugates is very specific. Deconjugation with arylsulfatase to remove sulphate groups is found not to be effective.

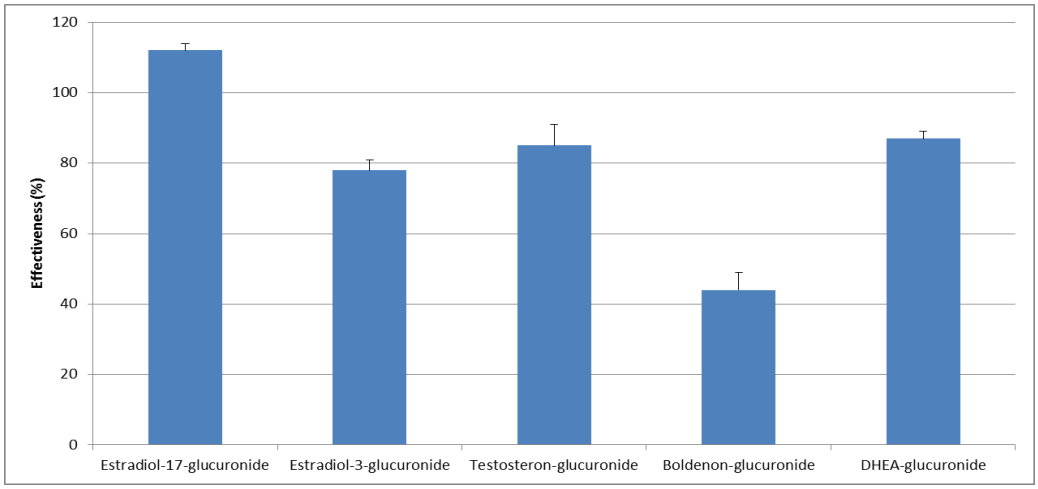

Figure 3. Effectivity of ß-glucuronidase to deconjugate glucuronide-conjugated steroids.

Chemical deconjugation

With methanolic sulphuric acid none of the conjugated sulphate steroids were deconjugated. By use of methanolic hydrochloric acid, all sulphate steroids in this study could be detected and quantified. Besides methanolic hydrochloric acid also acetyl chloride and methanolic hydrochloric acid from trimethylsilyl chloride were used for deconjugation. All three acids performed similar in this experiment.

For the other components, *e.g*. steroid glucuronides and compounds that possesses a glucuronide and sulphate, there is a significant difference in effectiveness of the deconjugation. With an ANOVA analysis it was shown that for DHEA there is an effect of the temperature and acid concentration for the deconjugation rate, *i.e*. the deconjugation is the highest at a high temperature and a low acid concentration or at a low temperature and a high acid concentration.

Furthermore, for estradiol significant differences were found in the effectiveness of the deconjugation for different temperatures, time and acid concentrations. Removal of substituents from estradiol appeared to be optimal at a high temperature, long solvolysis time and high acid concentration. Although the differences are not significant for other components, the high

temperature showed systematically a slightly greater effectiveness for the quantified components. It a concluded that the optimum temperature is 60°C at an acid concentration of 1 M.

The choice of type acid is less obvious. For a number of components, the effectiveness of the deconjugation is better with methanolic hydrochloric acid from acetyl chloride or methanolic hydrochloric acid from trimethylsilyl chloride and for some components is this better with methanolic hydrochloric acid. However, the differences are very small, the choice for which acid to use was made on the basis of other factors such as cost and laboriousness. Because it takes more time and money to make methanolic hydrochloric acid (methanolic hydrochloric acid from acetyl chloride and methanolic hydrochloric acid from trimethylsilyl chloride) it is more economical to buy methanolic hydrochloric acid.

Chemical deconjugation – optimisation time

In order to disclose the effect of time on the degree of deconjugation and degradation and unwanted conversion, the result of solvolysis with 1 M methanolic hydrochloric acid at 60° C was studied after 0 h, ½ h, 1 h, 2 h, 4 h, 6 h and 24 h (Figure 4).

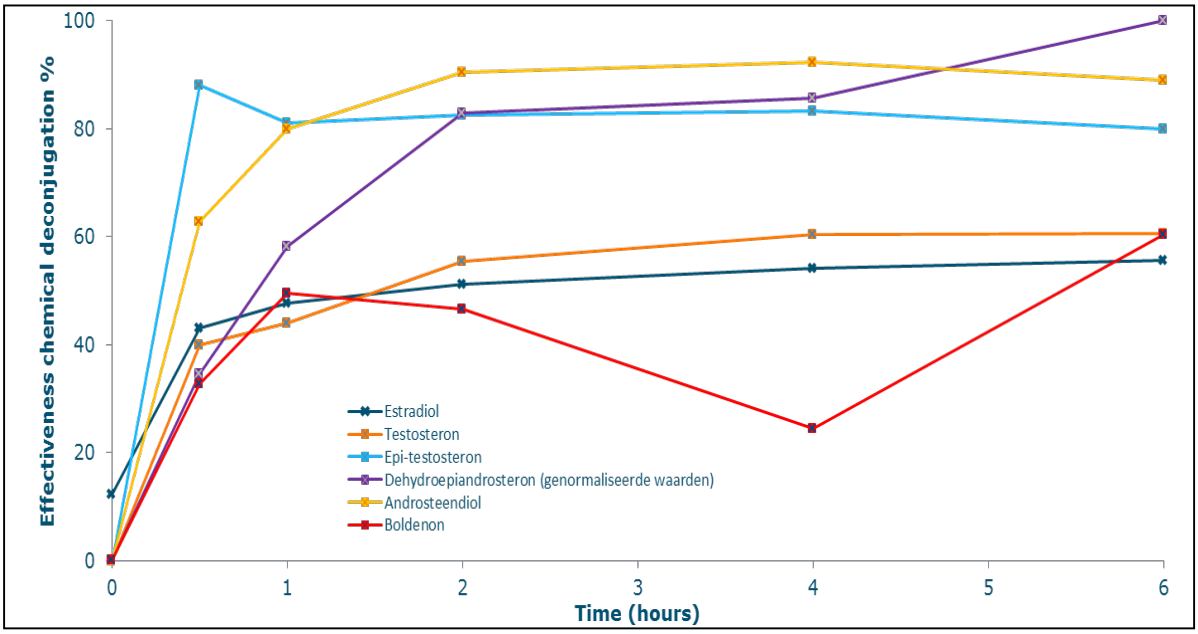

Figure 4. Chemical deconjugation by methanolysis at different time points. The percentage is expressed as free steroid scaled to 100% in case of deconjugation of all present conjugates. Steroids were conjugated with sulphate or glucuronide, or were conjugated with glucuronic acid and sulphate.

From Figure 4 it can be concluded that the effectiveness of deconjugation in the first hour is almost complete. From 6 to 24 h, the concentration of all components slightly decreased, with estradiol as an exception indicating that conjugates were not further liberated and that free components degraded at incubation times in excess of 6 h. For almost all components, the highest amounts of free component were highest at 2 h methanolysis. After 2 h, the increase in amount of free component was limited. For this reason, 2 h reaction time was found to be the optimal time for this method.

The steroids androstenediol and 17α-testosterone were deconjugated till approximately 80%. The conjugates of these steroids consist of a mixture of the conjugates androstenediol-disulphate and 17α-testosterone-sulphate. The steroids estradiol, 17β-testosterone and boldenone that were quantified were originating from sulphate and glucuronide of boldenone, a sulphate and glucuronide of 17β-testosterone and two sulphates, two glucuronide and a combination of sulphate and glucuronide of estradiol. This means that the deconjugation of sulphates with this method was effective and less effective for glucuronides.

Since the ultimate method should be effective for all components, a chemical deconjugation at a high temperature with 1 M methanolic hydrochloric acid for 2 h was chosen. Using these conditions, the effectiveness of the chemical deconjugation on the individual components is shown in Figure 5. The results show that the deconjugation of sulphates is generally effective as well of the 17-glucuronides. It is intriguing that deconjugation of a substituent at the 17-position occurs better than at the 3-position. A good example is 17β-estradiol-17-sulphate which had a higher deconjugation rate than 17β-estradiol-3-sulphate. For DHEA, the deconjugation of the sulphate on the 3 position is effective, the deconjugation of the glucuronide on the 3 position is not effective at all. This can be explained by the fact that the glucuronide on the 3-position is relative large

compared to a sulphate group and therefore steric hinders the removal of the glucuronide group. It is concluded that chemical deconjugation is effective for sulphates and for glucuronides at the 17-position and is not very effective for glucuronides at the 3 position.

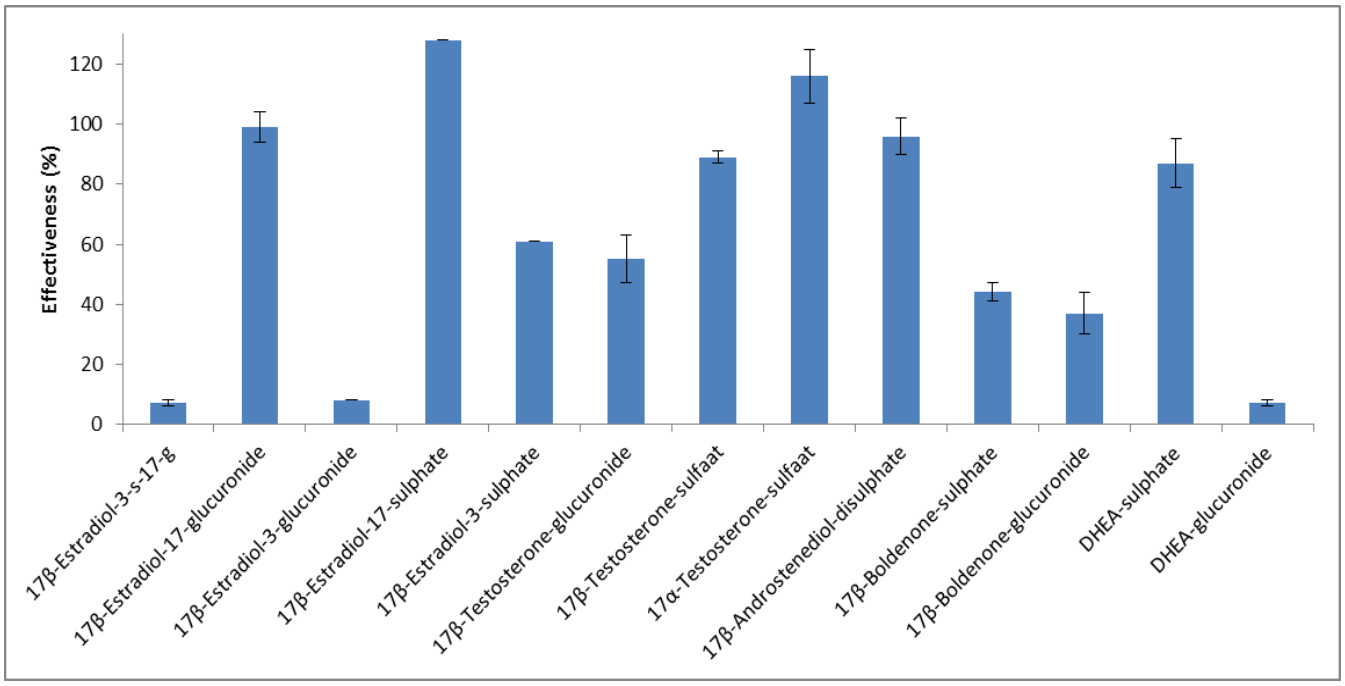

Figure 5. Effectiveness of the chemical deconjugation at 60°C with 1 M methanolic hydrochloric acid for 2 h.

Conclusions

In this study, optimization of enzymatic and chemical deconjugation were performed. The enzymatic deconjugation has shown that β-glucuronidase of *E. coli* works effectively for glucuronide conjugates of many different types of steroids. Enzymatic deconjugation of sulphate conjugates is not possible with enzymes. ß-Glucuronidase is most effective at 37°C, volume of 20 µL 5-20 MU mL^{-1} and incubation time of 15 min in 2 mL phosphate buffer (pH 7).

The chemical deconjugation is especially for sulphate conjugated steroids effective. Glucuronides can be deconjugated with this method as well. The position of the glucuronide in the steroid skeleton has a great influence on the effectiveness of the chemical deconjugation. For both sulphate as well as for glucuronidated steroids, the steroid sulphate or glucuronide on the 3-position is less effective than for steroids with the sulphate or glucuronide on the 17-position. Especially for glucuronide conjugated steroids the efficiency is low. Chemical deconjugation is most effective at 60°C with 1 M methanolic hydrochloric acid. The optimal time to perform chemical deconjugation is 2 h.

If on forehand the conjugation status is known, a choice can be made between an enzymatic or chemical deconjugation. If the conjugation status is unknown and a fast deconjugation is necessary, a chemical deconjugation is best option. If a full deconjugation of every steroid has to be obtained, deconjugation using a combination of enzymatic and chemical deconjugation has to be performed.

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QUANTITATIVE ANALYSIS OF STEROIDS, CORTICOSTEROIDS, RESORCYLIC ACID LACTONES IN URINE BY THE USE OF 96-WELL PLATES CLEAN-UP AND NARROW-BORE UHPLC-MS/MS

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Abstract

A new method for detection of residues at low concentration levels in urine was developed with the following goal: easy, fast and sensitive analysis of different classes of compounds, with reduced consumption of chemicals and disposables. The compounds analysed with this method are steroids, corticosteroids and resorcylic acid lactones. The sample clean-up consisted of a deconjugation step by adding β-glucuronidase to 500 µL urine, followed by a solid-phase extraction (SPE) clean-up with 96-well plates (Oasis® HLB). With the 96-well plates, less consumables and solvents are used compared to the classical SPE cartridges. Several washing steps were applied to obtain clean extracts after which the compounds were eluted and analysed with UHPLC-MS/MS. To achieve low detection levels with reduced solvent consumption, a narrow-bore column with an internal diameter of 1 mm was used. During the analysis, the MS operated in negative and positive ionisation mode. Overall, the method offers improved sample throughput: sample pre-treatment and analysis of 96 samples in less than 24 h.

Introduction

In Europe, the use of growth-promoters is strictly forbidden in the cattle production (1). A zero-tolerance policy is applied for these substances which means, in practice, that the analysis has to be performed at the lowest concentration possible (2). In a recent European Food Safety Authority opinion, it was assessed that with the current state-of-the-art, the detection limits should still be lowered by at least tenfold (3). In Europe growth-promoters are still being used, therefore it is desirable to improve current methods so that a larger number of compounds can be detected at lower detection limits (4).

To control the misuse of growth-promoters, routine analysis of urine samples is carried out with the use of LC-MS/MS or GC-MS/MS methods. In order to achieve the regulatory requirements with ever-decreasing limits of detection, new multi-methods are needed. Currently, the analysis of growth-promoters in urine typically consists of a deconjugation step with *Helix pomatia* enzymes, several LLE (liquid-liquid extraction) or few SPE (solid-phase extraction) steps, sometimes even HPLC-fractionation, followed by mass-spectrometric detection. To process 24 samples, this workflow costs approximately three working days and consumes lots of chemicals and organic solvents.

In this study, we completely redesigned our workflow to become more efficient and to decrease detection limits. The following changes were made. Based on a previous study (5), we adjusted the deconjugation step: instead of using a *Helix pomatia* enzyme preparation, only β-glucuronidase was used. This would only cleave the glucuronide groups but would not give any unwanted conversions as may occur when using *H. pomatia* preparations. Moreover, the deconjugation rate of sulphate groups with *H. pomatia* enzymes is very low which limits its effectiveness (5). To increase sensitivity, a narrow-bore column (ID = 1 mm) was used instead of the typical UHPLC columns with an ID of 2.1 mm. This would gain a factor four in sensitivity and lower the organic solvent consumption by 75%. LLE and single tube SPE steps were replaced by 96-well plate SPE, which increased the efficiency enormously and also, makes it easy to apply different washing steps in a short period.

In conventional LC-MS/MS methods, 5 mL of urine is processed. This amount of urine could introduce high amount of interfering compounds (such as salts, urea etc.) when a 96-well plate is applied. To avoid this, a smaller quantity of urine was processed. In theory, this would decrease the sensitivity. Instead, due to a more efficient sample clean-up and less ion suppression effects on the mass spectrometer, detection limits increase when a lower amount of sample is processed.

Here we present the successful inclusion of all these steps in a novel sensitive and fast method for the analysis of a group of classic growth-promoters in urine samples.

Materials and Methods

Chemicals

All chemicals, including standards and solutions, were with defined quality. Pure chemicals were *pro analysi* quality. The water was milli-Q quality. The following standards were used: α–trenbolone (NMI), 17ß–trenbolone (Steraloids), 17ß–Boldenone (Steraloids), 17α–Boldenone (EURL), isoflupredone (Research Plus), betamethasone (Steraloids), flumethasone (Sigma), triamcinolone-acetonide (Sigma), dexamethasone (Sigma), clobetasol (Glaxo Wellcome), ADD (androsta-1,4-diene-3,17-dione) (Steraloids), α-zeranol (Sigma), zearalanone (LGC Standards), zearalanone (TRC), zearalenone (Sigma), α-zearalenol (Sigma), ß-zearalenol (Sigma), 16α-OH-stanozolol (Cerilliant), 16α-OH-stanozolol (NMI), isoflupredone-d3 (d2 major)

(TRC), 17ß–trenbolone-d3 (EURL), 17ß–boldenone-d3 (EURL), α-zeranol-d4/α-zeranol-d4 (EURL), α-zearalenol-d4 (EURL), α-zearalenol-d7 (EURL), ß-zearalenol-d4 (EURL), ß-zearalenol-d7 (EURL), 16α-OH-stanozolol-d3 (EURL), triamcinolone-acetonide-d6 (EURL), dexamethasone-d4 (Cachesyn), zearalenone-d6 (TRC), α-zearalanol-d4/ß-zearalanol-d4 (EURL), 17ß-nortestosterone-d3 (EURL).

Sample Clean-up

For each sample, 25 µL internal standard solution (0.1 mg L^{-1}) was pipetted in separate polypropylene tubes. Under a gently stream of nitrogen at 40°C, the standard was evaporated until just dry. Of each sample, 500 µL urine was pipetted in the polypropylene tube and vortexed. Then, 500 µL phosphate buffer (pH 7.4) and 10µL ß-glucuronidase solution was added, and the mixture was vortexed. The pH was checked with pH indicator strips and, if necessary, adjusted to the pH 6.5 - 7.5. Samples were placed at 37°C for 16 h. An Oasis HLB 96-well plate was conditioned with 1 mL of MeOH per well, followed by 1 mL water. Afterwards, the samples were transferred to the SPE 96-well plate and each well was subsequently washed with 1 mL water and dried. Then, six consecutive washing steps were applied with six different wash solvents: wash solvent 1 (60% MeOH/2% HAc), wash solvent 2 (20% ACN/2% HAc), wash solvent 3 (20% ACN), wash solvent 4 (10% ACN/2% NH_3), wash solvent 5 (50% MeOH/2% NH_3), and wash solvent 6 (60% MeOH). Elution was performed with 1 mL ACN. The eluate was collected in a 96-well collection plate in which already 20 µL DMSO per well was present. The plate was placed in a 96-well plate evaporator and dried till only DMSO is left after which 30 µL 10% MeOH was added and the plate shaken.

Table 1. UHPLC gradient.

Time (min)	*Mobile phase A* (%)	*Mobile phase B* (%)
0.0	80	20
0.2	80	20
3.2	70	30
7.5	50	50
7.6	0	100
8.6	0	100
8.7	80	20
10.0	80	20

UHPLC analysis

Chromatographic separation was performed with a UPLC BEH C_{18}, 100 mm x 1.0 mm, 1.7 µm column. Mobile phase A was water/ACN/HCOOH/ammonium formate 1 M 900/100/0.02/2 (v/v/v/v) and mobile phase B was water/ACN/HCOOH/ammonium formate 1 M 100/900/0.02/2 (v/v/v/v), column temperature was kept at 60°C, vial tray temperature was 20°C. Injection volume was 5 µL. The flow rate was 0.15 mL min^{-1}. The gradient elution is given in Table 1.

MS-analysis

The MS settings are summarised in Table 2. The MS was operated in positive / negative switching polarity mode. In Table 3 the mono-isotopic masses of the precursor ions and corresponding product ions are listed.

Table 2. MS settings.

Parameter	*ESI, positive*	*ESI, negative*
LM 1 resolution	3.0	2.8
HM 1 resolution	15.0	15.0
Ion energy 1	1.0	1.9
LM 2 resolution	3.0	3.0
HM 2 resolution	15.0	15.0
Ion energy 1	1.0	1.3
Capillary voltage	3.0 kV	-3.0 kV
Source temperature	150°C	150°C
Desolvation temperature	400°C	400°C
Desolvation gas flow	800 L h^{-1}	800 L h^{-1}
Cone gas flow	150 L h^{-1}	150 L h^{-1}
Nebuliser gas flow	7 bar	7 bar
CID gas flow	0.18 mL min^{-1}	0.18 mL min^{-1}

Table 3. Monitored MRM's (= formic acid adduct)*

.	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cone Voltage (V)	Collision energy (eV)	ESI mode
Isoflupredon*	423.2	293.0	20	30	-
		347.1		15	
Isoflupredon-d3*	426.2	350.1	20	20	
Dexamethason/Betamethason*	437.2	307.0	20	30	-
		361.2		15	
Dexamethason-d4*	441.2	363.1	20	20	-
Flumethason*	455.2	305.0	20	30	-
		379.1		20	
17ß–Trenbolon/17α–Trenbolon	271.2	199.1	30	25	+
		253.2		20	
17ß–Trenbolon-d3	274.1	256.2	30	20	+
17ß–Boldenon/17α–Boldenon	287.2	121.1	25	25	+
		135.1	30	15	
17ß–Boldenon-d3	290.2	121.1	25	25	+
ADD	285.2	121.1	25	25	+
		147.1	20	15	
16α-OH-Stanozolol	345.2	81.1	30	45	+
		95.1		40	
16α-OH-Stanozolol-d3	348.3	81.1	50	45	+
Triamcinolon-acetonide	435.3	339.2	40	13	+
		415.3		8	
Triamcinolon-acetonide-d6	441.3	421.3	40	8	+
Clobetasol	411.2	355.1	20	13	+
		391.2		5	
Zearalenon	317.2	131.2	44	30	-
		175.1		24	
ß-Zearalenol/α-ZearalenolZeara-lanon	319.2	160.2	30	30	-
		275.2		21	
ß-Zearalenol-d4/α-zearalenol-d4	323.2	160.1	45	32	-
Zearalanon	319.2	205.2	54	22	-
ß-Zearalanol/α-Zearalanol	321.2	259.2	56	26	-
		277.2		20	
α-Zearalanol-d4/α-Zearalanol-d4/Zearalanon-d6	325.1	281.1	50	20	-

Results and Discussion

The method optimisation was carried out at each sample preparation step. First of all, the influence of the clean-up materials on the analytes recovery was investigated. When 96-well plates were used, it was determined that large losses occurred. During solvent-evaporation step, compounds may be adsorbed by the walls of the 96-well plate, which makes their reconstitution very difficult. This non-specific binding was plate-independent, and occurred in every type of plate used. To prevent the adsorption a small volume of DMSO as a "keeper" solvent was added.

An easy way to increase the sensitivity of a method is reducing the column dimension. From a theoretical point of view, the sensitivity should increase proportional with the reduction of the column diameter if the same mass amount of sample is injected. In our laboratory, we use 2.1 mm columns on routine basis. Switching to a column with ID of 1 mm would gain a theoretical factor four in sensitivity. It has to be noted that this factor four can only be reached if all LC connections are made properly and dead volume is kept to a minimum. In Figure 1 an example is given of α/ß-nortestosterone whereby the same amount was injected on a 2.1 mm and 1 mm column. The signal-to-noise ratio is a little more than a factor four better on the 1 mm column than expected from theory. The factor four was also reached for the other compounds in this study. In addition, the amount of organic solvents used was reduced, the flow was decreased from 0.6 mL min^{-1} on a 2.1 mm column to 0.15 mL min^{-1} on the 1 mm column, this is a reduction of 75% organic solvent.

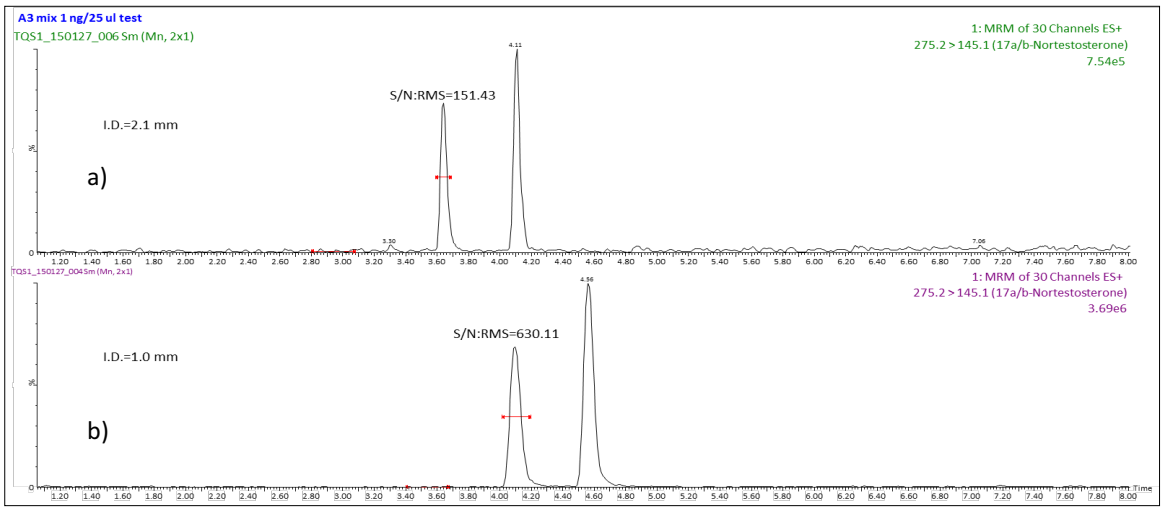

Figure 1. Extracted ion chromatograms of 17 α/ß-nortestosterone analysed with a) Acquity BEH C_{18}, 1.7 µm, 2.1 x 100 mm and b) Acquity BEH C_{18}, 1.7 µm, 1.0 x 100 mm.

Figure 2. Elution profile of a-trenbolone (blue bars) and ß-trenbolone (red) under different methanol/acetonitrile alkaline and acid conditions.

To increase the sample throughput and to adapt a more efficient workflow, SPE was performed on an SPE with 96-well plate format. Due to this efficient workflow, it is possible to test and to apply different washing steps. During method development several washing solvents were tested. The following combinations were investigated whereby the organic solvent was increased from 10 to 90 percent in steps of 10 percent: % MeOH/HAc (2%); % MeOH/NH_3 (2%); % MeOH; % ACN/HAc (2%);

% ACN/NH_3 (2%); % ACN. For each compound the corresponding elution profile was constructed (see Figure 2 for the washing profile of 17α/ß-trenbolone).

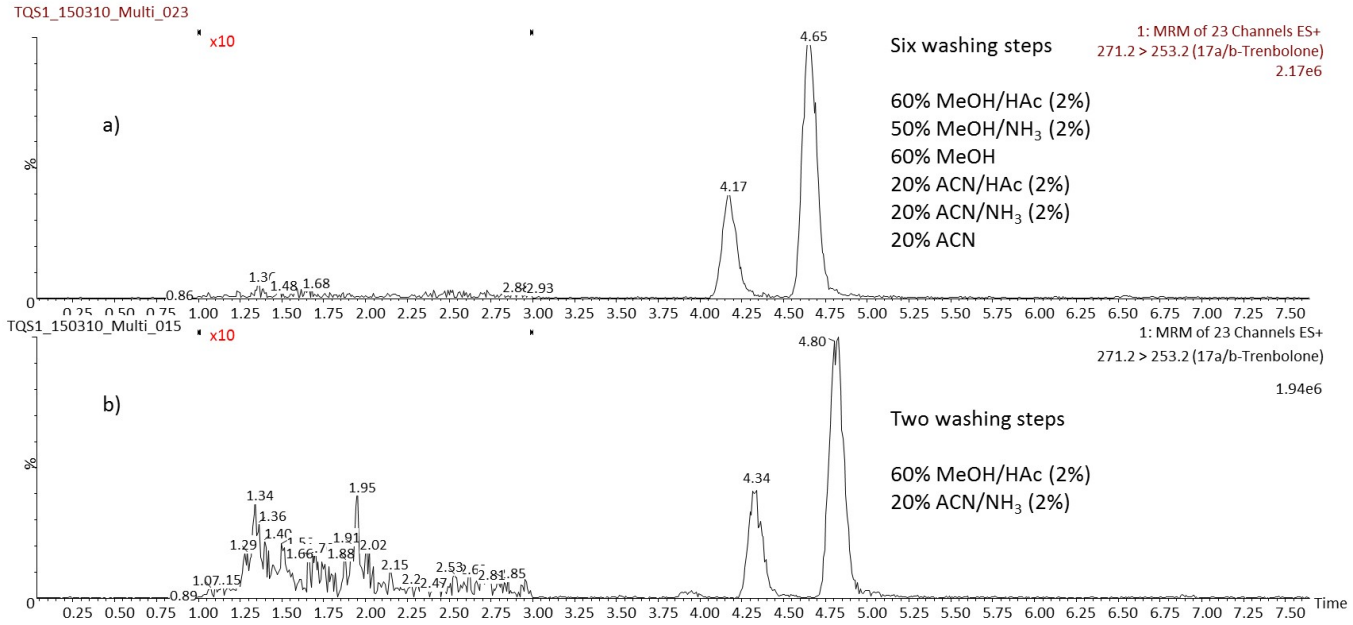

Figure 3. Extracted ion chromatograms of a/ß-trenbolone after application of two different washing procedures for the SPE 96-well plate clean-up. a) Use of six consecutive washing steps (60% MeOH/HAc (2%), 50% MeOH/NH_3 (2%), 60% MeOH,20% ACN/HAc (2%), 20% ACN/NH_3 (2%), and 20% ACN) and b) use of two washing steps (60% MeOH/HAc (2%) and 20% ACN/NH_3 (2%). The elution profile between 1-3 min is amplified by a factor 10 for clarity.

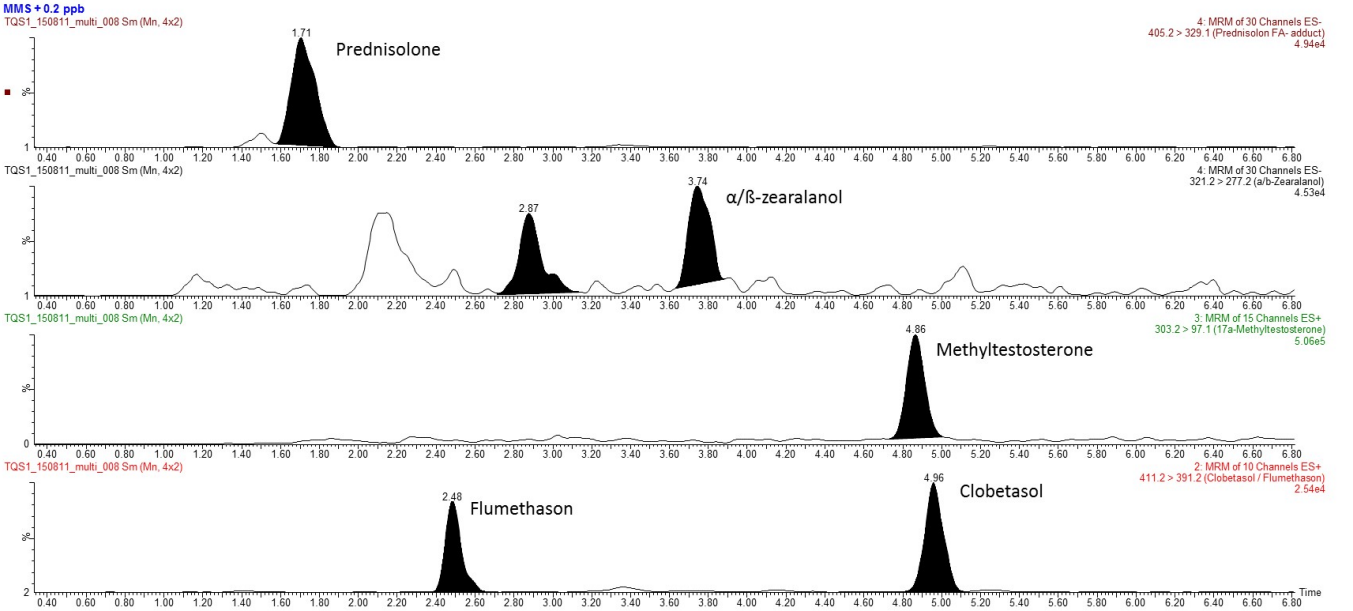

Figure 4. EIC of prednisolone, a/ß-zearalanol, methylprednisolone, flumethason and clobetasol in a urine sample spiked at 0.2 ng mL^{-1}.

For each condition the critical breakthrough washing condition was selected. The composition with the lowest percentage of organic modifier determines the critical breakthrough condition for that washing solvent for the whole method. The following critical washing conditions were determined: 60% MeOH/HAc (2%), 50% MeOH/NH_3 (2%), 60% MeOH, 20% ACN/HAc (2%), 20% ACN/NH_3 (2%), 20% ACN. After determination of these critical compositions, an experimental design was constructed to test the optimal number and sequence of the selected washing solvents. To limit the amount of experiments not all combinations were tested as some did not make sense, for example switching directly from an acidic to alkaline solvent. Also combinations of just a few washing steps were made to determine if that is sufficient for the clean-up of urine samples.

In Figure 3, an example is given of two combinations of washing steps. The chromatogram between 1 and 3 min was amplified 10 times to show the difference in background noise in the samples. Clearly the more rigorous washing had lower background what would also result in less pronounced matrix effect. The method was finally tested with spiked urine samples and an example for a few compounds at a level of 0.2 ng mL^{-1} is given in Figure 4.

To assess the performance of the method, 20 different *bovine* urine samples obtained from calves, cows, pregnant cows, bulls and mixed urines were spiked at 1 ng mL^{-1}. The results of this experiment are shown in Figure 5. For all compounds the accuracy was ≈100% and the covariation coefficient lower than 20%. This clearly demonstrates that the method can be have a wide scope of application for different types of urine samples.

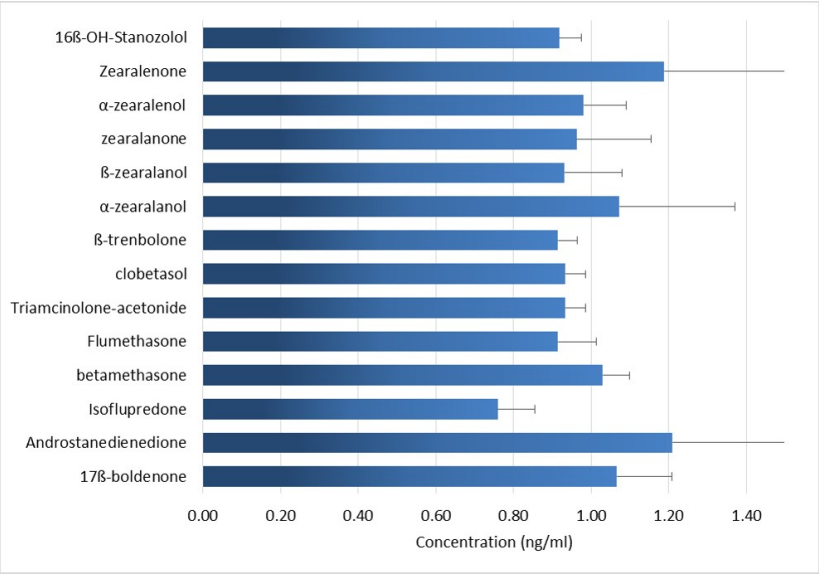

Figure 5. Average recovery (concentration ng mL^{-1}) and standard deviations of 20 different types of urine samples spiked at 1 ng mL^{-1}.

Conclusions

An easy, fast and sensitive method for analysis of different classes of compounds, with reduced consumption of chemicals and disposables was developed. The compounds analysed with this method are different steroids, corticosteroids and resorcylic acid lactones. Overall, this method offers improved sample throughput: sample pre-treatment and analysis of 96 samples in less than 24 h. The method will be further validated to determine its ruggedness and applicability. It will be implemented in routine analyses.

Acknowledgements

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MULTI-CLASS, MULTI-RESIDUE METHOD FOR VETERINARY DRUG ANALYSIS BY LC-MS/MS

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Introduction

Over 300 veterinary drugs are used in modern agriculture and residues are regulated for food raw materials of animal origin to ensure consumer's safety. Recently, the analytical approach for monitoring drug residues has rapidly evolved from single compound or family methods towards large multi-class-multi-residue methods by LC-MS/MS or LC-HRMS. A method devoted to the analysis of 110 compounds belonging to 18 different families is hereafter presented. Three main commodity groups were targeted including milk- and meat-based products and baby-foods. The method developed is a screening method by LC-MS/MS used for selection of samples with levels of veterinary drugs that may exceed the screening detection limit (SDL) with a given certainty. The result of a screening is either negative *i.e.* "< SDL (in µg kg^{-1})" or "suspect". All results found as suspect shall be verified by a full re-analysis from the original sample by a confirmatory quantitative method.

Materials and Methods

The method is based on a modified QuEChERS protocol. Briefly, a test portion is extracted with a mixture of water/acetonitrile/formic acid (80/20/0.1%). A Na_2SO_4/NaCl salt mixture (4/1, w/w) is then added and the sample is immediately shaken and centrifuged. At this stage the partitioned sample extract is diverted into two different clean-up procedures named "Full Screening" and "Avermectin".

- Full Screening: An aliquot of the upper acetonitrile phase is cleaned by dispersive solid-phase extraction (d-SPE) (Na_2SO_4/PSA/C18), evaporated and reconstituted in methanol-water (15+85) prior to injection onto LC-MS/MS.
- Avermectins: for avermectins specifically, another aliquot of the upper acetonitrile phase is also cleaned by d-SPE ($MgSO_4$/PSA/C18). The extract is then evaporated and reconstituted in methanol-water (80+20) prior to injection onto LC-MS/MS.

LC analysis was performed on an Acquity UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm column) heated at 40°C using an Agilent 1290 Infinity system. Mobile phase was constituted of water with 0.5 mM of ammonium formate and 0.1% of formic acid (solvent A) and methanol with 0.5 mM of ammonium formate and 0.1% of formic acid (solvent B). A gradient program was set-up as: [0-0.2 min] with 20% B isocratic, [0.2-2.9 min] linear gradient up to 100% B, [2.9-3 min] hold at 100%, [3-3.2 min] return to 20% B and [3.2-4.5 min] hold at 20% B. The flow rate was at 400 µL min^{-1}.

For MS detection, an AB Sciex QTrap 5500 was equipped with a Turbo V Ion Source. The block source temperature (TEM) was set at 550°C for full screening and at 350°C for the analysis of Avermectins. Analysis was performed using tandem MS in scheduled selected reaction monitoring (scheduled MRM) mode alternating two transition reactions for each compounds.

Table 1. Table 1. SDL obtained after qualitative validation.

Veterinary drug families	SDL (ng g^{-1})	Veterinary drugs	SDL (ng g^{-1})
1. Anthelmintics	10 to 15	Flumethrin	15
2. Avermectins	5	Virginiamycin (M1)	10
3. Benzimidazoles	10	Baquiloprim	15
4. Coccidiostats	15	Trimethoprim	15
5. Ionophores	2 to 5	Rifaximin	15
6. Lincosamides and macrolides	15	Novobiocin sodium	15
7. NSAID	5 to 15	Bacitracin A	50
8. Phenicols	0.3 to 10	Xylazine	15
9. Quinolones	10	Carazolol	5
10. Sulfonamides	5 to10	Chlorpromazine	15

Results and Discussion

A total number of 67 samples from all commodity groups were spiked at 1*SDL, 2*SDL, 4*SDL and 8*SDL. The method was validated for all analytes in terms of false positive (FP) and false negative (FN) rates. The qualitative criteria defined by CRL 2010/01/20 were fulfilled with FP rate < 10% and FN rate < 5%. The SDL concentration (in ng g^{-1}) achieved in milk-based products, meat-based products and baby foods can be consulted in Table 1.

Conclusions

A high throughput screening method was developed with fast sample preparation and low quality control requirements (no recovery, no linearity). The method was found applicable to a wide range of matrices and will allow rapid compliance testing and to generate occurrence data on the usage of veterinary drugs worldwide.

References

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ANALYTICAL STRATEGY BASED ON ISOTOPIC CLUSTER IDENTIFICATION AND MASS DEFECT TO HIGHLIGHT RELEVANT METABOLITES

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Abstract

Besides having access to adapted analytical tools, the efficiency of strategies implemented to control the use of forbidden compounds also relies on the selection of both the appropriate biological matrix and the relevant compounds to be monitored. Fishing such relevant compounds requires deep investigation of drug metabolism to establish which specific metabolites to be monitored, with good sensitivity, over the longest period of time. State-of-the-art metabolism study involves radio-labelled experiments that most of the time scientists cannot afford. Therefore, semi-targeted or untargeted approaches have been reported as efficient alternative strategies to highlight relevant metabolites. In the present study, an original semi-targeted approach to identify chlormadinone metabolites in calf urine samples has been developed, using chlorine (Cl) characteristics (mass defect, isotopic clusters) as common chemical feature of possible metabolites. The workflow can be divided in 5 steps: sample preparation, HRMS data acquisition (R = 35,000 at *m/z* 200), automatic peaks integration (centWave function of Xcms package under R), automatic Cl isotopic clusters identification (Excel® VBA macro) and interpretation (H/Cl Mass Defect-plot graphical representation and VBA macro tool). The strategy was successfully applied to urine samples collected from one calf orally treated with chlormadinone acetate, enabling detecting already described metabolites but also unrevealed ones.

Introduction

The past decade has witnessed spectacular advances in chromatography and high-resolution mass-spectrometry (HRMS), opening the way to untargeted full-scan footprints as a new methodological approach. As large datasets arise from such HRMS couplings, the current challenge for an efficient fishing lies in rapidly highlighting the signals of interest among large data sets resolving thousands of untargeted signals. For addressing this issue, and considering such interesting signals relate to halogenated ions, a direct approach consists in screening for halogenated ions produced in negative mode pending quite energetic conditions, typically negative chemical ionization (Byers *et al.*, 2014). More generally, two physico-chemical properties can be exploited as discriminating keys for data mining, as follows.

The first key is the mass defect (MD) engendered by heavy atoms such as halogens compared to major atoms from organic compounds (C, H, O, N). In this way, the higher the mass spectrometric resolution, the better the discrimination. An elegant mean to visualize the signals and help their interpretation is to plot the fractional part of the *m/z* against the *m/z* (MD plot). Such fractional part is frequently wrongly referred to as "mass defect" by authors dealing with mass spectrometric data and actually representing the nuclear binding energy. In extracts from biotic samples, signals with no particular MD – typically limited to C, H, O, N elements – are grouped in dense areas, while other/halogenated signals plot in different and more specific areas. The International Union of Pure and Applied Chemistry (IUPAC) standard is based on an exact mass of 12 for ^{12}C. In order to facilitate the reading of such visualization, one can adapt the IUPAC mass scale into a more appropriate one.

In 1963, Kendrick was the first to suggest an alternative based on an exact mass of 14 for $^{12}CH_2$ as reference (Kendrick, 1963). With this scale, ions series differing by one or more CH_2 groups (alkyl series) show the same fractional part. Thus, alkyl series plot on horizontal lines in the derived MD Kendrick-plots suggested by Hughey *et al.* (2001), which is largely used in the field of petroleomics (Marshall and Rodgers, 2004). Slight variations on this theme have been recently reviewed by Sleno in 2012. In particular, Taguchi *et al.* (2010) introduced a scale based on the substitution of hydrogen by chlorine. The conversion is performed by multiplying the *m/z* in the IUPAC scale by the ratio between the differences in mass between H and ^{35}Cl in the new scale (34) over the IUPAC scale (33.960128). In the corresponding H/Cl-scale MD plots, chlorinated homologue series (*e.g.* mono- to decachlorinated biphenyls) plot on horizontal lines. Since then, several studies referring to H/Cl-scale MD plots were reported in the field of halogenated contaminants (Jobst *et al.*, 2013; Pena-Abaurrea *et al.*, 2014). Conveniently, the H/Cl-scale also fits to brominated compounds.

The second key, restricted to chlorinated and brominated compounds, is the occurrence of characteristic isotopic clusters derived from the significant abundance of two natural and stable isotopes separated by 2 nominal atomic mass units, each for chlorine and bromine atoms. The T-SEN package, developed using the free and open source programming software R, includes a user database of organohalogenated compounds to assign and integrate target peaks in two-dimensional chromatograms (GCxGC-HRTOF data) based on ion ratios (Zushi *et al.*, 2013). For untargeted screening, automatic sorting for isotopic clusters in Visual Basic script language and backed to an instrument manufacturer software dealing with GCxGC-HRTOF data has been proposed, based upon theoretical ion ratios from halogen atoms and selected combinations only and using tolerances marginally leading to erroneous exclusions (Hilton *et al.*, 2010). Another way to proceed was proposed by Hashimoto

et al. in 2013 using dedicated software, again on GCxGC-HRTOF data, leading thus to the extraction of a subset of data based on the MD between isotopes (1.9971 for Cl, 1.9980 for Br) and some ion ratio criteria.

Based on such preliminary available knowledge and developments, the aim of the present work was to develop a new methodology capable of seeking unknown organohalogenated compounds in biological samples, in a context of chemical food safety. We developed a general fit-for-purpose data processing workflow supported by appropriate biocomputering solutions to filter potentially chlorinated and/or brominated signals within a complex dataset acquired by chromatography coupled to HRMS. First, chromatographic features are automatically extracted from the acquired data based upon existing xcms package in the open source programming R environment. Second, a VBA script pairs and classifies features according to retention time (RT) and MD between C, Cl and Br isotopes, regardless ion ratios. Third, visualization tools such as H/Cl-scale MD plots help interpreting and identifying or suggesting structures. Although the general workflow aspires to fit any HRMS dataset, we used a dataset obtained by LC-orbitrap with smooth electrospray ionization (ESI) to develop and assess the tool. The workflow has been applied to a dataset acquired from calf urine after exposure to chlormadinone acetate, allowing for investigating potential metabolites. More generally, such strategy may also be helpful in revealing degradation products from biological processes or industrial accidents, depending on the research question raised.

Materials and Methods

Chemicals

Solvents used as mobile phases (water, acetonitrile and methanol) were from Sigma-Aldrich (LC-MS ChromaSolv grade, Saint-Louis, USA). Ammonium acetate salts (Emsure® grade) as well as sodium methoxide were purchased from Merck (Darmstadt, Germany). Chlormadinone acetate (Figure 1) was purchased from LGC Standards (Teddington, UK).

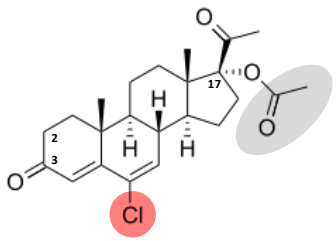

Figure 1. Chlormadinone acetate structure.

Biological samples

Chlormadinone acetate (50 mg, Mylan Laboratories, Canonsburg, PA, USA) was orally administered in a single intake to a calf in the frame of a faecal and urinary excretion kinetic study. As the excretion peak of deconjugated chlormadinone in urine was found at 5 h after administration (~100 ng mL^{-1}, data not yet published), urine collected just prior administration (control) and 5 h after injection were further investigated in the present study.

Sample preparation

Calf urine (20 mL) was purified according to a method, accredited against the ISO 17025 standard, and dedicated to the analysis of steroids in urine (Marchand *et al.*, 2000), pending slight modifications. Briefly, the progestogen fraction of interest was obtained following an enzymatic hydrolysis (*Helix pomatia*, Sigma-Aldrich), a solid-phase extraction step (ENVI-Chrom P, Sigma-Aldrich) and a liquid/liquid partitioning (NaOH *vs.* hexane/diethyl ether 70:30 v/v). The purified extracts were evaporated to dryness under a gentle stream of nitrogen and reconstituted into 50 µL methanol/water mixture (80:20, v/v).

LC-HRMS data acquisition

Extracts were analysed with an UltiMate 3000 UHPLC pumping system coupled to an Orbitrap Q-Exactive mass spectrometer fitted with a heated ESI source (HESI, Thermo Fisher Scientific, San José, CA, USA). External calibration was performed by infusing calibration mixtures for positive ion mode (Thermo Fisher Scientific). Instrument control and data processing were carried out by Chromeleon Xpress and Xcalibur softwares (Thermo Fisher Scientific). Chromatographic separation was achieved using reversed-phase chromatography on a Acquity UPLC® BEH column (100 mm × 2.1 mm, 1.7 µm) (Waters Corporation, Milford, MA, USA) kept at 50°C. Mobile phases consisted of 0.1% formic acid in water (A) and in acetonitrile (B). The gradient began with (A/B) 95:5 (v/v) for 1 min, then ramped linearly to 0:100 over 9 min to be maintained for 4 min and returned to 95:5 over 2 min, followed by equilibration for 4 min. The flow rate of the mobile phase was 0.6 mL min^{-1} and the sample injection volume was 2 µL. Samples were ionized in the positive mode with HESI parameters as follows: sheath gas flow, 40 arbitrary units (AU); auxiliary gas flow, 4 AU; capillary temperature, 300°C; source heater temperature, 350°C; spray voltage, 3.0 kV; S-lens radio frequency, 50 AU. HRMS data were acquired in full scan mode over the *m/z* range 100-650 at a

chosen resolving power of 35,000 full-width half maximum (FWHM) at *m/z* 200. The automatic gain control (AGC Target) was set at high dynamic range (5×10^5) and the maximum injection time was set to 500 ms.

Peak integration

Acquisition in full-scan mode coupled with HRMS generates huge data sets especially upon analysis of complex matrices treated without applying any drastic purification. Such large data set therefore requires automatic processing. The open access msConvert software (ProteoWizard) was used through the open source programming R environment to convert raw LC-HRMS data (.raw) to the open format .mzXML by adapting the script depending on the acquisition mode (negative or positive). Open raw datasets were then processed by the xcms package using the centWave peak detection algorithm (Tautenhahn *et al.*, 2008) to extract chromatographic features. This peak picking is based on the detection of "region of interest", corresponding to the selection of the same *m/z* (± tolerance) in several consecutive scans. Parameters were optimized for our specific analytical conditions for noise filtering and deconvolution to resolve co-eluting ions (method="centWave", ppm=30, prefilter=c(3,1000), snthresh=10, peakwidth=c(5,50), mzdiff=-0.01, integrate=1, noise=1,000, nSlaves=10). A table report in .csv file format was created where each feature was defined by an exact mass (*m/z*), a retention time (min) and a signal intensity (area).

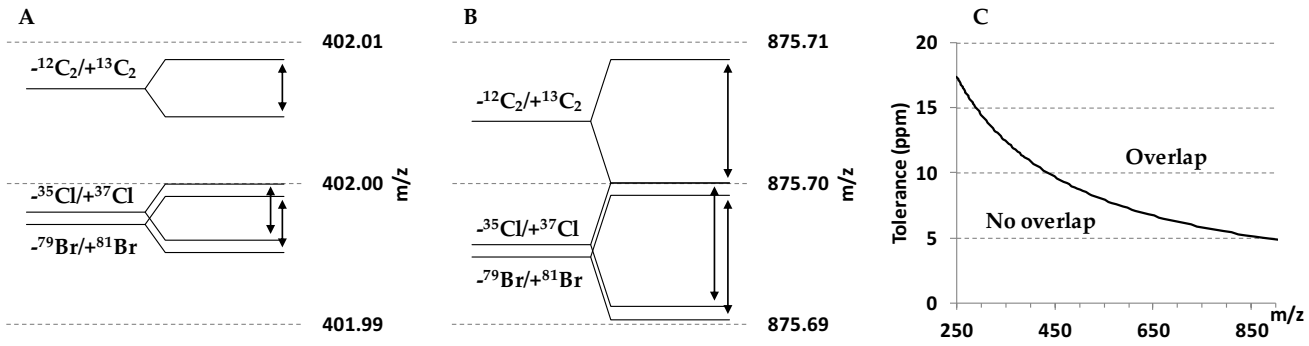

Figure 2. Schematic representation of tolerance bands when searching for Cl or Br contribution at M+2, starting from M at m/z 400.00 (A) and 873.70 (B) with a mass tolerance of 5 ppm, and areas of overlapping or not between ^{37}Cl or ^{81}Br and $^{13}C_2$ contributions as a function of m/z and tolerance (C). Arrow: tolerance band.

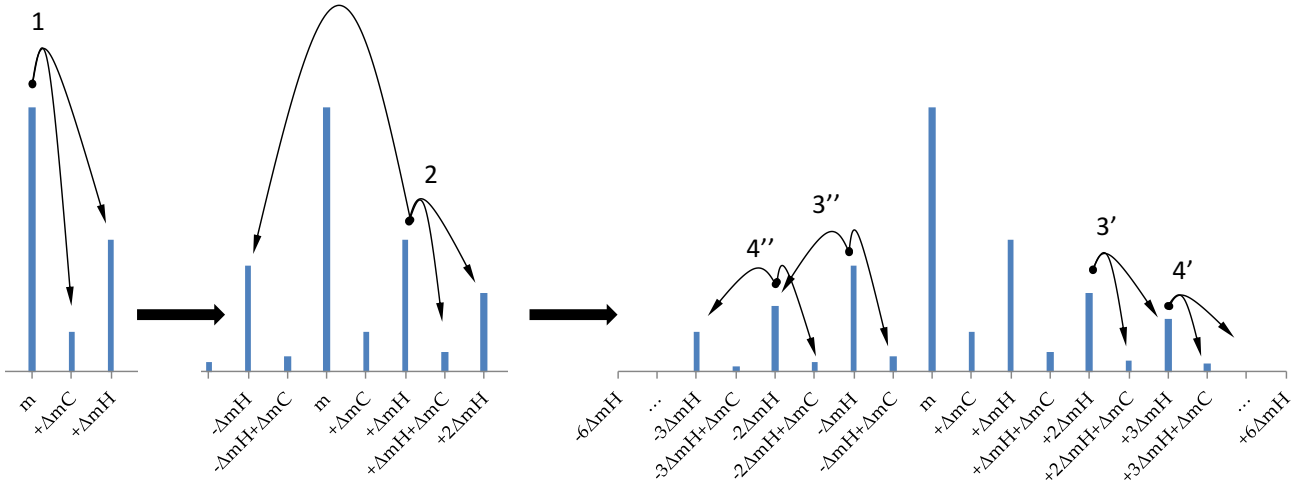

Figure 3. Schematic representation of the pairing process. At step 1, the mass m ion m is the most intense feature not yet paired or investigated. Step 2 occurs if M+2 was paired in Step 1. Steps 3' and 3" occur if M+4 and M-2 were paired in Step 2, respectively. Steps 4' and 4" occur if M+6 and M-4 were paired in Steps 3' and 3", respectively, and so on up to M+12 and M-12. m: exact mass of initial feature; ΔmC: mass variation from the substitution of ^{12}C by ^{13}C; ΔmH: mass variation from the substitution of ^{35}Cl by ^{37}Cl or ^{79}Br by ^{81}Br.

Pairing of Isotopic clusters

A group of several features can belong to the same compound due to the existence of different natural isotopes. A data treatment tool based on the mass defect (MD) and composed of a macro in Visual Basic for Applications (VBA) language was developed in order to pair features belonging to a same isotopic pattern (chlorinated and/or brominated) generated from an Excel spreadsheet. An iteration loop selects features in descending order of areas intensities. If the selected feature (M) is

not yet paired to a more intense feature, the script searches among the less intense features the ones compatible with a Cl or Br isotopic contribution at M+2 and with a ^{13}C contribution at M+1, pending a match on the RT and the exact mass with 1 s and 5 ppm tolerances by default, respectively. With such tolerance in ppm, which is related to the obtained resolution, no overlapping between ^{37}Cl or ^{81}Br and $^{13}C_2$ isotopic contributions occurs up to *m/z* 873, as illustrated in Figure 2.

Contributions from other heavy elements such as ^{34}S can however not be excluded. If an M+2 is paired, the macro subsequently searches for M+4 and M-2 from both Cl or Br isotope contributions, as well as for M+3 from ^{13}C contribution. If an M+4 or an M-2 are paired, M+6 and M+5 or M-4 and M-1 are respectively looked for, and so on up to M+12 and M-12 which is large enough to point out any larger isotopic cluster. With such event, the relative abundance of extreme isotopic combinations might be assumed too small to be detected. Figure 3 summarizes the pairing process. At the end of the iteration loop, all features are counted in three categories as unpaired, paired with an M+1 only (^{13}C) or paired with at least one M+2 (Cl or Br). The M+2 category of features is sorted as a list identified by base peak feature, RT and isotopic cluster span. A matrix table also details the number of clusters by minimum and maximum paired isotopic contributions.

Graphical functionalities and interpretation

For each sample, the H/Cl-scale MD plot with all features was drawn according to the procedure described in the introduction section. Display options allow narrowing the features selection to a RT range, a minimum area and/or the M+2 category. Mass accuracy is characterized using known compounds (*e.g.* internal/external standards). Then, paired isotopic clusters are manually investigated. VBA macros help extracting base peaks from a selected area on the MD plot, explicating all paired features to a base peak feature and highlighting selected clusters on the MD plot. Corresponding extracted ion chromatograms are then compared and integrated on the Xcalibur software in order to estimate the number of chlorine or bromine. Exact masses are adjusted as well and isotopic pattern can be completed at both ends. Indeed, isotopic contributions of low intensity are often missing after automatic peak picking. Elemental composition assignments were performed using Xcalibur software, considering the C, H, O, N, P, S, Cl and Br elements, according to the determined number of halogens as well as a consistent mass accuracy. Finally, structural hypotheses were suggested.

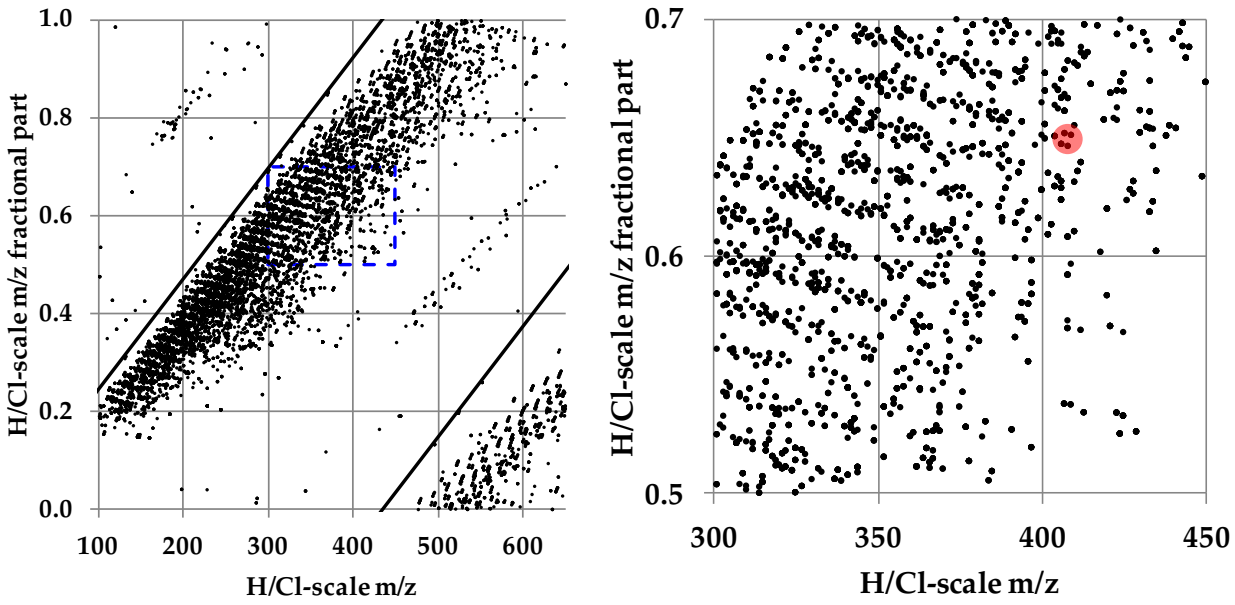

Figure 4. H/Cl-scale MD plot obtained for the calf urine sample extract with the set of 12,888 features (left) and expanded view of chlormadinone (CM) region (right). RT range: 1–10 min; Continuous line: baseline where the alkane series would theoretically plot ($[M+H]^+$); Blue dashed square: expanded region; Red circle: $[M+H]^+$ of CM acetate.

Results

Application to compounds related to chlormadinone (CM) in calf urine

Most of the compounds specific to the calf urine sample were eluted in the 3.5–7.5 min range. The centWave function resulted in 12,888 irredundant features between 1 and 14 min, from which 10,156 clusters were classified through the VBA macro tool. Among them, 7,680 were not paired, 2,342 contained an M+1 only while 108, 25 and 1 ranged from the M base feature up to M+2, M+3 and M+4 features, respectively. Plotting these data on an H/Cl-scale MD plot (Figure 4) highlights the

efficiency of the filtering script since a limited number of clusters emerge from the dense and poorly specific area (Figure 5), among them clusters corresponding to CM and CM acetate.

Manual review of M+2 and M+3 clusters showing a slightly negative slope and being absent from the control urine led to a list of 7 potential metabolites (Table 1), to which likely elemental compositions were assigned and corresponding structures hypothesized. 2-OH-CM and 3-OH-CM were previously described in human urine (Schindler *et al.*, 2003), more precisely α and β isomers for each one, and clusters probably corresponding to these isomers were observed in the present study (Figure 5). Although Extracted Ion Chromatogram (EIC) from 3-OH-CM reveals 2 peaks, as expected, EIC from 2-OH-CM reveals at least 3 peaks, suggesting that another carbon position might be hydroxylated. Probable dehydrogenation products from CM acetate, OH-CM and CM were also observed. At last, probable losses of H_2O and C_2H_4O from CM were observed, tentatively linked to carbon position 17. The mass accuracy derived from the base peak of these 9 chemical formulas was highly consistent (-0.4 ± 0.4 ppm), but structures (except CM) still require standard confirmation. Kinetics should also be established pending necessary verification/optimization in sample preparation and detection.

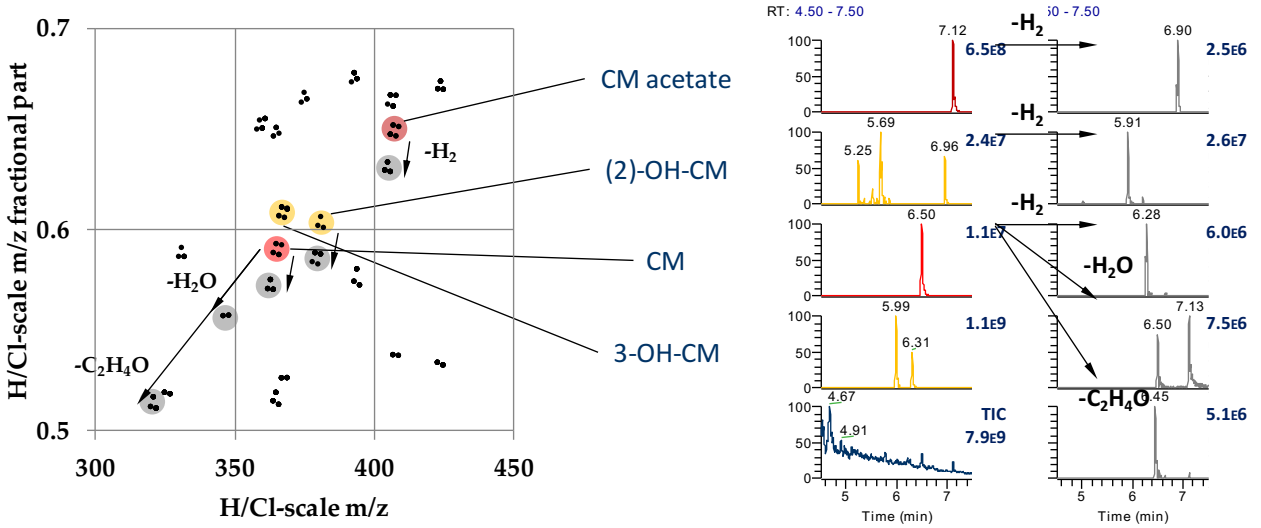

Figure 5. Expanded view of the H/Cl-scale MD plot obtained for the calf urine sample extract with the subset of features restricted to the M+2 category (RT range: 1-10 min, n=392; 134 clusters) (left), and total Ion current (TIC) and extracted ion chromatograms (EIC, 5 ppm) of base peak chromatographic peaks for chlormadinone (CM), CM acetate and suspected metabolites in calf urine extract (right). Red: $[M+H]^+$ of CM acetate and CM; Yellow: $[M+H]^+$ of 3-OH-CM and 2-OH-CM; Grey: other potential CM metabolites; Arrows: $-H_2$, $-H_2O$ and $-C_2H_4O$ vectors.

*Table 1. Tentative identification for selected positive isotopic clusters in calf urine sample. *: confirmed by analytical standard injection.*

IUPAC *m/z*	H/Cl *m/z*	Sum area	RT (min)	Suggested ion formula	Accuracy (ppm)	Suggested structure
405.1823	405.6473	1,385,338,117	7.12	$C_{23}H_{29}O_4Cl$	-0.95	* CM acetate
363.1719	363.5887	2,698,366,909	6.50	$C_{21}H_{27}O_3Cl$	-0.60	* CM
365.1879	365.6069	67,685,037	5.99; 6.31	$C_{21}H_{29}O_3Cl$	0.14	3-OH-CM
379.1670	379.6021	48,752,799	5.25; 5.54; 5.69; 6.96	$C_{21}H_{27}O_4Cl$	-0.12	2-OH-CM
403.1669	403.6295	4,416,102	6.90	$C_{23}H_{27}O_4Cl$	-0.46	CM acetate minus H_2
377.1514	377.5842	46,435,177	5.91	$C_{21}H_{25}O_4Cl$	-0.12	2-OH-CM minus H_2
361.1565	361.5709	9,368,730	6.28	$C_{21}H_{25}O_3Cl$	-0.14	CM minus H_2
345.1612	345.5573	33,461,193	6.50; 7.13	$C_{21}H_{25}O_2Cl$	-1.04	CM minus H_2O
319.1458	319.5120	9,596,488	6.45	$C_{19}H_{23}O_2Cl$	-0.42	CM minus C_2H_4O

Discussion

A VBA script was developed for pairing features according to the exact mass difference between Cl and Br isotopes in order to filter potential organo-halogenated clusters among full scan HRMS datasets. This macro appeared highly efficient for revealing such clusters prior to applying critical look through manual investigations. A key parameter was found to be the RT tolerance. For chromatographically resolved peaks, a RT tolerance of 1 s appeared appropriate.

Feature redundancies were also observed as an output of the peak-picking centWave function and were easily taken out. However, similar features showing almost same RT and *m*/*z* were also observed, with a highly dominant one, leading to multiple paring combinations for one cluster and then again redundant information. Such similar features are due to centWave function parameters, especially mzdiff. This parameter might be adjusted or a complementary algorithm could be developed to merge such similar features.

A second key parameter was found to be the *m*/*z* tolerance relative to two consecutive isotopic peaks. It depends on the instrumental precision indirectly linked to the accuracy. A general rule is still missing to avoid empirical grope. Such a rule would minimize chances of overlapping between isotope contributions (*cf.* Figure 2).

As regard to the time-consuming manual review of filtered clusters, much effort should be devoted to automation of replicate steps, for example selection of a cluster (already developed), display of corresponding EIC with adjustment of raw data *m*/*z*, screening for minor peaks at isotopic cluster ends, comparison of isotopic pattern with the hypothesis on number of Cl and Br atoms, monoisotopic peak *m*/*z* and elemental composition hypotheses. Additionally, a dereplication user database shall help in rapidly qualifying known organo-halogen compounds before focusing on unknowns, as already implemented for GCxGC-HRTOF datasets by Zushi *et al.* (2013). The efficiency of such dereplication strategy will depend on matching criteria as well as its incrementing, including theoretical and observed signals.

Finally, the proposed methodology proved to be powerful in filtering organo-halogen compounds from HRMS datasets, with application for screening of organo-halenated drugs and related degradation/metabolic products in this project. It could be applied or adapted to datasets obtained with couplings other than LC-ESI-Orbitrap (*e.g.* GC-APCI-TOF) in order to explore different fractions.

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DETECTING β-AGONISTS TREATMENTS IN FOOD PRODUCING ANIMALS: AN OVERVIEW OF ANALYTICAL POSSIBILITIES

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Abstract
β-Agonist drugs are synthetic molecules with therapeutic applications in human as well as in veterinary medicine. These compounds are banned for growth-promoting purposes, but their illegal use in food-producing animals is regularly suspected in Europe and their presence episodically pointed out in edible tissues imported from third countries. The presence of residues and their associated harmful effects on humans makes the control of such veterinary drug residues a key component in ensuring consumer protection. Although clenbuterol is still probably the most popular known, a wide range of other β-agonists are also supposed to circulate on the black market which constitutes a challenge for control laboratories. Besides well-described protocols of administration, some practices consisting in the use of low dose "cocktails" - that may exert a synergistic effect and exhibiting similar or better growth-promotion properties - have also been reported as additional challenge to be overcome by control laboratories.
This article overviews the various possibilities to demonstrate the administration of β-agonists in meat-producing animals. The choice of biological matrices of interest (meat, liver, lung, retina, urine...) together with the selection of the most appropriate analytical strategies (targeted, ion mobility, effect based approaches...) will be discussed, considering screening and confirmatory requirements.

Introduction
β-Agonist drugs are synthetic molecules used for therapeutic purposes in human as well as in veterinary medicine for their anti-asthmatic, bronchodilator, tocolytic and cardiotonic properties. However, and despite their ban for growth-promoting purposes [Dir 1996/22/EC], their illegal use in food producing animals is regularly suspected in Europe and in edible tissues imported from third countries. The presence of residues and their associated harmful effects on humans makes the control of such veterinary drug residues a key component in ensuring consumer protection [Dir 1996/23/EC]. The control of β-agonists misuse received extra-attention after outbreaks of food poisoning in 1990 in Spain caused by consumption of *bovine* liver. This was the first time that pharmacological residues present in slaughtered cattle were found to have caused acute intoxication in consumers. Although clenbuterol is still probably the most popular known β-agonist illegally used in farming, a wide range of other β-agonists exists which constitutes a challenge in detecting their use. Besides classical protocols of administration, some practices consisting in the use of "cocktails" composed of mixtures of low amounts of several substances that exert a synergistic effect and exhibiting similar growth promotion properties have also been reported as additional challenge to be overcome by control laboratories.

Emerging screening strategies based on biomarkers monitoring have proven their efficiency in highlighting in a generic way a range of β-agonists based practices, while confirmatory strategies targeting β-agonists residues in various relevant biological matrices (urine, serum, tissues, retina, hair) enable long-term detection of forbidden practices. During this study, we also evaluated the combination of the travelling wave ion mobility separation with a classic UHPLC-MS workflow. Finally, histology was evaluated as a powerful screening strategy to detect β-agonists misuse; its application should be encouraged to investigate microscopic changes in lungs of treated animals.

Materials and Methods

Animal experiments
*S*everal animal experiments have been designed to provide the study with matrices of interest. They involved various doses (1 to 10 µg kg^{-1} body weight) of β–agonists (clenbuterol, ractopamine, cimaterol, zilpaterol, mabuterol), different treatment lengths (6 to 21 days) and withdrawal periods (1 to 70 days) associated with the collection of a large range of *bovine* matrices (urine, serum, hair, retina, muscles, liver and lung).

Residues analysis
Matrices of interest for the study have been analysed according to validated and ISO17025 accredited methods. Sample sizes were as follows: urine 10 mL, hair 100 mg, retina 200 mg, serum 1 mL and tissues 20 g. The methods briefly consist in either a deconjugation step with *Helix pomatia* during 15h at 52°C (urine, serum and tissues) or hydrolysis with 1 M HCL for 4 h at 50°C (retina and hair samples), followed by a mixed mode SPE purification step (CSDAU, SDS, Peypin, France). Separation of

the compounds was performed on a C18 column (50 x 2,1 mm, 3 µm; Uptisphere 3HDO – Interchim, Montluçon, France) and MS identification using a triple quadruple (Agilent® 6410/Waters Xevo T-QS) in the SRM mode according to the 2002/657/EC requirements.

Ion mobility characterisation
Analyses were performed on a hybrid quadrupole/traveling wave ion mobility/orthogonal acceleration time-of-flight geometry instrument, (Synapt G2-S HDMS; Waters, Manchester, UK). Compounds were visualized with Advanced Chemistry Development software V12.01 (Ontario, Canada). Mass-spectra and mobilograms were analysed using MassLynx 4.2. Relevant pieces of information were extracted with DriftScope 2.7 and UNIFI 1.6.5. Collision Cross Sections (CCS) were calculated based on the ion mobility peak of singly protonated ions from poly[D]alanine.

Metabolomics
Urine samples (2 mL) were filtered over 10 KDa membranes under centrifugation at 10,000 *g* at 5°C for 20 min and normalised (30 mg mL^{-1}). Chromatographic separation was performed with a high performance liquid chromatography (HPLC) system (1200 Infinity Series from Agilent Technologies, Santa Clara, California, USA) on a Hypersil Gold C18 column (2.1 mm x 100 mm, 1.9 µm particle size, Thermo Fisher Scientific). Mobile phase consisted in water containing 0.1% acetic acid (A) and acetonitrile containing 0.1% acetic acid (B). Liquid-chromatography - high resolution mass-spectrometry (LC-HRMS) fingerprints were acquired on an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in positive heated electrospray ionization mode. Full scan mass-spectra were acquired from *m/z* 80 to *m/z* 1000 using a mass resolution of 30,000 FWHM at 400 *m/z* in centroid mode. Data were processed by the open-source XCMS software and analysed with SIMCA-P+ software to set up descriptive and predictive models and highlight candidate biomarkers.

Histology
At the slaughterhouse, the lungs of the clenbuterol-treated calves were examined and a sample from each lobe was taken. After the routine tissue processing and paraffin embedding procedures, 3 ± 2 µm sections were cut from the left caudal lobe of each animal. The slides were then stained with haematoxylin and eosin (HE) and Masson's trichrome stain in order to examine the tissue morphology and the increase of fibrosis due to the long-term stimulation of β-adrenergic receptors.

Results and Discussion

Residues determination
In order to take into account new potential growth promoting practices, such as the use of low concentration cocktails, and assess performances of current analytical strategies, a set of animals experiments involving various doses of β-agonists, different withdrawal periods and the collection of a large range of biological matrices have been designed. The analytical study led to the conclusion that muscle, liver, lung and kidney are not the most appropriate matrices to detect the various administrations, whereas urine, hair and retina were more relevant for control purposes at the farm (urine and hair) or the slaughterhouse (retina) (Figure 1).

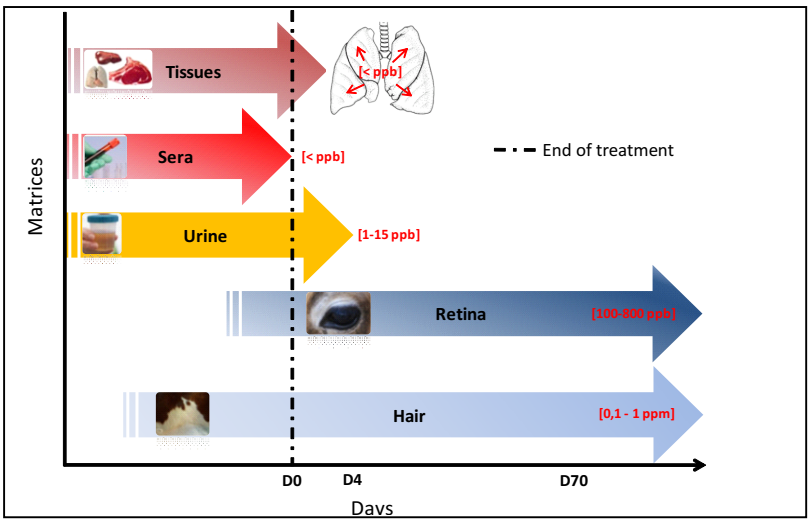

Figure 1. Comparison of biological matrices as for their relevance regarding β-agonists detection window time and maximum observed concentrations

Ion mobility characterisation

The traveling-wave ion mobility separation demonstrated its ability to efficiently separate compounds that cannot, or with difficulty, be separated with classical analytical workflows. The proof-of-concept has been performed on a range of aryleth-anolamine compounds; in particular, isobaric compounds (*e.g.* ractopamine and isoxuprine) could be easily separated when applying the drift time filter (Figure 2). A signal clean-up was further more observed in biological matrices, enabling enhanced specificity and sensitivity of the detection of β–agonists at trace levels in complex biological matrices (Beucher *et al*. 2015).

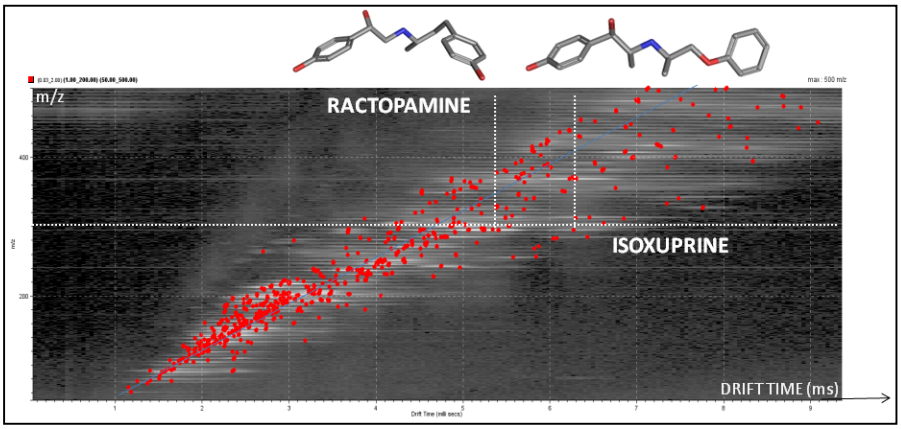

Figure 2. Mobilograms observed for a range of β-agonists compounds. Illustration of the separation efficiency for isobaric compounds.

Metabolomics

The study performed on urine samples collected in the frame of several independent experiments involving different animals, different β-agonists treatments and different parameters (doses, compounds mixture, treatment length), allowed highlighting biomarkers of interest and implementing a robust statistical model to predict samples from β-agonists treated *bovines* (Figure 3). Established model and biomarkers have been further validated through challenge tests involving independent experiments. Performances of the proposed model fit with EU requirements for screening methods (Dervilly-Pinel *et al*. 2015).

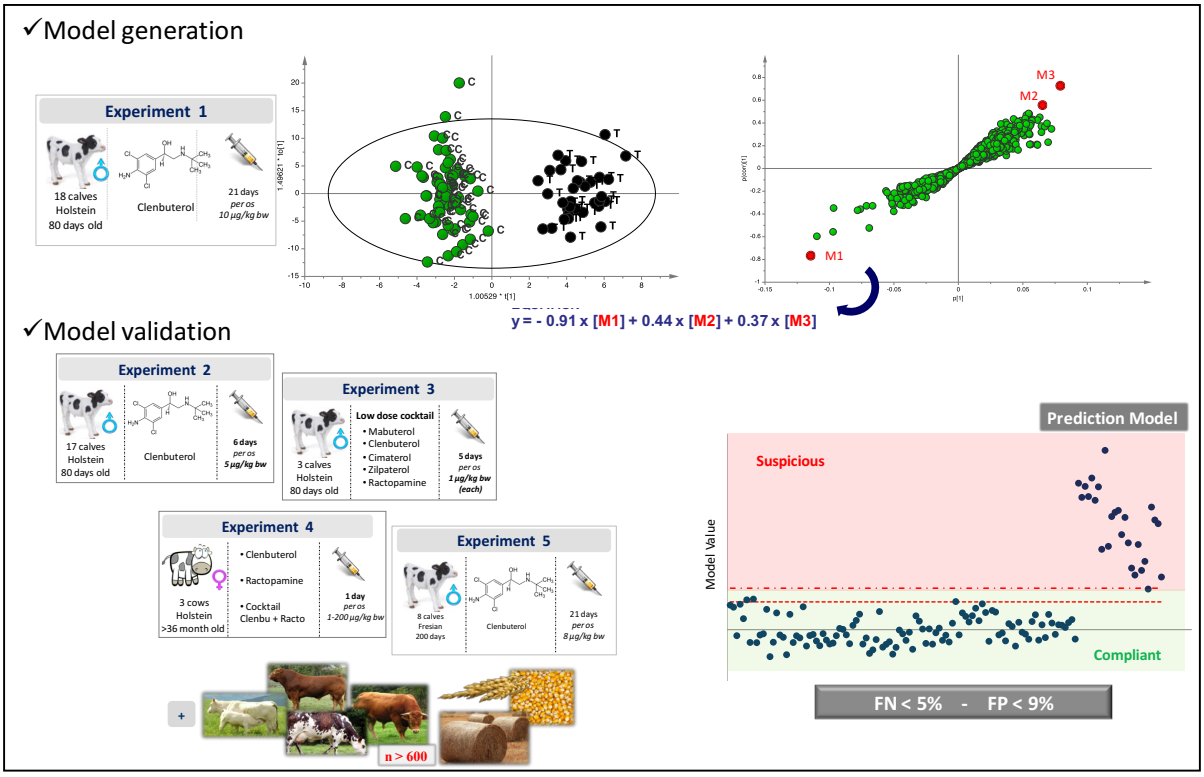

Figure 3. Generation and validation of metabolomics model to predict urine status regarding any β-agonist treatment in bovines.

Histology

The histological examination of β-agonists-treated calves' lung samples shows a mild to moderate hypertrophy of alveolar walls, with scant interstitial fibrosis (Figure 4), when compared to the untreated one. The trichromic special staining (Masson's staining) highlights an increased interstitial fibrosis in the clenbuterol treated calf's lung samples, associated with a moderate bronchiolar smooth muscle hypertrophy (Figure 5).

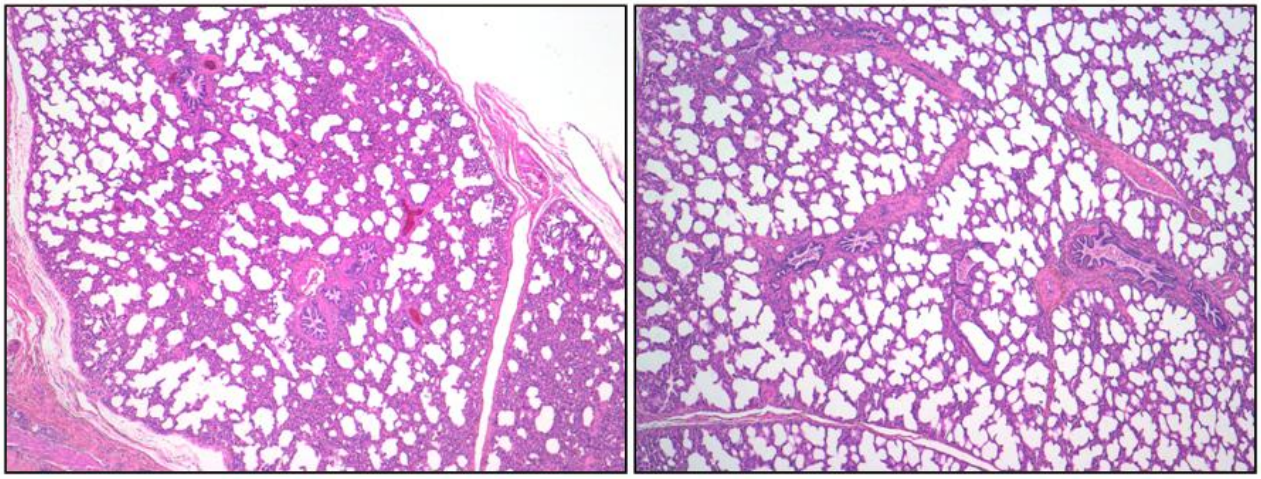

Figure 4. HE staining of lung samples from a clenbuterol-treated calf (left) and an untreated calf (right); HE 4X.

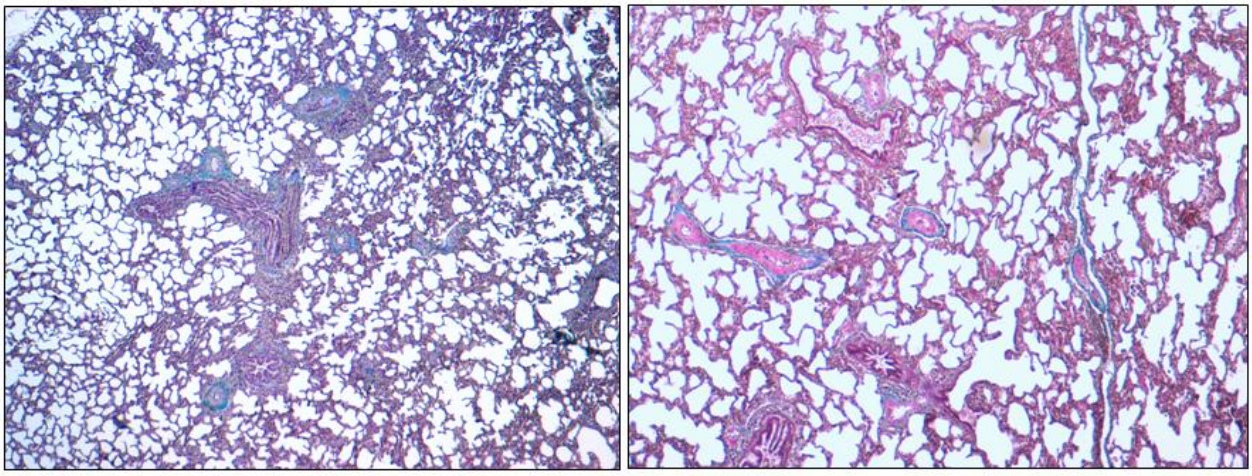

Figure 5. Masson's trichromic staining of lung samples from clenbuterol-treated calf (left) and untreated calf (right); 4X.

Conclusions

In order to take into account new potential growth-promoting practices, such as the use of low concentration cocktails, and assess performances of current or emerging analytical strategies, several animal experiments involving various doses of β–agonists, different withdrawal periods and the collection of a large range of biological matrices have been designed.

Screening approaches have been assessed as follows: the **metabolomics** strategy enabled highlighting and validating generic biomarkers to screen for a range of β–agonists treatments in male calves. The model has been officially implemented since 2013 in the frame of the French Monitoring and Control Plans. The **histological** examination of β-agonists treated calves' lung suggests that fibrosis (interstitial and peri-bronchiolar) and smooth muscle hypertrophy are the two main features of prolonged clenbuterol treatment. Further investigations are needed to obtain robust data about the histopathological changes after clenbuterol administration (immunohistochemistry procedures for the detection of β2-adrenoreceptor modulation).

Confirmatory approaches involving targeted residues measurements led to the conclusion that muscle, liver, lung and kidney are not the most appropriate matrices to detect the various administrations, whereas **urine, hair** and **retina** were more relevant for control purposes at the farm (urine and hair) or the slaughterhouse (*retina*). *Retina* in particular was found very effi-

cient even after long withdrawal periods since high amounts up to 800 µg kg^{-1} could be detected. The extra ion mobility dimension of separation investigated in different biological matrices resulted in a selective signal clean-up together with increased discrimination of ions presenting similar mass-to-charge ratio or retention times**.**

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ANALYTICAL STRATEGIES TO DETECT SELECTIVE ANDROGEN RECEPTOR MODULATORS ADMINISTRATION IN *BOVINES*

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Abstract
Selective androgen receptor modulators (SARMs) are a novel class of androgen receptor ligands. They are intended to exhibit the same kind of effects as androgenic drugs, like anabolic steroids, but be much more selective in their action, targeting particular tissues (muscle for example) without any undesirable effects on other tissues. While main applications of these synthetic substances are for therapeutic purposes, they will also have a high potential for misuse in veterinary practice and the sporting world. In order to guaranty the consumers with food from animal origin free from any residues of such compounds, analytical strategies are required to ensure safe food and also enable fair trade between producers.
In this context several animal experiments involving bovines administered with different SARMs (Enobosarm, Bicalutamide) have been conducted to provide the study with biological matrices. Both targeted and untargeted mass-spectrometric analytical strategies have been developed to highlight relevant metabolites or biomarkers of such treatments and establish detection time window. Different animal matrices (urine and faeces) have been investigated to select most appropriate matrix to be used for control purposes.

Introduction
Selective androgen receptor modulators (SARMs) are a novel class of androgen receptors (AR) ligands (Dalton 2010a). SARMS are known since 1998 (Dalton 1998) and have been investigated for therapeutic properties in cachexia treatment since 2005 (Gao 2010). SARMs action is directed on androgen receptors of muscles (growth and strength) and bones (development) (Kim 2005, 2013). SARMs belonging to the aryl-propionamide group have been the first ones to show an interesting agonistic activity with a tissue-selective action in the steroidal signalling pathway (Aikawa 2015, Bhasin 2009, Gao 2006, Narayanan 2008).

While SARMs is on the World Anti-Doping Agency (WADA) list of prohibited substances, cases of human doping and misuse by athletes have already been reported reported (Grata 2011, Canadian Sport Sanction Registry). SARMs may also exhibit a high potential for misuse in animal husbandry as growth-promoters to increase weight gain, improve carcass quality and reduce production costs (Dalton 2010b). While in Europe, growth-promoters are banned for use in food-producing animals since 1988 (Council Directive 88/146/EEC prohibiting the use livestock farming of certain substances having a hormonal action 1988), strategies to detect potential SARMs misuse in livestock production are urgently required to guaranty the consumers with food from animal origin free from any residues of such compounds. Since these compounds have recently emerged, they have not been investigated a lot and more studies are necessary to develop comprehensiveness of SARMs metabolism through *in vivo* approaches (Thevis 2011, de Rijke 2013, Hansson 2014, Beucher 2016).

In the current work two SARMs, namely enobosarm and bicalutamide, belonging to the aryl-propionamide group, were investigated to set up analytical strategies enabling detecting both parent compounds and their metabolites in *bovine* matrices (urine and faeces) after oral administration. The objectives were to identify the main metabolites and associated detection time windows in each matrix and to propose a validated method of analysis based on ultra high performance liquid chromatography coupled to a triple quadrupole mass spectrometer.

The novelty of this work relies (i) on the pharmacokinetic and metabolism study in the context of a daily administration in calves under conditions similar to those that may be used for a potential growth promoting practice, (ii) in the assessment of faeces as an alternative interesting biological matrix to monitor such compounds.

Materials and methods

Experimental design and samples
Eleven Prim'Holstein entire male weaned calves were purchased from the same producer and reared during three weeks. They were allotted into two homogeneous groups in terms of health status, mean age (81 days for the control group, 78.2 days for the treated group) and mean weight (86.2 kg for the control group, 87.2 kg for the treated group). Two experiments were performed with these animals as follows:

In experiment A, a group "treated" (n = 5) and a group "control" (n = 5) were designed to study the kinetic of enobosarm elimination after a long term oral administration at the concentration of 1 mg kg^{-1} feed (1 ppm). Enobosarm was administered with the feed over the 21-days experimental period (50 kg per calf, the amount of enobosarm administered was therefore 50 mg per calf for 21 days i.e. 2.38 mg/calf/day). Experiment B consisted in a high dose (200 mg) administration of enobosarm to a single calf to study the metabolism of enobosarm.

The calves were followed clinically every day, urines and faeces were collected on spontaneous urination and defecation ("free catch") at D-3, D-2 D-1 (three days before oral administration of enobosarm), D0, D0+3 hours, D0+5 hours (day of enobosarm administration), D1, D2, D3, D5, D7, D9, D11, D14, D18, D21 (ten days after oral administration of enobosarm), by the same operator. All samples were stored at -20°C until preparation and analysis.

Furthermore, one Montbeliard entire bull calf (190 kg) was reared during 2 weeks to study the kinetic elimination and the metabolism of bicalutamide after an oral administration at the concentration of 100 mg on one-shot. The animal was followed clinically every day. Urine and faeces were collected on spontaneous urination and defecation at D-3, D-2 D-1 (three days before oral administration of bicalutamide), D0, D0+2,5 hours, D0+6,5 hours (day of bicalutamide administration), D1, D2, D3, D5, D-6, D7, (seven days after oral administration of bicalutamide), by the same operator. All samples were stored at -20°C until preparation and analysis.

Residues Analysis

Four standards of SARMs (enobosarm, andarine, bicalutamide hydroxyflutamide) were purchased to prepare standards solutions at final concentration of 10 ng μL^{-1} in ethanol and stored at -20°C until use. Bicalutamide d4 was used as internal standard (1 ng μL^{-1}) and fluorometholone was used as external standard (1 ng μL^{-1}). Enzymatic hydrolysis was performed with a β-glucuronidase/aryl-sulfatase mixture isolated from *Helix pomatia*. A solid phase extraction (SPE) was carried out with Oasis HLB cartridges.

Faeces (previously freeze-dried) and urine samples were thawed and mixed with internal standard in buffer conditions. Next, enzymatic hydrolysis was realized or not, depending on conditions of the studies (kinetic or metabolism). Then SPE was applied and samples were evaporated and suspended in chromatographic solvent before injection.

Liquid chromatography hyphenated to a tandem mass spectrometry (LC-MS/MS) was conducted using ultra-high pressure liquid chromatography (UHPLC) on reversed-phase separation equipped with an Acquity UPLC BEH C18 column and connected with mass spectrometry detection in electrospray ionization (ESI) negative mode with a triple quadrupole (QqQ) Xevo TQ-S (Waters). In selected reaction monitoring (SRM) MS/MS (QqQ) mode, parameters were optimized for the following transitions of enobosarm (*m/z* = 388.1>118.0, 388.1>269.0, 388.1>185.0) and bicalutamide (*m/z* = 429.1>255.1, 429.1>185.0, 429.1>173.0). Ion chromatograms were recorded in full scan negative mode for metabolism investigation purposes.

Results and Discussion

Biological matrices collected during the animal experiments enabled establishing kinetics of excretion in urine and faeces for both bicalutamide and enobosarm. Samples were analysed with and without hydrolysis step to assess the metabolisation potential through phase II conjugates.

Urinary excretion of enobosarm

When no deconjugation step was applied, and considering the animal from exp. B, enobosarm was observed the most excreted (38.20 ng mL^{-1}) in urine 5 h after administration, and the concentrations subsequently decreased to be undetectable 5 days after administration. After applying a hydrolysis step in the sample preparation workflow, the concentrations of the total forms were detected between 0.25 and 155 ng mL^{-1} with a maximum at D1. Enobosarm could be detected with decreasing levels until nine days after administration (Figure 1a).

In the same time five calves of animal experimentation A received a low dose of enobosarm (2.38 mg/calf/day) from first to last day of the experiment (D0 to D21). The analysis of their urine samples allowed observing the behaviour of the molecule in the calves' body upon daily intake, in particular to investigate the issue of a possible accumulation of enobosarm in the body. Samples of two calves were analysed with and without hydrolysis to monitor their elimination kinetics. As expected, no enobosarm was detected before oral administration. During the 21 days of treatment the average level of enobosarm was found between 0.18 and 1.2 ng mL^{-1} when no deconjugation was applied, while total forms (after deconjugation) exhibited an average concentration between 0.4 and 2.8 ng mL^{-1}. In this case, the concentration of enobosarm in the urine started to increase very rapidly in the early hours and the first days of the experiment (between D0 and D2, D3) before to decrease slightly (between D2, D3 and D7) and reach a plateau at 1.7 ng mL^{-1} (between D7 and D21). Without hydrolysis this phenomenon was found less important (the plateau being reached rather quickly): the amount of enobosarm in urine increased in the hours following the first administration, between D0 and D+5 hours, at which time it reached a plateau at about 0.7 ng mL^{-1}. The enobosarm excretion profile after a daily intake is presented in Figure 1b.

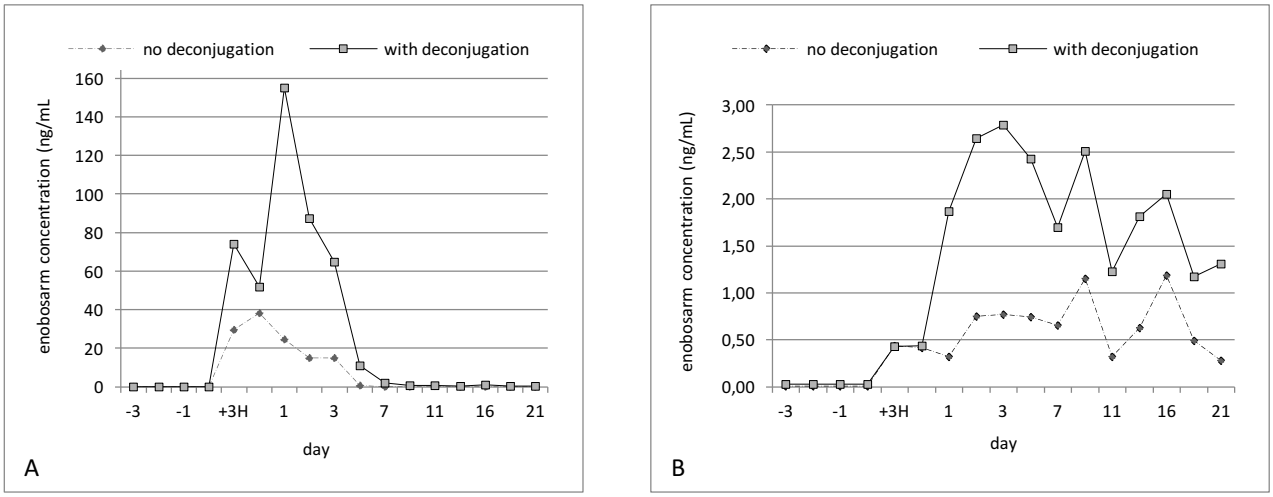


Figure 1. a) Enobosarm excretion in bovine urine. Kinetic of urinary elimination in a calf which received a high one-shot oral dose of enobosarm (200 mg/animal at D0). b) Enobosarm excretion in bovine urine. Kinetic of urinary elimination in a calf which received a daily intake oral dose of enobosarm (2.38 mg/animal/day during 21 days).

Faecal excretion of enobosarm

There was no difference between the kinetic profiles when deconjugation step or not was applied to the samples. Enobosarm was not detected before oral administration. Compared to urine, the concentration of enobosarm in faeces reached a very high level at D1 (53271 ng g^{-1}) and strongly decreased until five days after administration, and then decreased slowly remaining detectable until twenty-one days after oral administration (16 ng g^{-1}). The enobosarm excretion profile in faeces is showed in Figure 2.

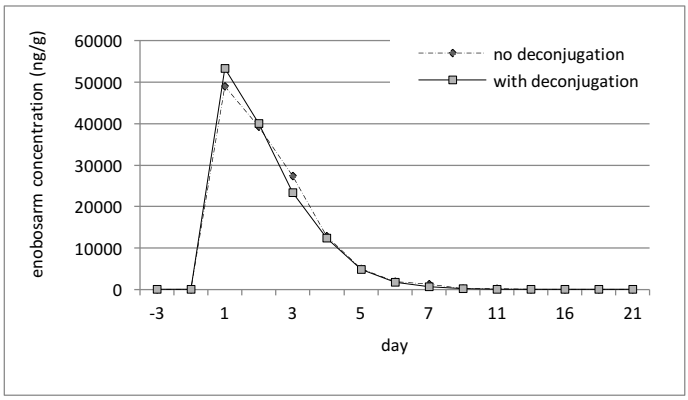


Figure 2. Enobosarm excretion in bovine faeces. Kinetic of faecal elimination in a calf which received a high one-shot oral dose of enobosarm (200 mg/animal at D0).

Urinary and faecal excretion of bicalutamide

As expected, no bicalutamide was detected in the sample before the treatment. After 4 hours following oral administration, bicalutamide was detected in urine and faeces. Bicalutamide free concentration increased very rapidly to reach a maximum at D1 (13.2 ng mL^{-1}). The concentration of bicalutamide in urine, after deconjugation step, reached a maximum at D1 (17.4 ng mL^{-1}). Both free bicalutamide and total forms showed the same profile, quick increase until D1 and slow decrease until D7. In faeces, bicalutamide's concentration were observed at very high levels compared to urine. It increased quickly with a maximum at D1 reaching 1,254 ng mL^{-1} for the free form and 1347 ng mL^{-1} for the total forms.

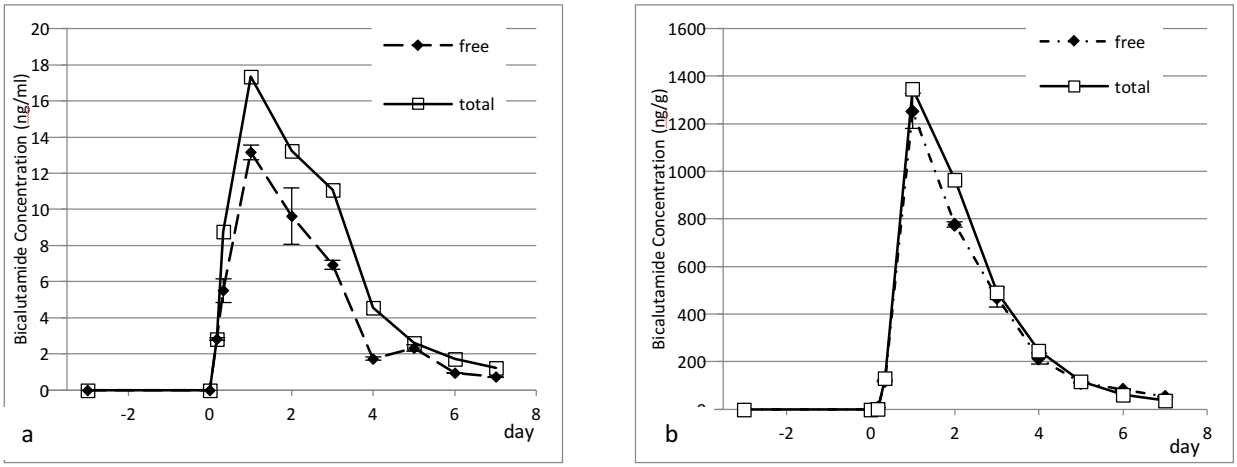


Figure 3. a) Bicalutamide excretion in *bovine* urine. Kinetic of urinary elimination in a calf which received a high one-shot oral dose of bicalutamide (100 mg/animal at D0). b) Bicalutamide excretion in *bovine* faeces. Kinetic of faecal elimination in a calf which received a high one-shot oral dose of bicalutamide (100 mg/animal at D0).

Metabolism investigation

To study enobosarm metabolism, the approach was to analyse and compare all mass spectrometric signals present in the non-deconjugated urine in order to identify specific signals related to enobosarm administration. Different acquisition modes (full scan, precursor ion scan, neutral loss) have been performed. Using an untargeted full-scan mode, and based on literature review for human metabolism, six ions $[M-H]^-$ exhibiting *m/z* ratio 201, 220, 287, 301, 564 and 580 were recorded for further investigation. Secondly, after applying a Precursor Ion scan mode, to identify precursor ions which product the same fragments as enobosarm or fragments descending from sulphate (*m/z* = 80, 97) and glucuronide (*m/z* = 113) forms, five ions [M-H]- of *m/z* ratio 287 (precursor *m/z* = 118 and 185), 404 (precursor *m/z* = 185 and 269), 484 (precursor *m/z* = 185 and 269), 564 (precursor *m/z* = 113, 118, 185 and 269) and 580 (precursor *m/z* = 185) were selected for further study. Finally, including the Constant Neutral Loss scan mode, to introduce the study of fragmentation loss of classical mammal group glucuronide (*m/z* = 192,180, 176), sulphate (*m/z* = 80, hydroxy (*m/z* = 16) and *O*-phenyl (*m/z* = 101), five ions [M-H]- of *m/z* ratio 281 (loss of *m/z* = 80), 287 (loss of *m/z* = 101), 463, 564 and 580 (loss of *m/z* = 176) were retained. The extracted ion chromatograms of the main metabolites of enobosarm are shown in Figure 3.

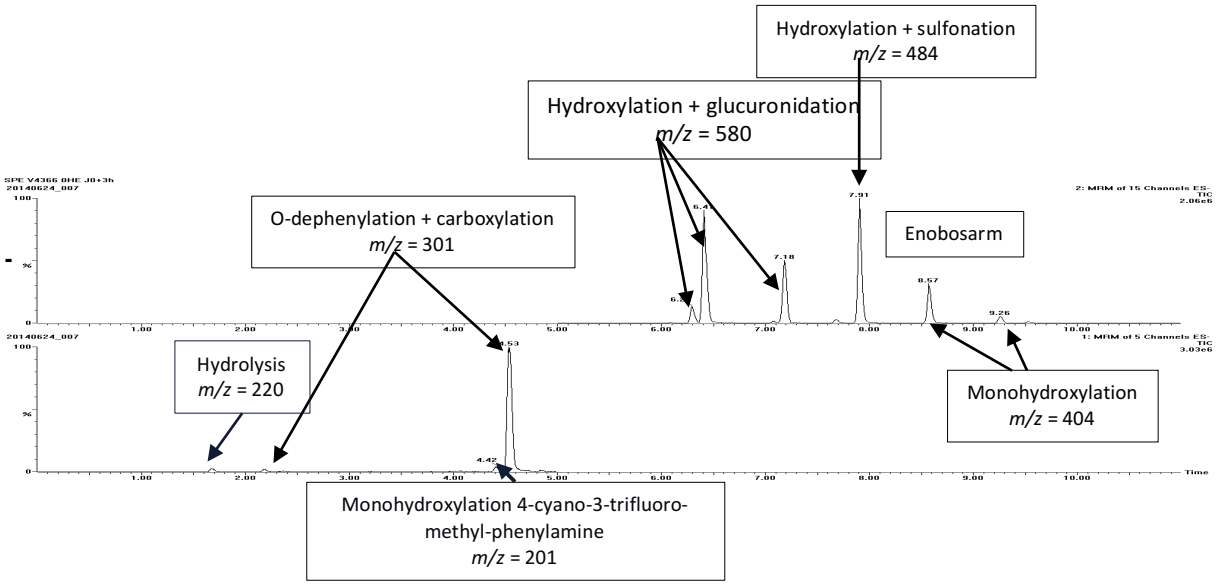


Figure 3. Full scan and extracted ion UHPLC-QqQ-MS/MS chromatograms of the main in vivo metabolites of enobosarm. These metabolites are identified in a calf urine without hydrolysis step. The urine was collected three hours after an oral administration of 200 mg of enobosarm.

Finally, in order to identify metabolites of interest a focus was made on six precursor ions and associated transitions. The second step realized in Product Ion Scan mode has strengthened the structural identification of potential metabolites of *m/z* ratio 201, 220, 301, 404, 484 and 580. Structures of metabolites derived from enobosarm in *bovine* are proposed in Figure 4.

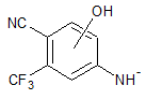
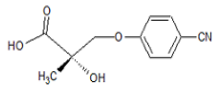
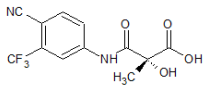
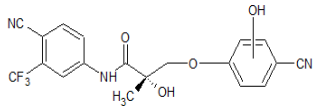
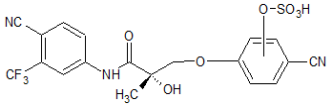
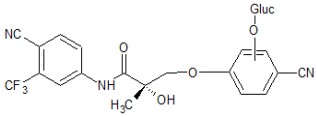
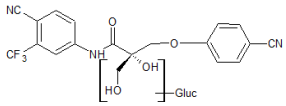
Supposed formula	Chemical structure proposed	Transition 1	Transition 2
$C_8H_4ON_2F_3$		201 > 154	201 > 181
$C_{11}H_{10}O_4N$		220 > 118	220 > 90
$C_{12}H_8O_4N_2F_3$		*301 > 257*	*301 > 113*
		301 > 185	
$C_{19}H_{13}O_4N_3F_3$		404 > 134	404 > 185
		404 > 134	404 > 185
$C_{19}H_{13}O_7N_3F_3S$		484 > 134	484 > 404
$C_{25}H_{21}O_{10}N_3F_3$		580 > 404	580 > 310
		580 > 404	580 > 269
		580 > 285	580 > 255

Figure 4. Proposed structures for the potential metabolites following an oral high dose administration of enobosarm in a calf.

The study of bicalutamide metabolism was also performed through Fullscan and Precursor Ion scan modes highlighting five potential metabolites: M1 (*m/z* = 605), M2 (*m/z* = 621), M3 (*m/z* = 445), M4 (*m/z* = 509) and M5 (*m/z* = 525), which elucidation is currently in progress.

Conclusions

Results obtained by complementary analytical strategies allowed demonstrating a strong faecal elimination of SARMS of interest and their metabolites after a high dose oral administration. The HPLC-ESI(-)-QqQ-strategy for the analysis of two SARMs of the class of aryl propionamides on urine and faeces matrices was successfully developed to propose the method for validation. The application of these methods allowed establishing different kinetics of elimination of SARMs in urine and faeces of calves. For enobosarm, this work highlighted the presence of a great amount of conjugated forms of the molecule in the urine, mainly metabolites of phase II reactions, sulfonated and glucuronide-conjugated. Enobosarm can be detected in urine with an upper concentration up to nine days when samples undergo hydrolysis. Faeces demonstrated to be the main matrix of excretion of enobosarm since values up to 500 times compared to urine could be detected, during 21 days.

To the best of the authors' knowledge, this is the first study of a daily administration of SARMs, in calves under conditions similar to those that may be used during a potential misuse practice. And this *in vivo* study has been used to complete the characterization of enobosarm and bicalutamide metabolites, future targets for control, resulting from the real metabolism of SARMs in fattening calves. Such results are particularly promising in the objective of setting up a confirmatory analytical strategy and a target matrix to highlight SARMs abuse in livestock animals. Next step is to develop a screening tool to detect any SARMs abuse on the basis of a metabolomics approach as already shown efficient for other families of growth-promoters (Dervilly-Pinel 2012, 2014).

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AMBIANT MASS SPECTROMETRY: HIGH THROUGHPUT STRATEGY FOR IDENTIFICATION AND QUANTIFICATION OF ANABOLIC STEROID ESTERS

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Abstract

Recent developments in ambient mass-spectrometry (AMS), such as Atmospheric Solids Analysis Probe (ASAP) and Direct Analysis in Real Time (DART) mass spectrometry open a whole new range of possibilities to screen for drug preparations. The potential of ASAP and DART for fast identification and quantification of 21 anabolic steroid esters (based on testosterone, estradiol, nandrolone and boldenone) has been evaluated. These compounds are known to be used both in human and in food-producing animals to enhance performances and to improve rate of growth, respectively. Using a triple quadrupole (QqQ) MS instrument, mechanism of ASAP ionization and fragmentation highlighted common neutral mass loss of 96.1 allowing thus rapid screening in minutes with minimal sample preparation. Ester identification is further achieved through an efficient 2 min workflow. Direct analysis in high resolution scan mode allowed steroid esters screening by accurate mass measurement (R= 60,000 mass error < 3ppm). Steroid esters identification was further supported by collision-induced dissociation (CID) experiments leading to the generation of two additional specific ions. These innovative high throughput approaches were successfully applied for the characterization of oily commercial preparations; and thus fit the needs of competent authorities in the fight against forbidden or counterfeited substances.

Introduction

Anabolic steroids are usually administered intramuscularly (IM) under their ester forms (synthetically produced) in lipophilic excipient providing longer biological availability and lasting effects. Depending on the length of the side chain, steroid esters show different release times. Thus, mixtures of long- and short-side chain esters are usually administered in order to reach constant doses of active molecules on a wide time scale. Several analytical strategies have been investigated to prevent anabolic steroid (esters) misuse. Forensic laboratories search for anabolic steroids (esters) in oily solutions or in tablets whereas anti-doping and food safety laboratories examine hair or blood samples for steroid ester contents as an unequivocal proof of misuse. With biological samples, the main challenge is to detect the xenobiotics of interest present at trace level in the considered matrix. Therefore, extended and time-consuming purification procedures have been developed, involving liquid-liquid extraction, solid phase extraction and sometimes a derivatisation step to enhance sensitivity of the detection.

The detection step of steroid esters is mainly performed by liquid chromatography coupled to mass-spectrometry (MS^n or HRMS) leading to efficient management of steroid esters issues. Nevertheless, the need for faster multi-residue approaches with both increased sensitivity and selectivity is crucial today: the quicker the analysis the more efficient the control. In particular, methods allowing rapid characterization of seized preparations are required. Atmospheric solids analysis probe (ASAP) was first introduced in 2005 by McEwen and co-workers (McEwen *et al*. 2005) and is derived from atmospheric pressure chemical ionization (APCI). ASAP can be used in conjugation with tandem mass-spectrometry (MS/MS) for fragmentation pathways studies or with high resolution mass spectrometry (HRMS) for accurate mass measurements and fingerprinting purposes.

In the present work, the rapid identification and quantification of anabolic steroid esters by ASAP has been assessed and mechanisms of ionization and fragmentation in both positive and negative modes were studied (Doué *et al*. 2014). In parallel, the potential of Direct Analysis in Real Time (DART) hyphenated with Orbitrap-MS for fast and simple identification and quantification of anabolic steroid esters, using isotope dilution, has been evaluated (Doué *et al*. 2014). This study was applied on a large set (n = 21) of steroid esters derived from testosterone, estradiol, boldenone or nandrolone. The applicability of ASAP and DART to the characterization of oily preparations was finally probed.

Materials and Methods

Chemicals and materials

The reference steroid esters including testosterone acetate (T Ac), testosterone benzoate (T Bz), testosterone cypionate (T Cy), testosterone decanoate (T Dc), testosterone heptanoate (T Hp), testosterone propionate (T Pr), boldenone acetate (B Ac), boldenone benzoate (B Bz), boldenone propionate (B Pr), boldenone undecylenate (B Un), nandrolone benzoate (N Bz), nandrolone decanoate (N Dc), nandrolone laurate (N Lr), nandrolone propionate (N Pr), nandrolone phenylpropionate (N PhPr), estradiol 17-cypionate (E2 17Cy), estradiol 17-enanthate (E2 17En), estradiol 17-propionate (E2 17Pr), estradiol 17-valerate (E2 17Vl), estradiol 17-decanoate (E2 17Dc), and estradiol 3-benzoate (E2 3Bz) were obtained from either Steraloids Inc. Ltd. (London, UK) or Sigma-Aldrich (St Louis, USA). Testosterone acetate-d3 (T Ac-d3), testosterone benzoate-d3

(T Bz-d3), testosterone decanoate-d3 (T Dc-d3) and testosterone heptanoate-d3 (T Hp-d3) were used as labelled internal standards (IS) and were kindly provided by RIKILT (Wageningen, The Netherlands).

Each steroid ester stock solution was prepared at 1 mg mL^{-1} by dilution in an appropriate volume of ethanol and stored at -20°C. The working standard solutions were prepared by diluting stock solutions in methanol. An oily commercially available solution Sustanon® (T Pr at 30 mg mL^{-1}, T PhPr at 60 mg mL^{-1}, T Isocaproate (T Iso) at 60 mg mL^{-1} and T Dc at 100 mg mL^{-1}) was used to assess performances of the developed AMS strategies.

Before analysis, oily solutions were diluted 1,000 fold in methanol. For quantification purposes, a mixture of the four labelled internal standards (T Ac-d3, T Bz-d3, T Dc-d3 and T Hp-d3) at 1 mg L^{-1} each (final concentration) was added before dilution.

ASAP-MS Instrumentation

The steroid esters were analysed using a Waters ASAP Probe on a XEVO® TQ-S (QqQ) instrument. For fragmentation pathways study and identification of unknown samples, the APCI probe temperature was set at 450°C whereas for quantification purposes a linear gradient of temperature was applied from 100 °C to 600°C in 15 s and held at 600°C thereafter. In positive mode, corona current was set to 3 µA, cone voltage to 45 V and source offset to 70 V, while in negative mode corona current was set to 5 µA, cone voltage to 35 V and source offset to 80 V. For scan, precursor ion scan, product ion scan or neutral loss scan, data were acquired in "Multi Channel Analysis" which consists in accumulating profile scans over a given period (*i.e.* 50 s). In selected reaction monitoring (SRM) experiments, collision energy (CE) was optimized for each compound and argon was used as collision gas at 0.15 mL min^{-1}.

DART-MS Instrumentation

The DART-MS system consisted of a DART-Standardized Voltage and Pressure (DART-SVP) ion source with a one-dimension transmission autosampler (IonSense, Saugus, MA, USA) coupled to a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A vapour interface (IonSense) was employed to hyphenate the ion source and the mass spectrometer. Both the DART-SVP source and the Orbitrap MS were operated in positive ionization mode. The optimized settings of the DART-SVP parameters were as follows: helium flow: 3.5 L min^{-1}; gas temperature: 300°C; discharge needle voltage: -5 kV and grid electrode: 350 V. Settings of the LTQ-Orbitrap hybrid mass spectrometer were as follows: capillary voltage: 15 V; capillary temperature: 250°C and tube lens offset: 100 V.

Table 1. Specific ions and common ions observed for the 21 selected steroid esters in scan and product ion scan of the corresponding $[M+H]^+$ or $[M-H]^-$ in both positive and negative mode ($APCI^+$ / $APCI^-$).

Steroid base	Compounds	Monoisotopic mass (g mol-1)	Scan and product ion scan of $[M+H]^+$		Scan and product ion scan of $[M-H]^-$	
			Specific ions API + (m/z)	Common ions API + (m/z)	Specific ions API - (m/z)	Common ions API - (m/z)
Testosterone	T Ac	330.2	331.2 $[M+H]^+$	289.2 $[T+H]^+$; 271.2 $[T+H-H_2O]^+$; 253.2 $[T+H-2H_2O]^+$; 175.1 ; 109.0 and 97.0	329.2 $[M-H]^-$ and 59.0 $[Ac]^-$	269.2 $[T-H-H_2O]^-$
	T Pr	344.2	345.2 $[M+H]^+$		343.2 $[M-H]^-$ and 73.0 $[Pr]^-$	
	T Bz	392.2	393.3 $[M+H]^+$ and 105.0		391.2 $[M-H]^-$; 121.0 $[Bz]^-$ and 77.0	
	T Hp	400.3	401.3 $[M+H]^+$ and 85.0		399.2 $[M-H]^-$ and 129.1 $[Hp]^-$	
	T Cy	412.3	413.3 $[M+H]^+$; 107.0 and 79.0		411.2 $[M-H]^-$ and 141.1 $[Cy]^-$	
	T Dc	442.3	443.4 $[M+H]^+$ and 105.0		441.2 $[M-H]^-$ and 171.1 $[Dc]^-$	
Estradiol	E2 17Pr	328.2	329.2 $[M+H]^+$	255.2 $[E2+H-H_2O]^+$; 159.1 ; 135.0 and 107.0	327.2 $[M-H]^-$ and 73.0 $[Pr]^-$	253.2 $[E2-H-H_2O]^-$
	E2 17Vl	356.2	357.2 $[M+H]^+$		355.2 $[M-H]^-$ and 101.0 $[Vl]^-$	
	E2 3Bz	376.2	377.2 $[M+H]^+$; 359.3 ; 105.0 and 77.0		375.2 $[M-H]^-$; 271.2 $[E2-H]^-$ and 121.0 $[Bz]^-$	
	E2 17Hp	384.3	385.3 $[M+H]^+$ and 85.0		383.2 $[M-H]^-$ and 129.0 $[Hp]^-$	
	E2 17Cy	396.3	397.3 $[M+H]^+$ and 79.0		395.2 $[M-H]^-$ and 141.0 $[Cy]^-$	
	E2 17Dc	426.3	427.3 $[M+H]^+$; 409.3 and 273.2		425.3 $[M-H]^-$; 271.2 $[E2-H]^-$ and 171.1 $[Dc]^-$	
Boldenone	B Ac	328.2	329.2 $[M+H]^+$	287.2 $[B+H]^+$; 269.2 $[B+H-H_2O]^+$; 173.1 ; 147.1 ; 135.0 and 121.1	327.2 $[M-H]^-$; 311.2 and 59.0 $[Ac]^-$	267.2 $[B-H-H_2O]^-$
	B Pr	342.2	343.2 $[M+H]^+$		341.2 $[M-H]^-$; 325.2 and 73.0 $[Pr]^-$	
	B Bz	390.2	391.2 $[M+H]^+$ and 105.1		389.2 $[M-H]^-$ and 121.0 $[Bz]^-$	
	B Un	452.3	453.3 $[M+H]^+$		451.3 $[M-H]^-$; 435.3 and 183.1 $[Un]^-$	
Nandrolone	N Pr	330.2	331.2 $[M+H]^+$ and 95.0	275.2 $[N+H]^+$; 257.2 $[N+H-H_2O]^+$; 239.2 $[N+H-2H_2O]^+$; 145.1 and 109.0	329.2 $[M-H]^-$ and 73.0 $[Pr]^-$	/
	N Bz	378.2	379.2 $[M+H]^+$ and 105.0		377.2 $[M-H]^-$; 121.0 $[Bz]^-$ and 77.0	
	N PhPr	406.3	407.3 $[M+H]^+$ and 105.0		405.3 $[M-H]^-$ and 149.0 $[PhPr]^-$	
	N Dc	428.3	429.3 $[M+H]^+$; 155.1 ; 95.0 and 81.0		427.3 $[M-H]^-$ and 171.1 $[Dc]^-$	
	N Lr	456.4	457.4 $[M+H]^+$ and 95.0		455.4 $[M-H]^-$ and 199.1 $[Lr]^-$	

Results and Discussion

Fragmentation patterns using ASAP in both positive and negative modes

All steroid esters were analysed independently in both positive and negative scan modes (range from 50 to 500 *m/z*) with a QqQ MS instrument. In order to study the fragmentation of the selected steroid esters, product ion scan experiments on the $[M+H]^+$ and the $[M-H]^-$ ions, at 20 and 30 eV (collision energies) respectively, were performed (Table 1).

In APCI+, the most common fragmentation pattern observed for all the esters was the loss of the ester side chain. Within the same steroid family, several common ions could be observed resulting from the fragmentation of the steroid itself. In $APCI^-$,

the only shared ions were the free steroid with a loss of water for testosterone, estradiol and boldenone; no common ions could be observed within nandrolone esters family. In negative mode, the direct observation of the ester side chains whatever the steroid moiety considered (*e.g*. 59.0 *m/z* for the acetate, 171.1 *m/z* for the decanoate, etc) was found interesting since it immediately informs on the presence of such ester based compounds. Combining negative and positive modes therefore gives access to valuable complementary pieces of information: steroid moiety in positive mode and ester side chains in negative mode.

Development of a simple ASAP workflow to identify steroid esters

It was observed for the 21 compounds of interest a common neutral loss of 96.1 in APCI+ resulting from the following transitions: 271.2 > 175.1 (T base), 269.2 > 173.1 (B base), 257.2 > 161.1 (N base) and 255.2 > 159.1 (E2 base). All steroid esters were analysed with neutral loss scan mode and showed the expected ions (*i.e*. 271.2 *m/z* for T esters, 269.2 *m/z* for B esters, 257.2 *m/z* for N esters and 255.2 *m/z* for E2 esters). Thus, this first approach allowed detecting the presence of steroid esters in just a few minutes. Since no information concerning the identification of the steroid esters was provided, two additional steps were proposed: (i) precursor ion scan mode in APCI+ of the detected specie(s) in order to obtain the mass of the $[M+H]^+$ and (ii) product ion scan mode in APCI- of the corresponding $[M-H]^-$ to identify the ester side chain. The resulting workflow is summarized in Figure 1.

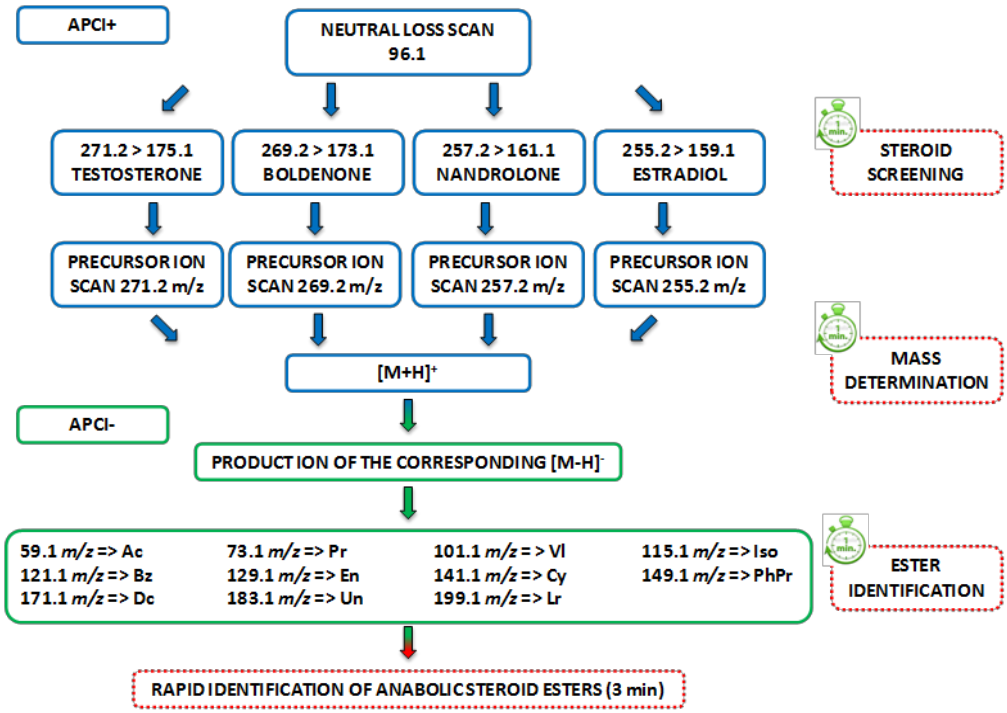

Figure 1. Schematic workflow proposed to identify steroid esters in less than 3 min.

Following such workflow, each steroid ester was identified in only 3 min. Corresponding mass spectra for B Bz are presented in Figure 2. As can be observed, the neutral mass loss spectrum in APCI+ showed only one intense peak at 269.2 *m/z* corresponding to the boldenone moiety with a loss of water. The presence of boldenone in the solution is thus detected. Then, precursor ion scan of 269.2 *m/z* in positive mode allowed detecting $[M+H]^+$ species at *m/z* 391.3 and thus determining the mass of the compound (390.3 g mol^{-1}). Finally, product ion scan in negative mode of the corresponding found molecular mass further allowed ester moiety identification (121.1 *m/z* corresponding to benzoate ester (Bz)).

Identification of steroid esters using DART-HRMS

All steroid esters were analysed separately in high resolution scan mode (R = 60,000 - FWHM at *m/z* 400). External mass calibration appeared as very stable and thus eliminated the need for addition of standards typically required for mass calibration. Corresponding measured mass and mass errors (expressed in ppm) are presented in Table 2. DART-HRMS provided exact mass measurement with a high accuracy since the higher mass error with external calibration was found at 2.8 ppm. CID experiments of the protonated molecules were then carried out to further support steroid ester identification. The two most abundant observed product ions are reported in Table 2.

Applying the DART-HRMS tool to the characterization of the Sustanon solution, four ions were detected in high resolution scan mode, corresponding to $[T\ Pr+H]^+$; $[T\ Iso+H]^+$; $[T\ PhPr+H]^+$ and $[T\ Dc+H]^+$ at 345.2420 *m/z*; 387.2888 *m/z*; 421.2731 *m/z*

and 443.3514 *m/z*, respectively (Figure 4). CID experiments revealed each time the presence of the testosterone moiety through the detection of either $[T+H]^+$; $[T+H-H_2O]^+$ or $[T+H-2H_2O]^+$.

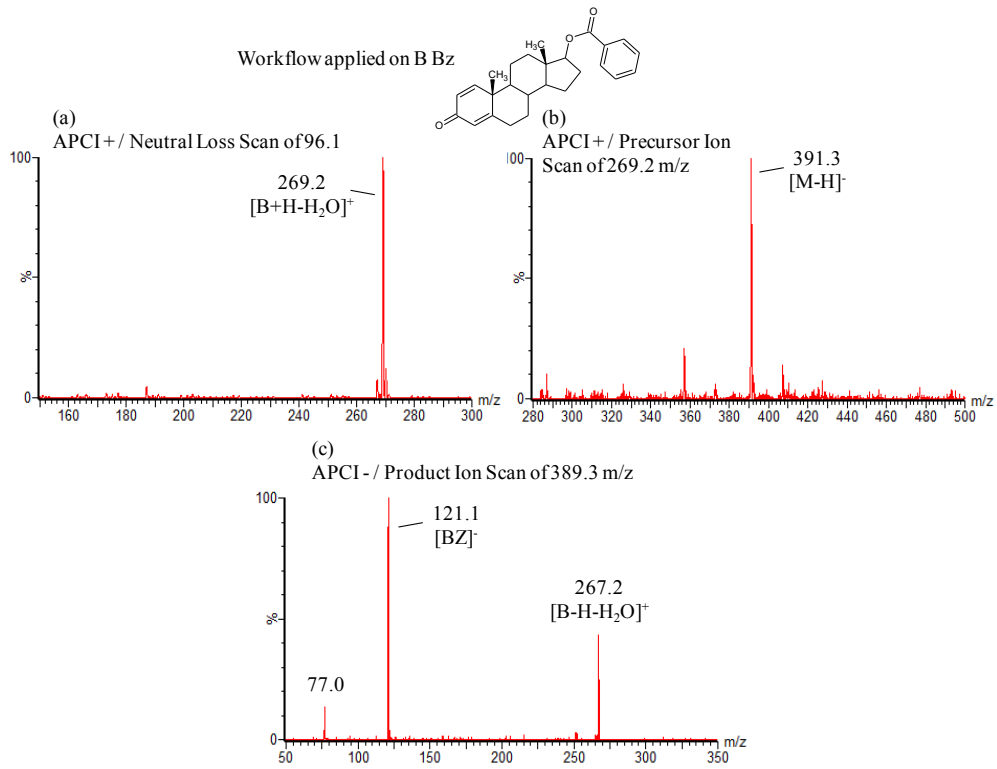

Figure 2. Workflow applied on B Bz at 10 µg mL^{-1} in MeOH. A) Neutral mass loss spectrum of 96.1, B) precursor ion scan mass spectrum of 269.2 m/z, and C) Product ion scan spectrum of 389.3 m/z.

Table 2: High resolution scan mode and collision induced dissociation (CID) experiments for the analysis of the 21 steroid esters. Measured mass and mass error of $[M+H]^+$ and product ions.

		High resolution scan mode		Collision induced dissociation (CID) experiments	
Steroid base	Compound	Measured mass of $[M+H]^+$ (*m/z*)	Mass error (Δ ppm)	Measured mass of product ions (mass error Δ ppm)	Collision energy CID (eV)
Testosterone	T Ac	331.2264	1.2	289.2153 (3.1); 271.2050 (2.4)	15
	T Pr	345.2416	2.3	289.2155 (2.4); 271.2051 (2.2)	15
	T Bz	393.2417	1.9	271.2049 (2.7); 253.1945 (2.4)	20
	T Hp	401.3043	1.7	271.2051 (2.1); 253.1946 (1.9)	20
	T Cy	413.3040	2.6	271.2050 (2.5); 253.1945 (2.4)	20
	T Dc	443.3512	1.8	271.2049 (2.8); 253.1944 (2.6)	15
Estradiol	E2 17Pr	329.2109	0.6	273.1845 (1.4); 255.1743 (0.2)	15
	E2 17Vl	357.2418	1.8	273.1834 (5.6); 255.1750 (-2.7)	20
	E2 3Bz	377.2104	1.9	273.1864 (-5.5); 255.1734 (3.7)	20
	E2 17Hp	385.2733	1.2	273.1864 (-5.3); 255.1733 (4.0)	20
	E2 17Cy	397.2742	-1.1	273.1853 (-1.4); 255.1752 (-3.3)	20
	E2 17Dc	427.3195	2.8	273.1834 (5.5); 255.1748 (-1.8)	15
Boldenone	B Ac	329.2107	1.4	287.1991 (5.0); 269.1887 (4.7)	15
	B Pr	343.2266	0.4	287.1990 (5.5); 269.1885 (5.6)	20
	B Bz	391.2260	2.1	287.1995 (3.8); 269.1889 (4.0)	20
	B Un	453.3354	2.0	287.1994 (4.2); 269.1889 (4.0)	20
Nandrolone	N Pr	331.2264	1.1	257.1899 (0.5); 239.1795 (0.1)	15
	N Bz	379.2262	1.5	257.1919 (-7.3); 239.1785 (4.0)	20
	N PhPr	407.2571	2.4	257.1916 (-6.1); 239.1793 (0.4)	20
	N Dc	429.3357	1.5	257.1888 (4.5); 239.1785 (4.1)	20
	N Lr	457.3668	1.8	257.1888 (4.5); 239.1795(0.0)	20

Application to the analysis of oily preparations

Both ASAP and DART workflows were applied on Sustanon® solution (mixture of T Pr, T PhPr, T Iso and T Dc). As presented in Figure 3 for the ASAP workflow, all testosterone esters could easily be identified according to the developed strategy.

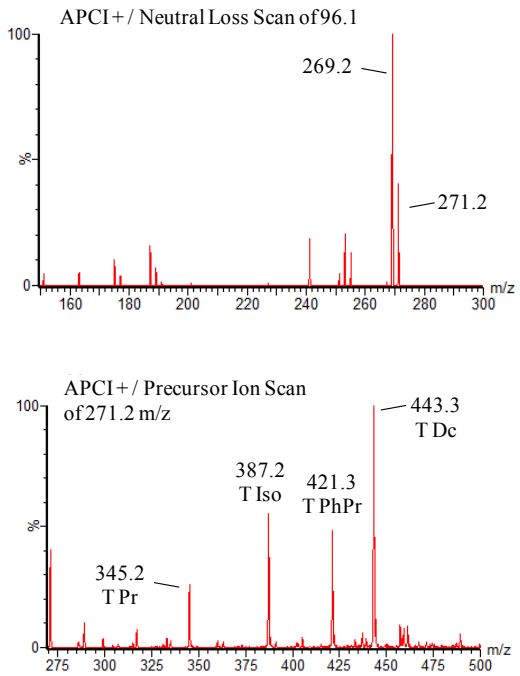

Figure 3: ASAP workflow applied on Sustanon® solution diluted by a factor 1000 in MeOH. (up): Neutral mass loss spectrum of 96.1 m/z. (down): Precursor ion scan mass spectrum of 271.2 m/z.

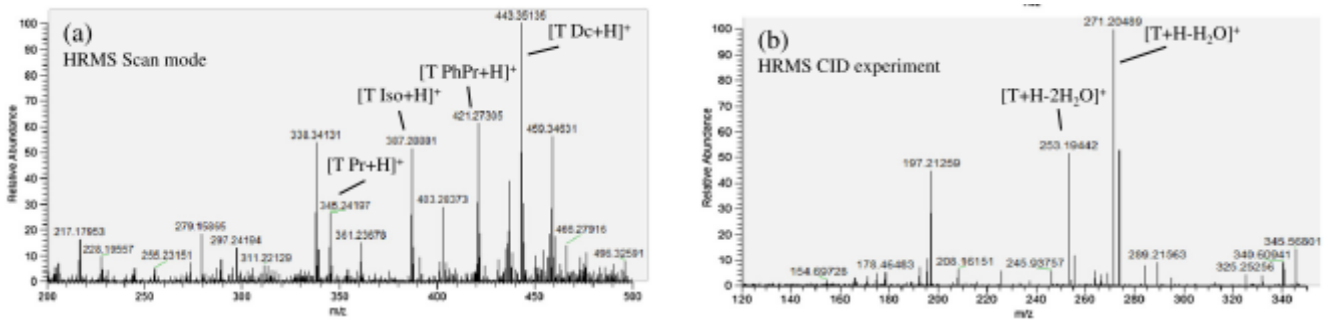

Figure 4. High resolution scan mass spectrum (a) and collision-induced dissociation (CID) of 443.3 m/z mass spectrum (b) for Sustanon oily solution (T Pr: testosterone propionate; T Iso: testosterone isocaproate; T PhPr: testosterone phenylpropionate; T Dc: testosterone decanoate and T: testosterone).

Conclusion

This study demonstrated the benefits of AMS strategies for high throughput identification and quantification of anabolic steroid esters in oily preparations. DART-HRMS further exhibits several advantages as follows: reduced sample size required (below 10 µL); high throughput (analysis of 10 samples in less than 5 min), fully automated process; high mass accuracy of the pseudo-molecular ion useful for determination of formula weight and molecular formula and CID spectrum of the protonated molecules $[M+H]^+$ which provides additional structural information. Quantitative data have been obtained thanks to the use of suited labelled internal standards.

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DETERMINATION AND CONFIRMATION OF SERMS, ANTI-ESTROGENS AND AROMATASE INHIBITORS IN *BOVINE* AND *PORCINE* URINE USING LC-MS/MS

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Abstract

Anti-estrogens and aromatase inhibitors (SERMs) are prohibited in human sports doping. For fattening purposes, they also form a risk in animal husbandry. A method was developed and validated using LC-MS/MS for the determination and confirmation of SERMs, anti-estrogens and aromatase inhibiters in *bovine* and *porcine* urine. This method was used in a survey of more than 200 *bovine* and *porcine* urine samples from Dutch farms. In 18 out of 103 *porcine* urine samples (17%) and 2 out of 114 *bovine* samples (2%) formestane (aromatase inhibitor) was detected. This can be an indication of a possible abuse of formestane, but from human doping control it is known that formestane can in some cases be of natural origin. To discover if the detected formestane in *porcine* and *bovine* urine is from either endogenous or exogenous origin, the samples should be analysed using GC-c-IRMS. Furthermore, studies on the possible endogenous origin in farm animals are urgently needed.

Introduction

Selective estrogen receptor modulators (SERMs) are non-steroidal therapeutic compounds which bind with the estradiol receptors in the body. Dependent of the compound and the target organ, this binding can enhance or reduce the effect of 17ß-estradiol (Dutertre and Smith, 2000). Anti-estrogens are compounds which bind to estradiol receptors in the body and thus block the estradiol binding. Because of this, anti-estrogens reduce the effect of 17ß-estradiol. Aromatase inhibiters inhibit the enzyme aromatase, which is a key enzyme in the production of estradiol in the body.

Since 2010 the World Anti-Doping Agency (WADA) prohibited the use of hormone antagonists and modulators, including SERMs, anti-estrogens (AE) and aromatase inhibitors (AI). The reason for this prohibition is their potential abuse in human sports (boosts free testosterone) and their ability to treat and prevent the side effects produced from anabolic androgenic steroids abuse (Mazzarino *et al*., 2011). There is a chance that abuse in livestock production takes place as well, and a validated method is needed for the detection of these compounds in *bovine* and *porcine* urine. There are, however, no MRL or Reference points of Action (RPA) values for SERMs, anti-estrogens and aromatase inhibiters in *bovine* urine and *porcine* urine.

This paper describes the development and the validation of a LC-MS/MS method for the determination and confirmation of SERMs, anti-estrogens and aromatase inhibiters in *bovine* and *porcine* urine. Furthermore, a survey was performed of more than 200 *bovine* and *porcine* urine samples from Dutch farms.

Materials and Methods

Materials

Methanol (MeOH) and Acetonitrile (ACN) were obtained from Actu-All (Oss, The Netherlands). Sodium hydroxide, acetic acid, formic acid, sodium hydrogen carbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate, tert-butyl methyl ether (TBME) were obtained from Merck (Darmstadt, Germany) and ß-Glucuronidase *E. coli* K12 from Roche (Indianapolis, USA). Potassium carbonate was obtained from Boom BV (Meppel, The Netherlands).

Milli-Q water was prepared using a Milli-Q system with a resistivity of at least 18.2 MΩ cm^{-1} (Millipore, Billerica, MA, USA).

The reference standards anastrozole, exemestane, letrozole, tamoxifene, toremifene, fulvestrant, DL aminoglutethimide, clomiphene, 4 hydroxytamoxifen, raloxifene, formestane, mesterolone and tamoxifen-$^{13}C_2$, ^{15}N were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dromostanolone was obtained from Steraloids (Newport, USA) and androsta-1,4,6-triene-3,17-dione from Carbosynth (Compton,UK). Testolactone and 4-hydroxycyclofenil were obtained from LGC (Teddington, UK) and 1α-methyl-5α-androstan-3α-ol-17-one and 2α-methyl-5α-androstan-3α-ol-17-one were obtained from NMIA (West Lindfield, Australia). Methyltestosteron-D_3 was obtained from EURL (Wageningen, The Netherlands).

Stock solutions were prepared in MeOH at 1,000 mg L^{-1}. Dilutions were prepared in MeOH.

Tamoxifen-$^{13}C_2$ was used as internal standard for tamoxifene, toremifene and clomiphene. Methyltestosteron-D_3 was used as internal standard for all other compounds.

Sample preparation

Sample preparation was based on a method of De Rijke *et al*. (2013) for the detection of selective androgen selective modulators (SARMs) (De Rijke *et al*., 2013). To 2 mL of a urine sample 750 µL 0.2 M Na_2HPO_4 / NaH_2PO_4 buffer was added. The pH was adjusted to 6.5 – 7.5 using dilutions of NaOH or HAc. After addition of 20µL β-glucuronidase *E. coli* K12 the sample was incubated for 1 h at 50°C. To the sample 100 mg of a mixture of K_2CO_3/$NaHCO_3$ (2/1) was added. After vortexing the sample was extracted with 4 mL TBME during 15 min by means of head-over-head. The sample was centrifuged during 15 min at 4,600 *g*. Two mL of the upper layer was evaporate under a gentle stream of nitrogen at 40°C until just dry. The residue was dissolved in 300 µL H_2O /ACN 70/30 and transferred into a LC-MS/MS sample vial.

Table 1. Components and their corresponding compound group

Compound	Compound group	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Raloxifene	SERM	474.1	112.1	30	25
			84.2		40
Tamoxifen	SERM	372.3	72.2	40	13
			129.1		30
Toremifene	SERM	406.1	72.2	40	12
			205.2		22
4-hydroxytamoxifen	SERM	388.2	72.2	40	12
			129.2		25
Fulvestrant	AE	607.3	467.1	40	25
			493.2		25
Clomiphene	AE	406.2	86.2	40	25
			100.2		10
Mesterolone	AE	305.3	269.3	40	15
			95.2		30
Dromostanolone	AE	305.4	269.2	40	15
			95.2		30
4-hydroxycyclofenil	AE	297.2	133.1	30	20
Anastrozole	AI	294.2	225.2	40	35
			209.0		35
Exemestane	AI	297.2	121.1	35	18
			149.1		15
Letrozole	AI	286.2	217.2	25	15
			190.1		35
DL-aminogluthimide	AI	233.2	188.1	40	15
			146.1		25
Formestane	AI	303.2	113.1	45	25
			173.1		18
androsta-1,4,6-triene-3,17-dione	AI	283.2	97.2	40	15
			265.1		10
testolactone	AI	301.0	121.1	30	10
			147.1		10
1α-methyl-5α-androstan-3α-ol-17 one**	AAS*	287.3	269.2	30	10
			161.1		15
2α-methyl-5α-androstan-3α-ol-17 one***	AAS*	287.3	269.2	30	10
			161.1		15

AAS: anabolic androgenic steroids, **: metabolite of mesterolone, *: metabolite of dromostanolone*

LC-MS/MS analysis

The LC-system consisted of a Waters autosampler and Acquity binary pump equipped with a Acquity UPLC BEH analytical column of 100 x 2.1 mm, 1.7 µm at a temperature of 60°C. Gradient elution was used with mobile phase 5 mM HCOOH (A)

and 5 mM HCOOH in ACN (B) at a flow rate of 0.6 mL min^{-1}. After injection of 5µL the mobile phase was hold at 100% A during 0.5 min followed with a gradient in 7.5 min to 100% B and back to 100% A after a hold of 1 min at 100% B. The total run time of the analysis was 12 min.

Detection was carried out using a Waters Xevo TQ-S mass spectrometer in the positive mode. The MS setting were: capillary voltage 3.0 kV; source temperature 150°C; desolvation temperature 600°C; cone gas flow 150 L h^{-1}; desolvation gas flow 600 L h^{-1}.

The SERMs, anti-estrogens and aromatase inhibiters were fragmented using argon as CID gas. The selected reaction monitoring transitions are given in Table 1 with the used cone voltage and collision energy settings.

Method validation

A full validation was carried out according to Commission Decision 2002/657/EC. The following parameters were determined: linearity, trueness, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCß), selectivity, robustness and stability. The validation was carried out on three different days with 21 different *bovine* urines and additionally one day with 7 different *porcine* urines. A summary of the validation results for *bovine* and *porcine* urine is given in Tables 2 and 3.

Table 2. Bovine *urine, summary of validation results*

Component	Detect from µg L^{-1}	Quantification from µg L^{-1}	Confirmation from µg L^{-1}	CCα	CCß
Anastrozole		1.25	1.25	0.85	1.71
Exemestane	5.0	10.0	5.0	7.0	14.0
Letrozole	1.25		1.25	<1.25	<1.25
Raloxifene	0.05			<0.05	<0.05
Tamoxifen		1.25	1.25	0.45	0.90
Toremifene		2.5	2.5	2.37	4.74
Fulvestrant	5.0		5.0	<5.0	<5.0
DL-Aminoglutethimide	2.5		2.5	<2.5	<2.5
Clomiphene (total)**		1.25	1.25	0.82	1.63
4-Hydroxytamoxifen peak 1*	0.625	1.25	0.625	<0.0625	<0.0625
4-Hydroxytamoxifen peak 2 *	0.625	1.25	0.625	<0.0625	<0.0625
Formestane	12.5			<12.5	<12.5
Mesterolone	12.5			<12.5	<12.5
Dromostanolone	12.5			<12.5	<12.5
Androsta-1,4,6-triene-3,17-dione		12.5		11.0	23.8
Testolactone		12.5		10.9	21.8
4-Hydroxycyclofenil (1 ion)	50			<50	<50
1α-methyl-5α-androstan-3α-ol-17 one	25	50		<25	<25
2α-methyl-5α-androstan-3α-ol-17 one	25			<25	<25

**= not known which peak cis or trans. Distribution of cis/trans in standard is 50/50; ** = cis and trans are not separated.*

Survey

To gain insight in the current situation of possible abuse of SERMs, anti-estrogens and aromatase inhibitors a survey was performed. For this survey 114 *bovine* urines and 103 *porcine* urines from Dutch farms were analysed using the method described above. In the *bovine* urine samples no SERMs, anti-estrogens and aromatase inhibitors were detected. In 18 of the *porcine* urine samples and 2 of the *bovine* samples formestane, an aromatase inhibitor was detected with indicative levels from 15 to 72 µg L^{-1}. Because no confirmation and quantification was possible with the LC-MS/MS method, a selection of the samples was analysed using GC-MS/MS. In the selected samples (n=3) formestane was confirmed according 2002/657/EC and quantification was performed using an external calibration curve of formestane. Results of the three selected samples are shown in Table 4.

From studies with human urine it is known that formestane can be of endogenic origin (De la Torre *et al*., 2014; Piper *et al*., 2012; Polet *et al*., 2013) and thus can be found at low levels in human urine. Limited studies show exogenous formestane detection in sports horse urine (Leung *et al*., 2013), but no literature is published of formestane findings in *bovine* or *porcine* urine. Studies to answer this question will have to be undertaken. Subsequently, in order to assess whether the formestane found in the *porcine* urine samples is endogenous or exogenous, the urine samples should be analysed using a suitable GC-c-IRMS method.

Table 3. Porcine urine, summary of validation results

Component	Detect from µg L^{-1}	Quantification from µg L^{-1}	Confirmation from µg L^{-1}	CCα	CCß
Anastrozole		1.25	1.25	0.72	1.43
Exemestane		5	5	3.47	7.47
Letrozole		1.25	1.25	0.92	1.83
Raloxifene	0.05		0.05	<0.05	<0.05
Tamoxifen		1.25	1.25	0.44	0.89
Toremifene		2.5	2.5	1.49	2.98
Fulvestrant	5		5	<5.0	<5.0
DL-Aminoglutethimide	2.5			<2.5	<2.5
Clomiphene (total)**	1.25	3.75		<1.25	<1.25
4-Hydroxytamoxifen peak 1*	0.625		0.625	<0.625	<0.625
4-Hydroxytamoxifen peak 2 *	0.625		0.625	<0.625	<0.625
Formestane	12.5			<12.5	<12.5
Mesterolone	12.5			<12.5	<12.5
Dromostanolone	12.5		25	<12.5	<12.5
Androsta-1,4,6-triene-3,17 dione	12.5		12.5	<12.5	<12.5
Testolactone	12.5		12.5	<12.5	<12.5
4-Hydroxycyclofenil (1 ion)***					
1α-methyl-5α-androstan-3α-ol-17 one	25		50	<12.5	<12.5
2α-methyl-5α-androstan-3α-ol-17 one	25		25	<12.5	<12.5

= not known which peak cis or trans. Distribution of cis/trans in standard is 50/50; ** = cis and trans are not separated; *= due to interference determination not possible.*

Table 4. Porcine urines samples with (indicative) amount of formestane and confirmation with GC-MS/MS.

Sample	LC-MS/MS indicative amount (µg L^{-1})	GC-MS/MS (µg L^{-1})
1	17	9
2	72	33
3	61	24

Conclusions

An LC-MS/MS method was developed for the determination and confirmation of selective estrogen receptor modulators (SERMs), anti-estrogens and aromatase inhibitors in *bovine* and *porcine* urine. Additionally, the method was validated according Commission Decision 2002/657/EC. A survey on more than 200 Dutch *bovine* and *porcine* urine samples was performed to gain insight in the current situation of possible misuse of the compounds in Dutch animal husbandry. In 18 out of 103 *porcine* urine samples (17%) and 2 out of 114 *bovine* samples (2%) formestane (aromatase inhibitor) was detected. Studies on the possible endogenous origin are urgently needed.

Acknowledgements

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ABUSE OR CONTAMINATION? RATIO DETERMINATION OF CLENBUTEROL ENANTIOMERS TO DISTINGUISH BETWEEN DOPING USE AND MEAT CONTAMINATION

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Abstract
Clenbuterol has been detected in urine of several athletes during routine doping control in the past years. Athletes claimed that the clenbuterol was ingested by eating contaminated meat. Providing analytical prove for this claim is difficult. Clenbuterol consists of two enantiomers, *R*(-)- and *S*(+)-clenbuterol. Currently available human and veterinary preparations were found to consist of a racemic mixture of both enantiomers. When clenbuterol is administered to animals this ratio may change in meat and other edible products of the animal. It is hypothized that consumption of contaminated meat with an altered ratio will also change the ratio in the urine of the athlete. To prove this a controlled human trial was performed in which subjects were administered clenbuterol by ingestion of drug preparations or by eating contaminated meat and liver. Urine samples of the subjects were collected and analyzed with chiral LC-MS/MS and SFC-MS/MS methods to measure the ratio of *R*(-)- and *S*(+)-clenbuterol. Results show a *S*-clenbuterol proportion in the urine samples of the volunteers dosed with drugs and meat of around 0.50. This is comparable with the proportion *S*-clenbuterol in the drug preparations and administered meat itself. The urine samples of the volunteers who ate liver on the other hand are *S*-clenbuterol enriched which is consistent with the ingested liver samples itself. The results of the study show a potential distinction between human urine in which clenbuterol is detected due to drug administration (doping) and contaminated animal liver products.

Introduction
In recent years clenbuterol, a prohibited substance in sports, was detected during routine doping controls in urine of several athletes (Thevis *et al*. 2013). The majority of them claimed clenbuterol came into their body via consumption of meat which was contaminated with clenbuterol. Gaining solid proof of this contamination is difficult.

Clenbuterol is a chiral molecule existing as two enantiomers, *R*(-)- and *S*(+)-clenbuterol. Commercially available human and veterinary preparations consist of a racemic mixture of both enantiomers (Waldeck and Widmark, 1985). Literature shows that this ratio may change in meat when clenbuterol is administered to animals (Smith 2000). After application of racemic clenbuterol to chicken and swine, *S*-clenbuterol was found to be enriched in corresponding animal tissues. Also, according to documentations, pharmaceutical preparations are prepared with racemic clenbuterol. Ingestion of different enantiomeric compositions may lead to different enantiomeric compositions of clenbuterol in athlete's urine samples and therefore allow for a distinction between the consumption of contaminated meat and the illegal administration of drugs (Sterk *et al*. 2011, Parr *et al*. 2013).

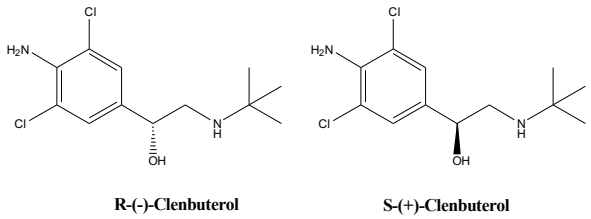

Figure 1. Chemical structures of R-(-)-Clenbuterol and S-(+)-Clenbuterol.

The hypothesis of the project is that when consuming clenbuterol-contaminated meat (ratio ≠ 1:1), the ratio of the clenbuterol enantiomers in the human urine also changes. In this way a distinction can be made between clenbuterol administered by preparation and clenbuterol that has entered the body due to consumption of contaminated meat. This was investigated by analysing contaminated meat samples, as well as urine samples from volunteers who consumed clenbuterol contaminated meat, liver or clenbuterol preparations. Analysis was performed using a chiral separation by LC-MS/MS and SFC-MS/MS. Results are presented in this contribution.

Materials and Methods

Animal trial

An animal trial (EC2012-14) was performed in which two calves were treated with the veterinary drug Ventipulmin® (racemic clenbuterol) for 22 days through oral administration. The dose of the administration for both animals was 10 µg kg^{-1} body weight per day. The calves were slaughtered and meat and liver samples were collected and sealed in edible portions. One of the liver samples and one of the meat samples were analysed to determine the proportion of *S*-clenbuterol.

Human trial

A controlled administration trial (KEBN-14-1-DK) was organised in which four male human subjects per administration group participated. Clenbuterol was administered as pharmaceutical drug via contaminated meat or liver (non-racemic clenbuterol). The drug which was used was Spiropent® which consists of racemic clenbuterol. It was administered orally. Meat was minced, prepared as meatballs and grilled. Liver was cut into smaller pieces and grilled. Meat and liver was eaten by the subjects. Multiple urine samples were collected daily before and up to eight days after administration of the drug or eating meat and liver.

Table 1: Number of human subjects which participated in the human trial. Subject number, type of treatment, concentrations in meat and liver and total administered concentrations of the different treatments are show.

Source of Clenbuterol	Number of subjects	Subject # in trial	Concentration of clenbuterol	Administered total amount of clenbuterol	Proportion *S*-clenbuterol
Muscle meat	4	1; 5; 9; 10	1.7 µg kg^{-1}	0.84 µg	0.51
Liver	4	3; 4; 7; 11	42 µg kg^{-1}	8.4 µg	0.64
Spiropent® tablet	4	2; 6; 8; 12	20 µg $tablet^{-1}$	20 µg	0.50
R-clenbuterol reference standard	1	13	1 µg mL^{-1}	10 µg	0.02
S-clenbuterol reference standard	1	14	1 µg mL^{-1}	10 µg	0.98

Analysis of urine, meat and liver

Meat samples were prepared for analysis by extracting the sample with acetonitrile followed by solid phase extraction (SPE). Urine samples were prepared for analysis by extraction on an Extrelut® NT-3 column. In both methods the dried extracts were reconstituted with 100% methanol before analysis.

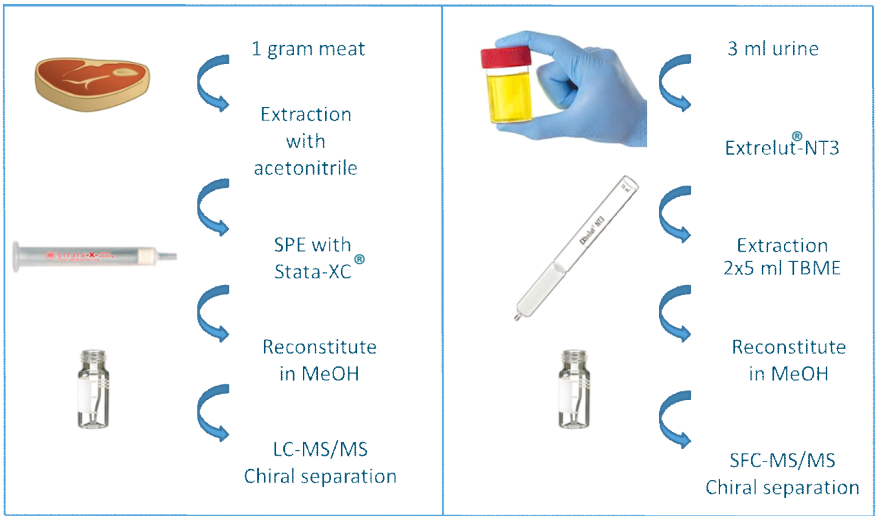

Figure 2. Scheme of meat and urine sample preparation.

Instrumentation

The analysis of the preparations, urine, meat and liver samples were all carried out with an Astec Chirobiotic V2 chiral analytical column (Supelco, Sigma-Aldrich, Deisenhofen, Germany). For preparation, meat and liver analysis: 150 mm x 2.1 mm; 5 µm particle size. For urine analysis: 150 mm x 4.6 mm; 5 µm particle size.

The preparations were analysed with a Waters ACQUITY UPLC in combination with a Waters PDA detector directly followed by a Waters SYNAPT G2-S QTOF MS detector. LC-Conditions: 0.5 mL min^{-1} of isocratic methanol, triethylamine, acetic acid (99.98: 0.013: 0.007, v:v:v) at 30°C. Fixed wavelength UV-detection at 244 nm for Clenbuterol and 292 nm for the internal standard propranolol.

The urine samples were analysed with a Waters ACQUITY UPC² in combination with a Waters TQD triple quadrupole MS detector. LC conditions: 2 mL min^{-1} of CO_2 with addition of modifiers of 0.1% NH_3 (25%, aq.) and 0.1% formic acid in methanol with a linear gradient of 50% to 60% of modifier in 4 min at 50°C. The MS was operated in MRM mode performing the following experiments: 277.2→203.2; 277.2→168.1; 277.2→132.1, and for the internal standard d_9-Clenbuterol 286.2→ 204.1 using a cone voltage of 30V, a collision energy of 17V, and a capillary voltage of 3.0 kV. Argon was used as collision gas at 0.25 mL min^{-1}, and nitrogen as cone gas (50 L h^{-1}) and desolvation gas (700 L h^{-1}).

The meat and liver samples were analysed with a Waters ACQUITY UPLC in combination with a Waters Xevo TQ-S MS detector. LC conditions: 0.5 mL min^{-1} of isocratic methanol, 2.5 mM ammonium formate at 40°C.

The MS was operated in MRM mode performing the following experiments: 277.2→203.2 (15 V); 277.2→168.1 (30 V); 277.2→132.1 (30 V) and for the internal standard d_6-clenbuterol 283.2→204.2 (15 V) (respective collision energies in brackets). Cone voltage was 20 V and capillary voltage 3.0 kV. Argon was used as collision gas at 0.18 mL min^{-1}, and nitrogen as desolvation gas (600 L h^{-1}) and cone gas (150 L h^{-1}).

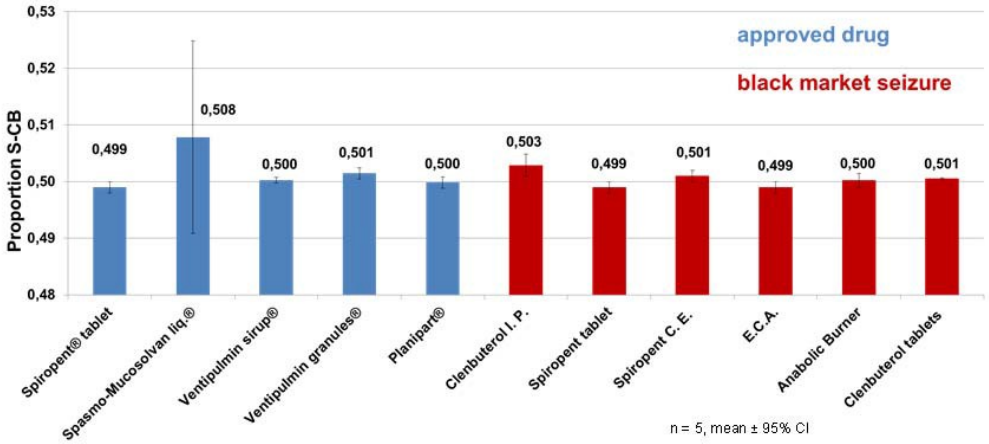

Figure 3. Proportion of S(+)-Clenbuterol in registered pharmaceutical preparations and 'black market' obtained preparations

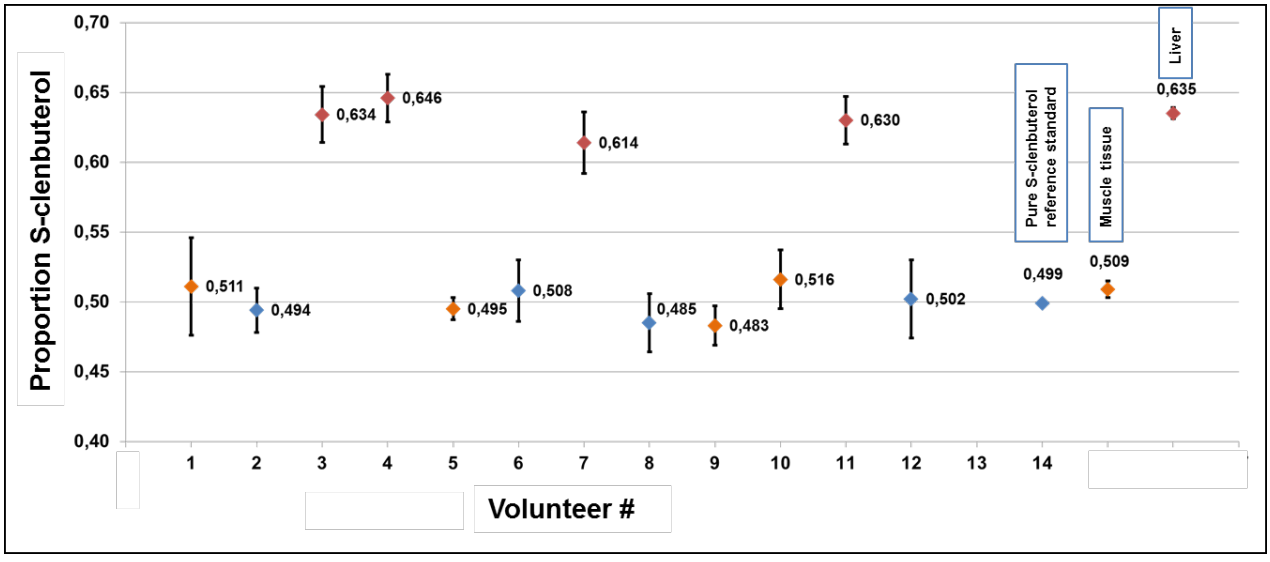

Figure 4. Proportion of S-clenbuterol in the urine samples of the subjects (1-12), pure reference standards (13-14), the contaminated muscle tissue and the contaminated liver.

Results and discussion

Different preparations were analysed to verify the proportion *S*-clenbuterol. Five approved pharmaceutical preparations were tested and six preparations from the black market were tested. The samples were analysed in five-fold on a UHPLC-UV system. Figure 3 shows the proportion *S*-clenbuterol in the analysed pharmaceutical preparations. All preparations, approved as well as 'black market', show a proportion *S*-clenbuterol around 0.50, so no enantiomer enrichment has been found in preparations.

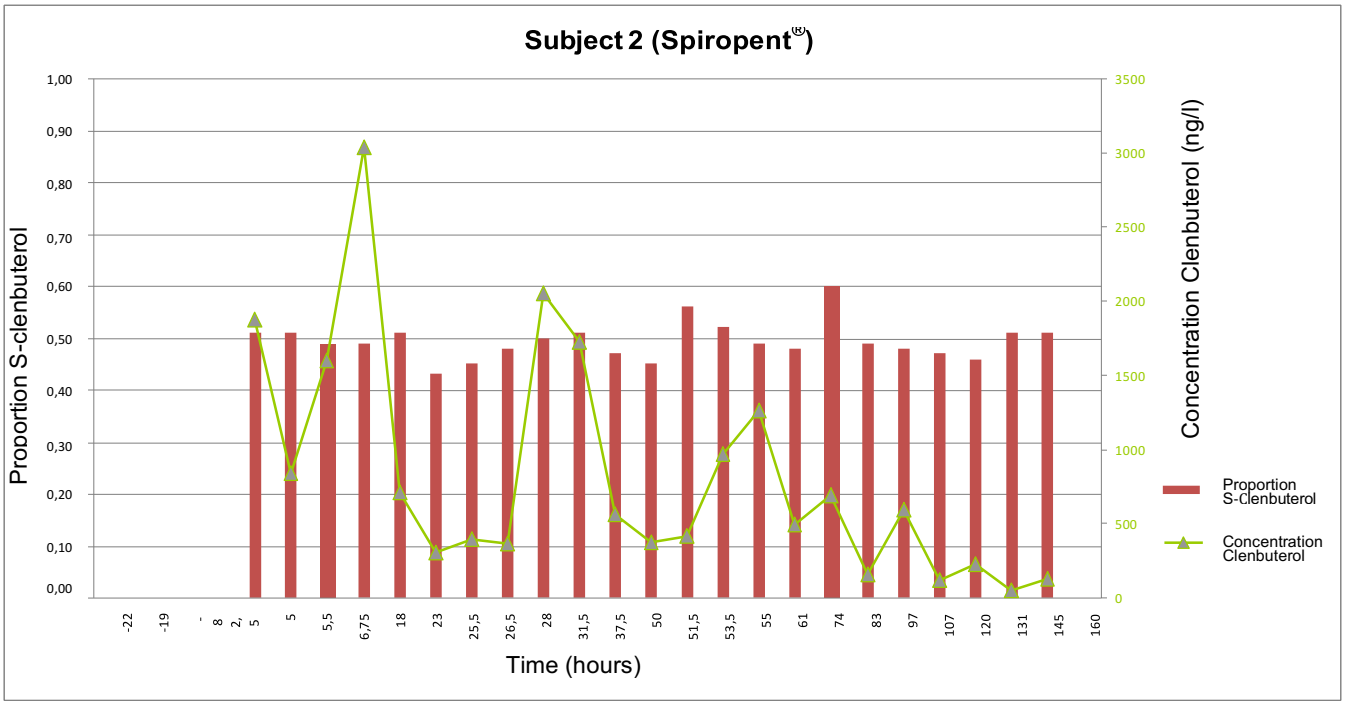

Figure 5. Excretion curve of a volunteer who consumed Spiropent®. The concentration of clenbuterol as well as the proportion S-clenbuterol is plotted against the time after administration in hours.

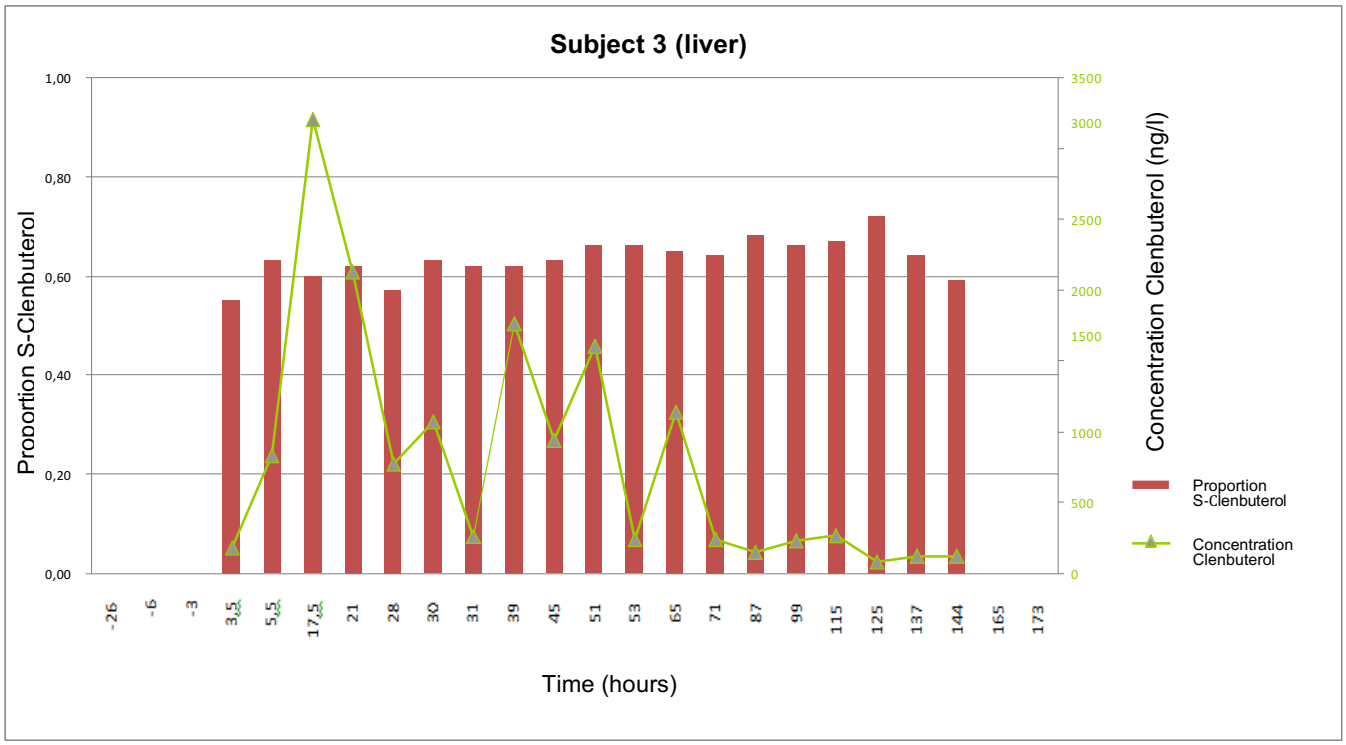

Figure 6. Excretion curve of a volunteer who consumed incurred clenbuterol liver. The concentration of clenbuterol as well as the proportion S-clenbuterol is plotted against the time after administration in hours.

Figure 4 shows the proportions *S*-clenbuterol in the analysed urine samples of the volunteers (1 to 14, Table 1) as well as the incurred muscle tissue and incurred liver. The urine samples of the subjects which were administered clenbuterol via preparation or via the meat samples resulted in *S*-clenbuterol proportions around 0.50. This is consistent with the found S-clenbuterol proportions in the preparations (see Figure 3) and the administered meat (proportion *S*-Clenbuterol = 0.51, see Figure 4). The urine samples of the subjects which consumed clenbuterol incurred liver gave *S*-clenbuterol proportions around 0.63, which is consistent with the S-clenbuterol proportions of the liver sample itself (see Figure 4).

Excretion curves of all subjects were constructed. Figures 5 and 6 show excretion curves of two volunteers as example: one to whom Spiropent® tablet was administered (Figure 5) and one by consuming incurred liver (Figure 6). The proportions *S*-clenbuterol in the urine samples of the volunteer who consumed Spiropent® shows values of around 0.50, which indicates no enrichment. The proportions *S*-clenbuterol in the urine samples of the volunteer who consumed incurred liver showed values of around 0.60, which indicates a *S*-clenbuterol enrichment.

Conclusions

Analysis of approved as well as of 'black market' preparations containing clenbuterol confirmed that they consist of a racemic mixture of *R*(-)- and *S*(+)-clenbuterol. This implicates that hitherto no *R*(-)- or *S*(+)-enriched clenbuterol is available on the market.

An animal experiment was performed in which two calves were administered Ventipulmin®, a clenbuterol containing preparation. Meat and liver of the calves were collected and used for a controlled human administration trial. Both meat and liver contained clenbuterol. Liver was enriched with *S*-clenbuterol, meat samples did not show enrichment for *S*-clenbuterol. Urine of the human subjects before and after consumption was collected and analysed for *R*(-)- and *S*(+)-clenbuterol. Clenbuterol was found in the urine samples of all subjects who consumed liver, meat or preparations. No *S*-clenbuterol enrichment was found in the urine of the preparation volunteers and the urine of the meat-consuming volunteers. However, the urine of the volunteers who consumed liver showed enriched *S*-clenbuterol. From this study it can be concluded that when consuming liver from animals treated (illegally) with clenbuterol enriched in *S*-clenbuterol, the enantiomeric composition in human urine samples changes compared with that found after administration as racemic source. A distinction can be made between the origin of the clenbuterol in the urine of athletes with clenbuterol either originating from pharmaceutical preparation (doping) or from consumption of contaminated animal liver products with altered composition. It is also shown that the proportion *S*-clenbuterol is stable during the excretion phase and does not change due to elimination of clenbuterol.

Acknowledgements

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PROFICIENCY TEST VETERINARY DRUGS: IMPLEMENTING THE ZSAR METHOD

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Abstract

DUCARES organizes proficiency tests (PT) in the food and feed area. The PT program exists since 1988, is ISO/IEC: 17043 accredited and has veterinary drugs proficiency tests at three levels: carry-over, additive and high. Most PT providers do not calculate the performance of participants that report results that are below the limit of quantification (LOQ), the so-called truncated results (< results). ISO:17043 states "If proficiency testing provider issues statements of participation or performance, they shall contain sufficient information to not be misleading." Therefore, PT providers are stimulated by accreditation bodies to provide information about the participant's performance for truncated results.
The new ISO standard 13528:2015 describes three options how to deal with truncated results in statistical calculations: removal of truncated values, retaining the truncated values but removal of the '<' sign and replacing the truncated results with half of the limit value. Although truncated results were removed from the statistical dataset, it is possible to give a performance indication with z-score ranges. The Z-Scores Arrow Range (ZSAR)-method is based on a z-score ranging between the lowest possible (result = zero) and highest possible z-score (limit value). The ZSAR results are shown graphically, with arrows in the distribution plot.

Introduction

From the 1980s on more and more strict requirements for product quality have caused the demand for laboratory testing to increase. Reliable laboratory testing with unambiguous results is of crucial importance. In addition, open economic borders within the European Union have, since 1992, led to increasing competition between laboratories. Proficiency tests are an important tool to evaluate the performance of laboratories.

From the view of public health and food safety, it is essential to obtain reliable information regarding the quality of food and feed and the presence of additives, contaminants and banned substances. DUCARES (Utrecht, NL) organizes proficiency tests (PT) in the food and feed area worldwide. Their PT scheme is ISO/IEC 17043 [1] accredited and was formerly known as the Agricultural Laboratories Quality Service (KDLL) scheme of the Product Board of Animal Feed (PDV). Nowadays DUCARES is appointed by the Dutch government to conduct PT accreditation program manure 'AP05', organises regular, bilateral and custom-made schemes, member of (inter)national working groups (NEN, Eurachem, Securefeed and GMP^{+}) and is recognized by RvA, QS, CNAS and NATA. To ensure a good comparability between laboratories, participants may use recommended methods in the DUCARES PT scheme, although the use of other (in-house) methods is similarly allowed.

In this proceeding, the z-score arrow ranges (ZSAR) method is implemented in the proficiency testing 'Veterinary drugs'. The ISO/IEC 17043 standard states that "if the proficiency testing provider issues statements of participation or performance, they shall contain sufficient information to not be misleading" [1].

Most PT providers, however, do not calculate the performance of participants who report results in the form "<x" to indicate that the result is below the limit of quantification (LOQ), so-called truncated results ("< results"). On the other hand, the providers are stimulated by accreditation bodies to provide information about the performance of participants who report truncated results.

Materials and Methods

ZSAR Method

The z-score range between the lowest possible z-score, z_{min}, derived from a result of 0 and the highest possible z-score, z_{max}, are calculated using the truncated result (x) reported by the laboratory in:

$z_{max} = (x - x^*) / s^*$

$z_{min} = -x^* / s^*$

Where x^* is the robust mean and s^* is the robust standard deviation of the participants' results [4]. In the distribution plot, the interval between z_{min} and z_{max} is highlighted by a double-headed arrow.

The information provided by the ZSAR method [2] gives the participating laboratory a good indication whether its truncated result comprises satisfactory score ($|z| \leq 2$) or is outside acceptability ($|z| > 2$), so that, if necessary, the participant can take corrective actions.

The ZSAR method was compared with the ISO 13528 [3] concerning truncated results. The ISO 13528 standard [3] describes three approaches for analysis of censored data:

a) Removal of truncated values

b) Retaining the values x after removal of the '<' sign

c) Replacing the truncated results with half of the limit value (x/2).

The examined example dataset (salinomycin in pig feed) contained results of two laboratories that provided truncated results.

Results and Discussion

The example dataset of salinomycin in pig feed (Table 1) was evaluated with the ZSAR method in view of three approaches of truncated results (<x) according to ISO 13528. This dataset contains two truncated results (<x).

Table 1. Evaluation of z-scores of participants' results of a PT on salinomycin in pig feed, with the ZSAR method in view of three approaches of truncated results (<x) according to ISO 13528.

PT scheme	DUCARES		ISO 13528		ISO 13528		ISO 13528	
Laboratory	ZSAR ($z_{min} \leftrightarrow z_{max}$)		removal of "<x" values		removal "<" sign		half the limit value	
no.	Result reported	*z*-score	Result	*z*-score	Result	*z*-score	Result	*z*-score
122	3.07	-0.6	3.07	-0.6	3.07	-0.6	3.07	-0.6
197	3.11	-0.4	3.11	-0.4	3.11	-0.3	3.11	-0.3
209	3.2	0.3	3.2	0.3	3.2	0.3	3.2	0.3
223	<6.0	-21.4 : 19.2			6.0↕	19.1	3.0	-1.0
258	3.192	0.2	3.192	0.2	3.192	0.3	3.192	0.3
301	3.325	1.1	3.325	1.1	3.325	1.2	3.325	1.2
343	3.123	-0.3	3.123	-0.3	3.123	-0.2	3.123	-0.2
372	<3.0	-21.4 : -1.1			3.0	-1.0	1.5	-11.1
379	3.14	-0.2	3.14	-0.2	3.14	-0.1	3.14	-0.1
411	2.99	-1.2	2.99	-1.2	2.99	-1.1	2.99	-1.1
415	3.37	1.4	3.37	1.4	3.37	1.5	3.37	1.5
423	3.25	0.6	3.25	0.6	3.25	0.6	3.25	0.6
461	2.85	-2.1	2.85	-2.1	2.85	-2.0	2.85	-2.0
466	2.662↕	-3.4	2.662↕	-3.4	2.662↕	-3.3	2.662↕	-3.3
553	3.384	1.5	3.384	1.5	3.384	1.6	3.384	1.6
581	3.159	0.0	3.159	0.0	3.159	0.0	3.159	0.0
595	3.24	0.5	3.24	0.5	3.24	0.6	3.24	0.6
604	2.97	-1.3	2.97	-1.3	2.97	-1.2	2.97	-1.2
635	3.33	1.1	3.33	1.1	3.33	1.2	3.33	1.2
667	3.06	-0.7	3.06	-0.7	3.06	-0.6	3.06	-0.6
n	*17*		*17*		*18*		*18*	
*x**	*3.163*		*3.163*		*3.153*		*3.153*	
*s**	*0.148*		*0.148*		*0.149*		*0.149*	

The z-score ranges of the truncated results are given as lowest z-score (z_{min}) ↔ highest z-score (z_{max}). All results are expressed in mg kg^{-1}.
x is the robust mean and s* the robust standard deviation, according to ISO 5725-2 [4].*
↕ *result is an outlier.*

Although the robust mean (x*) and the robust standard deviation (s*) for all the approaches are comparable, the contribution comes from different results, caused by outlier results of the various approaches.

The ZSAR method shows that for two laboratories (223 and 372) the *z*-score ranges comprise intervals of satisfactory, questionable and unsatisfactory scores. The truncated result (<3.0 mg kg^{-1}) of laboratory 372 is close to the robust mean (3.16 mg kg^{-1}).

The truncated results for laboratory 223 generates contradictory z-scores, an unsatisfactory score of 19.1 (removal "<" sign) against a satisfactory score of -1.0 (half the limit value).

The truncated results for laboratory 372 generates contradictory z-scores, a satisfactory score of -1.0 (removal "<" sign) against an unsatisfactory score of -11.1 (half the limit value).

The ISO13528 approaches provide contradictory results, it may be possible that unsatisfactory truncated results will not be noticed by the laboratory. In this case the laboratory may not take corrective actions, whilst the graphical ZSAR method may trigger the participant to review their performance and their underlying methods.

A typical example of the ZSAR method distribution graph of salinomycin in pig feed is presented in Figure 1.

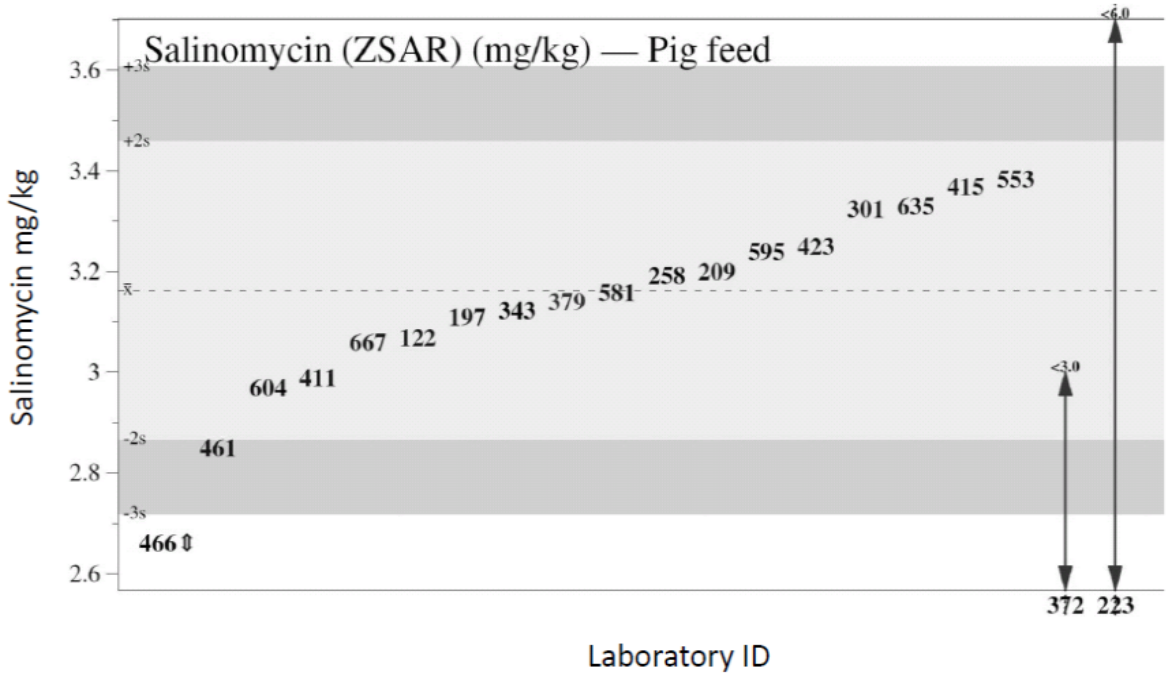

Figure 1. Salinomycin (mg kg^{-1}) in pig feed, a graphically presentation of the ZSAR method.

For example, for laboratory 372 one may be tempted to conclude that the truncated value probably indicates a satisfactory result below LOQ near the robust mean. The ZSAR range shows however that this is the case, but the ISO 13528 'half the limit value' approach does not.

The laboratory 223 *z*-score ranges between satisfactory and unsatisfactory. This laboratory should take corrective actions because their LOQ is almost two times higher than the robust mean and must examine the results carefully.

Conclusions

The ISO13528 approaches could provide contradictory results in contrast to the ZSAR graphical method which provide clear, unambiguous and indicative z-scores in such cases and may be an additional and valuable approach besides those in the standard ISO 13528.

It can be concluded that the z-scores of truncated results must be studied carefully when applying ISO 13528 methods. In certain cases, the ISO 13528 approaches may result in misleading z-scores for truncated results.

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GAS CHROMATOGRAPHY-MASS SPECTROMETRY/COMBUSTION/ISOTOPE RATIO MASS SPECTROMETRY (GC-MS/C/IRMS) AS A CONFIRMATORY ANALYSIS FOR THE ADMINISTRATION OF NATURAL STEROID HORMONES IN *BOVINES*

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Abstract
The use of steroid hormones as growth-promoting agents in stock farming is prohibited in the European Union. However, when it comes to synthetic analogues of endogenously produced steroid hormones, detection of abuse remains difficult. A method to differentiate between endogenous androgens and estrogens, and synthetic analogues thereof, in *bovine* urine samples, was developed, using gas-chromatography coupled to both mass-spectrometry and combustion/isotope ratio mass spectrometry in parallel (GC-MS/C/IRMS). 5-Androstene-3β,17α-diol (AEdiol) was used as the endogenous reference compound (ERC), 17α-estradiol (αE2) as the estrogen metabolite, and 17α-testosterone (αT) and etiocholanolone (Etio) as the androgen metabolites. The difference between the $\delta^{13}C_{VPDB}$ values of the ERC and the metabolites, allows to differentiate between treated and untreated animals. The results obtained after application of this method for official control purposes since 2014, are used to extend and evaluate the initially performed validation study. Finally, a progesterone metabolite, 5β-pregnane-3α,20α-diol (PD), was added to the existing method, to include the investigation of abuse of progestagens as well. Unexpectedly, the analyses of samples from *bovines* treated with esters of testosterone and estradiol revealed an impact of the treatment on the $\delta^{13}C_{VPDB}$ values of PD.

Introduction
The use of growth-promoters in stock farming is strictly prohibited within the European Union, according to Council Directive 96/22/EC. As a result, monitoring the abuse of steroid hormones in large scale surveillance programs for food safety reasons is mandatory for all member states, and the analytical guidelines are laid down in Commission Decision 2002/657/EC. However, these monitoring programs are based on the use of either GC-MS or LC-MS, which does not allow the unambiguous detection of synthetic analogues of naturally occurring steroid hormones in urine.

In the late 90's, it became clear that using gas-chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), it is possible to elucidate the origin of these compounds in *bovine* urine samples based on the measured $^{13}C/^{12}C$ ratio, expressed as $\delta^{13}C_{VPDB}$ values (Ferchaud *et al.*, 1998). To be able to detect the abuse, both endogenous reference compounds (ERCs) and metabolites of the targeted steroid hormones are measured. Since the ERC is not influenced by the targeted compounds, a substantial $\delta^{13}C_{VPDB}$ difference between ERC and metabolite, the $\Delta^{13}C_{VPDB}$ value (‰), can be measured. However, for food safety purposes, this technique has only been applied scarcely until now due to the extensive sample preparation and specialized equipment required for this analysis (Buisson *et al.*, 2005; Hebestreit *et al.*, 2006; Janssens *et al.*, 2013).

To monitor the abuse of synthetic analogues of endogenous androgens and estrogens in Belgium, gas-chromatography coupled to both mass-spectrometry and combustion/isotope ratio mass spectrometry in parallel (GC-MS/C/IRMS) has been used under accreditation since the beginning of 2014, measuring 5-androstene-3β,17α-diol (AEdiol) as the (ERC), 17α-estradiol (αE2) as the estrogen metabolite, and 17α-testosterone (αT) and etiocholanolone (Etio) as androgen metabolites in *bovine* urine samples. However, no methods are currently available to monitor abuse of synthetic analogues of endogenous progestagens. Therefore, 5β-pregnane-3α,20α-diol (PD) was added to the existing method, to serve as progesterone metabolite.

In the current study, the use of the results obtained from the official control samples to reinforce the method validation is discussed, as well as some interesting preliminary observations regarding the use of PD as progesterone metabolite.

Materials and Methods

Chemicals
All reagents and solvents were of analytical grade and were supplied by Sigma-Aldrich (Bornem, Belgium). Liquid chromatography solvents were of LC- and HPLC-grade from Biosolve (Valkenswaard, The Netherlands). Used steroids were obtained

from Steraloids (Wilton, NY, USA). SPE C_{18} cartridges were from Achrom (Zulte, Belgium). Escherichia coli K12 β-glucuronidase was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Samples
A number of 162 urine samples, provided by the Belgian competent authorities for official control purposes, were used to provide the data for the current study. Additionally, samples were used from a bull and a heifer, treated with a single intramuscular injection of 1.194 mg kg^{-1} 17β-testosterone propionate (βTP) and 0.276 mg kg^{-1} 17β-estradiol-3-benzoate (βE2B). Samples were collected before and until 24 days after administration. The animal experiment was performed at the Faculty of Veterinary Medicine of Ghent University (B) in line with the guidelines of the ethical committee.

Sample preparation
Ten mL of urine was hydrolysed for 15 h at 37°C and pH 6.8, using β-glucuronidase. Afterwards, the samples were brought onto a C_{18} solid-phase extraction (SPE) column, conditioned with 6 mL methanol and 6 mL H_2O. After washing with 6 mL H_2O and 5 mL H_2O/acetonitrile (ACN) (80/20; v/v), samples were eluted with 8 mL H_2O/ACN (10/90; v/v). Next, two subsequent liquid-liquid extractions (LLEs) with 4 mL *n*-pentane/diethyl ether (92.5:7.5; v/v) at different pH (pH 14 and pH 5.2) were performed, separating estrogenic and androgenic/gestagenic steroids. By two subsequent HPLC-purification steps, using reversed phase and normal phase, respectively, five separate fractions containing the isolated targeted analytes (αE2, AEdiol, Etio, αT, and PD) were obtained. After acetylation overnight at room temperature with pyridine and acetic anhydride, the extracts were injected into the GC-MS/C/IRMS system.

HPLC-UV
The first system was a Waters Alliance 2690 coupled to a UV-detector (diode array detector, DAD), operated between 205 and 235 nm, and an automated fraction collector. The system was equipped with a C_{18} functionalized precolumn (Kinetex Security Guard Ultra C18 for 2.1 mm ID) and a C_{18} functionalized column (Kinetex XB-C18; 250 mm x 4.6 mm; 5 µm). An isocratic method was used with a mobile phase, made of H_2O/MeOH (95/5; v/v) (solvent A) and MeOH (solvent B), held at a constant composition (A:B; v/v) of 35:65, and a rinsing phase at the end of the run. The flow rate was set at 0.8 mL min^{-1}, column temperature at 50°C and the injected volume was 100 µL.
The second Waters Alliance 2690 system was equipped with two diol functionalized columns (LiChrospher Diol; 250 mm x 4 mm; 5 µm) in series. A constant mobile phase composition of isooctane/isopropanol (85/15; v/v) was used, with a rinsing phase at the end of the run. The used flow rate was 1 mL min^{-1}, column temperature 40°C and the injected volume 100 µL.

GC-MS/C/IRMS
A Thermo Trace GC Ultra gas-chromatograph, equipped with an Optima 17MS column (Machery Nagel – 30 m; 0.25 mm i.d.; 0.25 µm df) and a Thermo Scientific TriPlus autosampler, was used. After GC, the sample was split by means of two SilFlows (^{TM} SGE Analytical Science) to a Thermo DSQ II single quadrupole mass-spectrometer at one end, and to a Thermo MAT 253 isotope ratio mass spectrometer, via the Thermo Scientific GC Isolink, at the other end. Gas flows were regulated using the Thermo Scientific Conflo IV interface. Eight µL was injected at 20 µL sec^{-1} in programmed temperature vaporizer mode, with an initial injector temperature of 100°C, which was held for 0.10 min with a vent flow of 20 mL min^{-1}. After raising the temperature at 8°C min^{-1}, it was held at 320°C for 2 min during sample transfer on column. The initial GC oven temperature was 110°C, which was held for 1.5 min. The temperature was then subsequently raised to 220°C at 30°C min^{-1}, 270°C at 6°C min^{-1} and to 300°C at 2°C min^{-1}. Finally, the temperature was raised to 330°C at 50°C min^{-1} and held for 3 min. The carrier gas was helium at a constant flow rate of 1.2 mL min^{-1}. The temperature of the transfer line was 300°C. The single quadrupole MS was operated in full scan mode (*m/z* 50 to 400). The combustion furnace was set at 950°C and was oxidized prior to each series of analyses for 1 h. The CO_2 reference gas was calibrated with a steroid mixture, and carbon isotope ratios of the compounds were expressed relative to Vienna Pee Dee Belemnite (VPDB). The shift of the $\delta^{13}C_{VPDB}$ value due to the formation of acetates is corrected as follows:

$$D_{OH} = D_{OAc} + 2m\,(D_{OAc} - D_{Ac})/n$$

D_{OH} is the $\delta^{13}C_{VPDB}$ value of the non-derivatised steroids, D_{OAc} the $\delta^{13}C_{VPDB}$ value of the acetylated steroids, D_{Ac} the $\delta^{13}C_{VPDB}$ value of the acetylating reagent, n the number of carbon atoms in a molecule and m is the number of hydroxyl groups to be acetylated.

Results and Discussion

Method validation
The extensive initial method validation included determination of the intermediate precision, trueness, absence of isotope fractionation caused by the sample preparation, specificity and the linear range for every targeted analyte. Additionally, it was assessed whether the method allowed to differentiate between samples from treated and non-treated animals. Details

on the successfully performed validation experiments and results for the androgens and estrogens can be found in previous publications (Janssens *et al.*, 2013; Janssens *et al.*, 2015), and the results for PD were highly similar (under revision for publication).

To reassess the intermediate precision over a longer period of time, the data from the compliant and spiked quality control samples, QC-neg and QC-pos, respectively, were evaluated. QC-neg was measured 14 times over a time span of eight months, and provided intermediate precisions of 0.86‰, 0.85‰ and 0.76‰ for AEdiol, αT and Etio, respectively. QC-pos was measured 16 times over a time span of ten months, and provided intermediate precisions of 0.82‰, 0.78‰ and 0.70‰ for AEdiol, αT and Etio, respectively. Since the initial validation experiment consisted of eight measurements over a time span of three months, the results obtained through the QC-samples provide a better estimation of the intermediate precision.

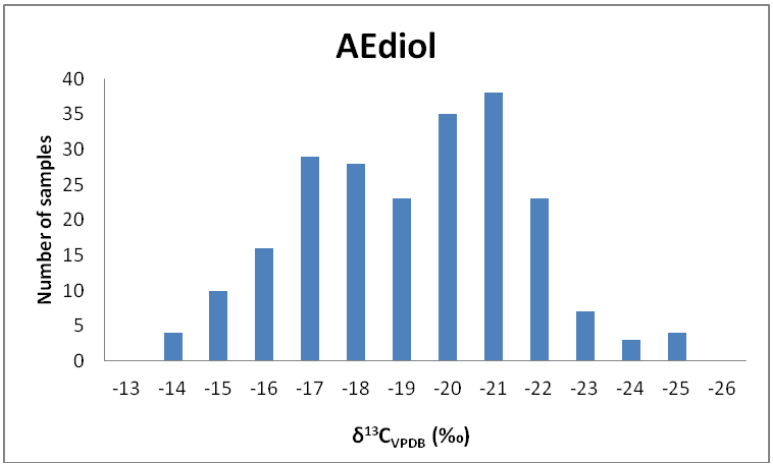

Figure 1. Distribution of the $\delta^{13}C_{VPDB}$ values of 5-androstene-3β,17α-diol (AEdiol), measured in official control samples from Belgian cattle.

Data from official control analyses

During method validation, results of samples from a compliant control population of approximately 20 animals, were used to determine a non-compliance threshold $\Delta^{13}C_{VPDB}$ value, calculated as the mean value plus three times the standard deviation (μ + 3 x SD). This way, the threshold $\Delta^{13}C_{VPDB}$ values were set at 3‰ for αE2, αT and PD, and at 4‰ for Etio, using AEdiol as the ERC. To re-evaluate the thresholds for the androgens, the data from the official control samples with compliant results were used, which provided a significantly larger control population. For both Etio (n = 115) and αT (n = 66), the recalculated threshold $\Delta^{13}C_{VPDB}$ value remained well below 3‰ for both compounds.

When evaluating the obtained measurements for AEdiol from the official control samples (n = 162), the $\delta^{13}C_{VPDB}$ values ranged from -14.12‰ to -25.97‰, around an average of -19.73‰ (Figure 1).

Impact of a testosterone/estradiol treatment on PD

PD is the ERC of preference for human doping control (WADA, 2016). To evaluate if PD could fulfil a similar function in case no progesterone was administered, nine urine samples from a bull and 11 from a heifer treated with a single intramuscular injection containing 17β-testosterone propionate (βTP) and 17β-estradiol benzoate (βE2B), from an animal experiment which was published in detail for androgens and estrogens (Janssens *et al.*, 2015), were analysed using the extended method, including PD.

When looking at the $\Delta^{13}C_{VPDB}$ values of all the metabolites together, using AEdiol as the ERC, as illustrated in Figure 2, it becomes clear that the $\delta^{13}C_{VPDB}$ values of PD are significantly affected by the βTP/βE2B treatment, even up to the point where the compliance threshold of 3‰ for PG treatment is crossed, which would indicate a PG-treatment. In the bull, the impact of the treatment on the $\delta^{13}C_{VPDB}$ values is even more significant than for the androgen metabolites. These findings were highly unexpected.

Conclusions

After performing the analysis for the detection of abuse of synthetic analogues of endogenous androgenic and estrogenic steroid hormones in routine for several years, the method proved to be robust and reliable. The above described results clearly indicate the importance of the selection of a suitable ERC, and the results of the quality control samples are clearly a valuable addition to the validation experiments.

By collecting the data of the ERC in the analysed samples, a clear image can be formed regarding the distribution of the $\delta^{13}C_{VPDB}$ values in the Belgian beef cattle population. The average value of approximately -20‰ indicates that the detection of abuse of steroid preparations is not significantly hindered by the feeding regime.

Finally, due to the unexpected impact of the βTP/βE2B treatment on PD, elevated $\Delta^{13}C_{VPDB}$ values of the ERC–metabolite combination AEdiol-PD, are not specific for progesterone treatment. However, the elevated $\Delta^{13}C_{VPDB}$ values are still indicative of steroid treatment. There is currently no explanation for the observed phenomenon, and it needs to be stressed that additional animal experiments are required to confirm and explain the observations.

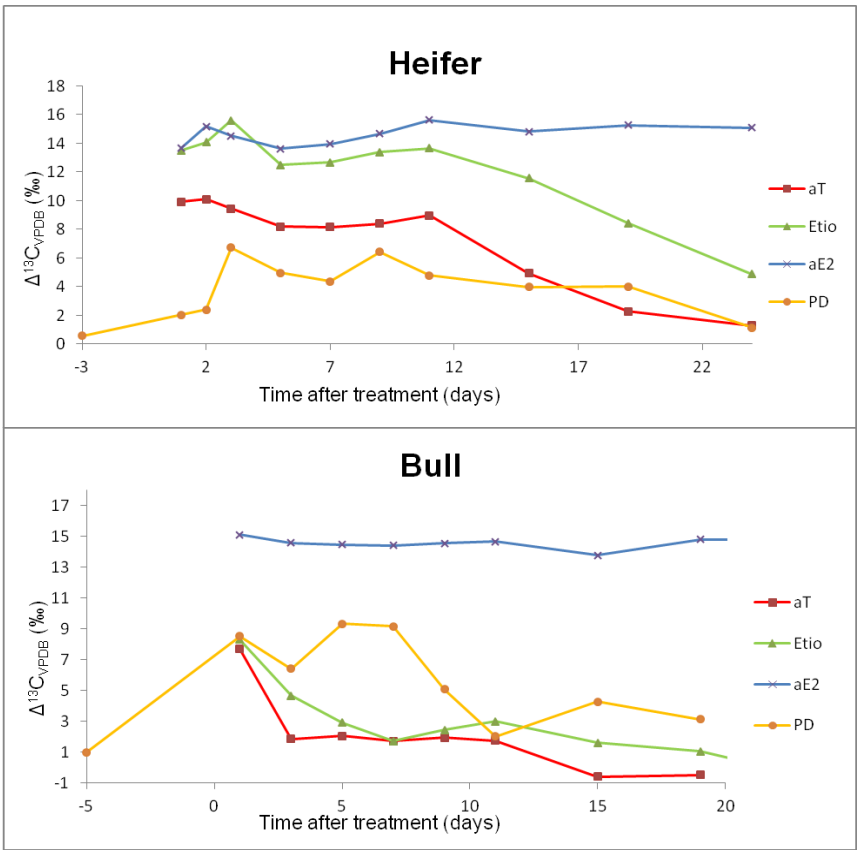

Figure 2. $\Delta^{13}C_{VPDB}$ values for 17α-testosterone (aT), 17α-estradiol (aE2), etiocholanolone (Etio) and 5β-pregnane-3α,20α-diol (PD), with 5-androstene-3β,17α-diol (AEdiol) as ERC, in urine samples from a bull (lower) and a heifer (upper), treated with a single intramuscular injection of 17β-testosterone propionate and 17β-estradiol benzoate.

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HILIC-UHPLC-ESI(+)-HRMS BASED METABOLOMICS AS A PREDICTIVE TOOL FOR DETECTION OF ANABOLIC ABUSE IN *BOVINE* ANIMALS

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Abstract
The illegal administrations of anabolic agents in meat-producing animals are reported to continue, even though the use of these has been banned within the EU. In this context, our project aimed to prove that an untargeted high-resolution mass spectrometry-based metabolomics analysis of urine could be used as a predictive tool for detection of anabolic steroid abuse in *bovine* animals. Urine samples (collected from calves pre- and post- boldenone undecylenate administration) following a minimal sample extraction procedure were analysed using HILIC-UHPLC-(ESI+)-HRMS. Statistical analysis of acquired fingerprints led to discrimination between samples collected pre- and post-steroid administration, and the selection of ions contributing most significantly to discrimination between animal treatment groups. The developed model presents good descriptive and predictive capabilities enabling usage as a tool for the classification of *bovine* urine samples of unknown status. To date a range of *bovine* urine samples of known and unknown treatment status originating from animals of varying breed, gender, age, geographical region and diet were randomly analysed using the established model to assess the relevance and robustness of the model upon variable factors.

Introduction
The use of anabolic agents in meat producing animals is prohibited in the EU for more than 20 years (EC, 1988) but illegal administrations are continuously being revealed mainly due to economic benefits. Moreover, the current testing systems to detect drug abuse are compromised by the use of endogenous hormones, designer drugs and low-dose chemical cocktails but also by the limited number of samples which can be tested routinely for drug residue presence. In this context, alternative means of detecting the abuse are urgently needed and metabolomics, an emerging field of "omics" research, offers potential as a new screening strategy. Metabolomic-based analysis, focusing on the identification of metabolite profiles representative of biological responses to exogenously administered agents, combines fingerprinting technology and chemometrics. This emerging strategy has recently demonstrated the efficiency for identifying unique metabolic profiles in animal biofluids following xenobiotic exposure (Mooney *et al.*, 2009; Dervilly-Pinel *et al.*, 2015; Jacob *et al.*, 2015; Kouassi Nzoughet *et al.*, 2015). The objective of the current study was to determine if untargeted high-resolution mass spectrometry-based metabolomic profiling of urine could be used as a predictive tool for detection of anabolic steroid abuse in cattle and thus serve as an alternative means to tackle illegal practices in food safety surveillance. Moreover, the goal was to employ hydrophilic interaction liquid chromatography (HILIC) to provide sufficient retention and separation of polar analytes.

Materials and Methods

Animal study design
Urine samples (n = 102) were collected from calves (n = 6) pre- and post- 1 mg kg^{-1} boldenone undecylenate administration (four days before treatment until day 35, respectively) and stored at -70°C prior to extraction.

Sample preparation
A minimal sample preparation procedure was applied to urine samples in order to generate as informative metabolic profiles as possible. Briefly, cattle urine samples (V = 500 µL) were filtered through a 10 kDa MWCO (Sartorius Stedim Biotech GmbH Vivaspin 500 centrifugal tubes, modified PES, 500 µL, Germany) under centrifugation at 15,000 *g* at 10°C for 30 min to remove high molecular weight proteins. Filtrates (V = 400 µL) were then freeze-dried for 48 h and dry mass of each sample was weighed so that all samples were reconstituted in 0.1% HCOOH in H_2O (v/v) by vortexing (5 min) at the same concentration level (40 mg mL^{-1}) for normalization purposes. To improve the peak shape and separation in HILIC analysis, samples were diluted 1:3 (v/v) with H_2O:MeCN (1:2, v/v). Following filtration through 0.2 µm PVDF centrifugal filter units (Ultrafree®-MC-GV, Durapore®, Merck Millipore Ltd. Cork, Ireland) at 12,000 *g* at 4°C for 2 min, 1.25 µL was injected onto the UHPLC-HRMS system.

Quality control (QC) samples

The quality control sample was obtained by pooling aliquots of the individual study samples in order to achieve a representative bulk sample. Pooled samples (QC) were injected at specific intervals throughout the entire analytical run to evaluate chromatographic reproducibility and MS performance. Moreover, a number of pooled samples were injected at the beginning of each run to condition/equilibrate the analytical platform.

HILIC-UHPLC-(ESI+)-HRMS fingerprinting

Replicate injections (n = 3) of urine sample from each individual animal were randomly analysed using an Acquity I Class UPLC® system coupled to a Xevo® G2-S QTof mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionisation source operating in positive mode (ESI+). LC separation (Graham *et al.*, 2013) was performed on Acquity UPLC® BEH HILIC analytical column (2.1 × 100 mm, 1.7 µm) (Waters) maintained at a temperature of 45°C and a flow rate of 0.6 mL min^{-1} over a 13-min run time. A binary gradient system comprising mobile phase A, 10 mM $HCOONH_4$ pH 3.30 and mobile phase B, 0.2% HCOOH in MeCN (v/v) was used. The gradient was as follows: (1) 0.0 min, 95.0% B, (2) 1.0 min, 95.0% B, (3) 8.0 min, 60.0% B, (4) 8.5 min, 60.0% B, (5) 10.0 min, 10.0% B, (6) 10.1 min, 95.0% B, (7) 13.0 min, 95.0% B. The UHPLC-HRMS system was controlled by v4.1 MassLynx software (Waters). Q-Tof-MS data was acquired in resolution mode. The capillary voltage was set at 1.0 kV. Sampling cone was set at 30 V. The desolvation and source temperatures were set at 450 and 120°C, respectively. Nitrogen was employed as the desolvation and cone gases, which were set at 850 and 100 L hr^{-1}, respectively. Mass spectra data was acquired in continuum mode using the MS^E function (low energy set at 4 eV and high energy ramped from 20 to 35 eV) over the range m/z 50 – 1200 Da, with a scan time 0.1 s. A leucine-enkephalin ($[M+H]^+$=278.1141 Da and $[M+H]^+$=556.2771 Da) lock mass calibrant was infused throughout at 5 µL min^{-1}.

Data analysis

Acquired MS data was subsequently imported into Progenesis® QI (Waters) software and processed using the following workflow. A set of adducts was pre-selected including: $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$, $[2M+Na]^+$, $[2M+K]^+$, $[M+2H]^{2+}$, $[M-H_2O+H]^+$. Retention time alignment was followed by peak picking (with an absolute ion intensity ≥ 200 arbitrary units, a peak width ≥ 0.03 min and retention time limits: 0.35 – 8.00 min), deconvolution, normalization (to all compounds) and quantification of compound ions. The results were exported as a two dimensional table in which rows and columns represented analysed samples and the normalised peak areas as an accurate mass (m/z) and retention time (min) pair. Multivariate analysis of the processed data was performed using SIMCA-P v.14.0 (Umetrics, Umeå, Sweden). Clustering of the pooled samples (QC) was assessed using Principal Component Analysis (PCA) to evaluate the platform stability. The analysis of fingerprints led to the detection of 1,260 ions out of which 805 were kept with RSD < 30% and the number of missing values no more than 20% across 102 samples. Before multivariate statistical analysis, pareto scaling was applied to all variables in order to reduce the relative importance of large values, but keep data structure partially intact (Van den Berg *et al.*, 2006). Furthermore, the data was log-transformed (base 10) to reduce heteroscedasticity and thus to make classification and clustering more effective (Van den Berg *et al.*, 2006).

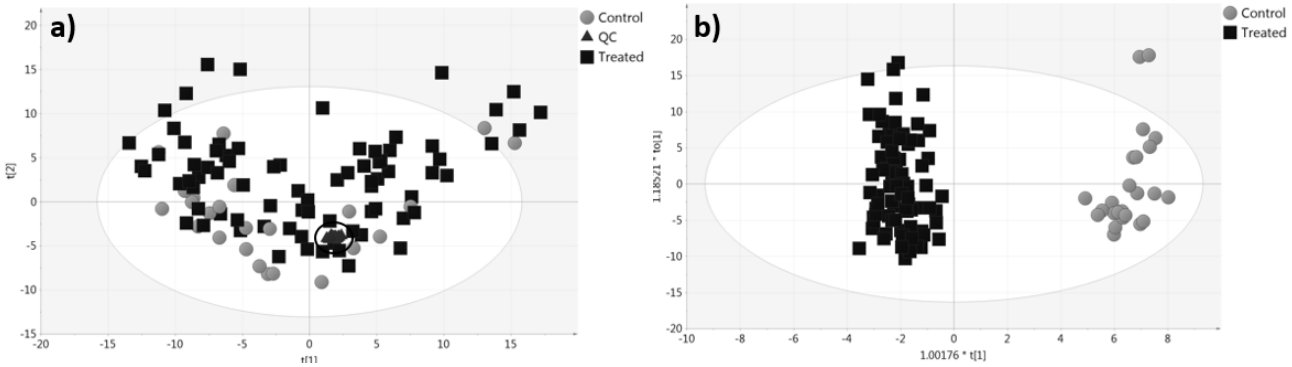

Figure 1. Score plots for a) PCA and b) OPLS-DA model based on the training dataset containing 805 ion variables.

Results and Discussion

As can be observed in Fig. 1a no significant differences are noted in a non-supervised analysis (PCA). Meanwhile, pooled samples (QC) are tightly clustered in the PCA plot (Fig. 1a) confirming the robustness of the UHPLC-HRMS platform and the reliability of the data. It is also the prerequisite for further statistical analysis and biological conclusions. Supervised multivariate analysis was performed and an OPLS-DA model (Orthogonal Partial Least Squares Discriminant Analysis), which separates the control samples from the treated ones, was built with the fitness of R2(Y) = 0.964 and Q2 = 0.837. The score plot (Fig. 1b) of the model demonstrates that urine samples of the two opposing classes have been well separated. The ions which contributed to the separation of the two opposing classes and those simultaneously satisfying the following four conditions were

identified as “features of interest”: 1) I*p*(cov)I and I*p*(corr)I were among the top ranking ones; 2) the Variable Importance in the Projection (VIP) score greater than one; 3) the jackknife 95% confidence interval, with reference to VIP scores resulting from cross-validation, exclusive of the value of 0; 4) unpaired student's *t*-tests identified the metabolite as significant at the 5% significance level. Statistical analysis of acquired urinary metabolomics fingerprints, followed by a visual examination of the chromatograms in order to eliminate potential artefacts, facilitated the selection of ions contributing most significantly to discrimination between samples collected pre- and post- boldenone undecylenate administration, and consequently enabled the selection of 38 candidate markers of steroid treatment (Fig. 2a). The fitness of the OPLS-DA model based on these selected features was R2(Y) = 0.926 and Q2 = 0.873 after performing 7-fold cross validation. The Y-axis intercepts of R2 = (0.0, 0.221) and Q2 = (0.0, -0.47) for 999 rounds of permutation tests, and $p = 1.93 \times 10^{-36}$ from CV-ANOVA both confirmed the robustness of the established model.

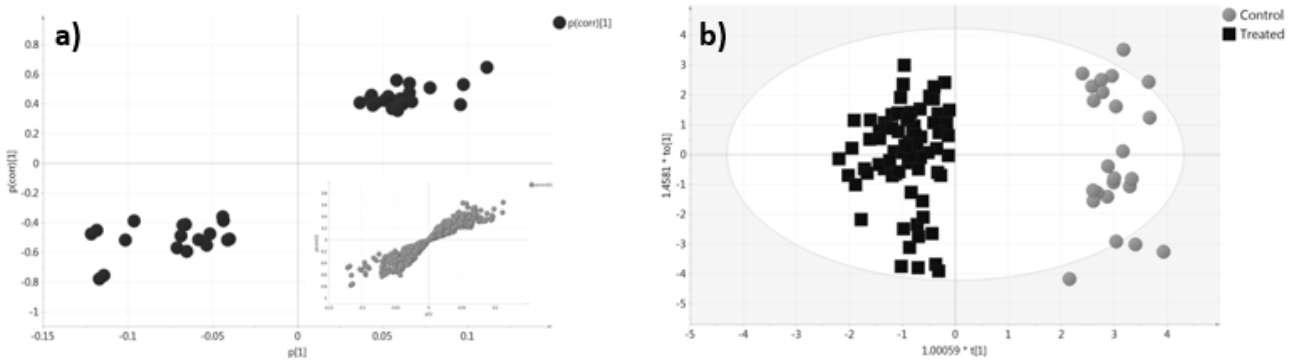

Figure 2. a) S-plot associated to the OPLS-DA model built on 805 variables (insert) illustrating distribution of the selected features. b) Score plot for OPLS-DA model based on 38 selected ions.

Testing sample set

To date 155 *bovine* urine samples of known and unknown treatment status originating from animals of varying breed, gender, age, geographical region and diet were randomly profiled and the acquired datasets were analysed using the established model based on 38 selected ions to assess the relevance and robustness of the model upon variable factors. All samples coming from known untreated animals were classified without misallocation. In the case of samples coming from animals of unknown treatment status, all of them were classified as “untreated”.

Conclusions

The new model presents good descriptive and predictive capabilities and thus can be employed as a predictive tool for the classification of urine samples of unknown status. It could serve as a new and alternative screening methodology to detect fraudulent anabolic treatment in cattle. The model has been partially validated to establish the rate of false positive results and additional experiments are being carried out to assess the rate of false negative results. Ongoing work is focused on the identification and biological interpretation of selected urinary metabolite markers utilised within constructed model.

Acknowledgements

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STUDY OF MATRIX EFFECT ON SULFONAMIDE ANALYSIS BY HPLC-MS/MS

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Abstract
The matrix effect (ME) is defined as the degree of ion-suppression or ion-enhancement in liquid-chromatography mass-spectrometry. This effect occurs during quantitative measurement specifically when using electrospray ionization. The signal intensity may be suppressed by high mass flows and co-elution of specific compounds. Alternatively, the signal can be enhanced by the accumulation of positively charged ions or neutralizing charge of molecules targets. Different methods are used to evaluate ME. In the present study, we determined the ME for four pharmaceutical substances (sulfadiazine, sulfamerazine, sulfamethazine and sulfamethoxipiridazine) in muscle of five species: chicken, pig, *bovine*, sheep and goat. The evaluation was performed by post-column infusion method that provided a ME profile for the entire chromatographic run time. An ABSciex quadrupole mass-spectrometer (QTrap-4500, ABSciex) was used as analytical detector. The system was interfaced by a Z-Spray ESI source that operated in the positive ionization mode.
To evaluate the ME, blank samples for each species were used. These extracts were spiked at different concentration levels for each substance using the same quantity of solvent and extract. We found comparable ME profiles for the sample extracts for all the studied compounds in the five species.

Introduction
The Matrix Effect (ME) is defined as the degree of ion suppression or ion enhancement during liquid-chromatography mass-spectrometry. This effect occurs during quantitative measurement specifically when using electrospray ionization. It is a widely recognized phenomenon. The signal intensity may be suppressed by high mass flows and co-elution of specific compounds, *e.g*. high concentrations of sugars, proteins, lipids, salts, amines, glycopeptides, phosphocholines or metabolites. Alternatively, the signal can be enhanced by the accumulation of positively charged ions or neutralizing charge of molecules (Rossmanna *et al*. 2015; Marchia *et al.* 2010; Barcellos 2015). To reduce ME, protein participation, solid-phase extraction (SPE), liquid-liquid extraction, sample dilution or flow reduction are used.

Evaluation of ME is achieved through two major techniques providing complementary information. First, qualitative results are obtained with a post-column infusion system which is based on the infusion of an analyte solution of interest between the column and the MS detector, leading to a constant baseline. Blank samples extracted with the tested sample preparation procedure are injected into the system. The presence of ME is highlighted by baseline alteration in a time window. MS responses of analytes eluting within this region will be altered (signal suppression or enhancement), inducing irreproducible and non-quantitative results.

The second method determines if the presence of matrix interferences causes a problem during the sample preparation step and/or during the analysis. The analysis is based on the comparison of three different samples: a neat standard, a biological sample spiked prior the extraction and a biological sample spiked after the extraction.

In the present study, we determined the ME for four pharmaceutical substances (sulfadiazine, sulfamerazine, sulfamethazine and sulfamethoxipiridazine) in muscle of five species, namely chicken, pig, *bovine*, sheep and goat. The evaluation was performed by the post-column infusion method that provided a ME profile for the entire chromatographic run time.

Materials and Methods
All chemicals and solvents were at least analytical –reagents grade and were purchased from local analytical suppliers. Methanol, acetonitrile, ethyl acetate, formic acid and LC quality water were obtained from Merck (Germany). The sulphonamides standard (sulfadiazine, sulfamerazine, sulfamethazine and sulfamethoxipiridazine) were supplied from Sigma – Aldrich.

For each species, two grams of blank muscle was extracted with 10 mL ACN/H_2O. A sample aliquot was loaded onto an SPE Strata X column conditioned with 3 mL MeOH and H_2O prior to use. The column washed with 1 mL water, eluted with 3 mL MeOH/ ethyl acetate (1:1). The eluate was evaporated using N_2. Dry extracts were dissolved in 25 µL ACN, 225 mL H_2O with 0.1% formic acid. These extracts were spiked with each substance at different concentrations but always using the same quantity of solvent and extract.

An Agilent 1260 HPLC apparatus equipped with a Symmetry C-18 3.5 µm column (2.1 x 100 mm) was coupled to an ABSciex quadrupole mass spectrometer (QTrap 4500, ABSciex) as the analytical detector. The system was interfaced by a Z-Spray ESI source that operated in the positive ionization mode. Multiple reaction monitoring (MRM) in positive electrospray mode (ESI+) was used for all compounds. For each analyte, two transitions were monitored. At a flow rate of 400 µL min^{-1}, 10-µL

samples were introduced for analysis. Gradient elution was employed with mobile phases comprising Solvent A: 0.1% aqueous formic acid and Solvent B: ACN fortified with 0.1% formic acid. The gradient used for chromatography is summarised in Table 1. The system was controlled with Analyst 1.6.1 software.

Matrix effect was calculated as percentage using the following formula:

$$ME = \frac{B}{A} \times 100\%$$

Where A is the peak response (area or height) of a standard in solvent, and where B is the corresponding peak for a standard spiked into in a blank matrix extract.

Table 1. Gradient program for chromatography.

Total time (min)	Flow rate (µL min^{-1})	Solvent A (%)	Solvent B (%)
2	400	60	40
3	400	55	45
5	400	50	50
7	400	60	40

Table 2. MS/MS parameters and ion transitions.

Name	Q1 (Da)	Q3 (Da)	Time (ms)	DP (volts)	CE (volts)	CXP (volts)
Sulfadiazine 1	251	108.1	27	33	35	11
Sulfadiazine 2	251	156	27	33	33	17
Sulfamerazine 1	265	156	27	13	23	9
Sulfamerazine 2	265	172	27	13	24	12
Sulfamethazine 1	279	186.1	27	60	26	17
Sulfamethazine 2	279	124	27	60	35	14
Sulfamethoxipiridazine 1	281	156.1	27	30	23	10
Sulfamethoxipiridazine 2	281	108	27	30	36	9

Results

In order to evaluate the matrix effect, the calibration curve in solvent (ACN/H_2O (50:50) containing 0.1% formic acid) was compared with the calibration curves in muscle from *bovine*, chicken, sheep, swine or goat. The concentrations were 10, 25, 50, 75 and 100 µg L^{-1} (*cf*. Figures 1 and 2). The ME results obtained for each species are shown in Figure 5.

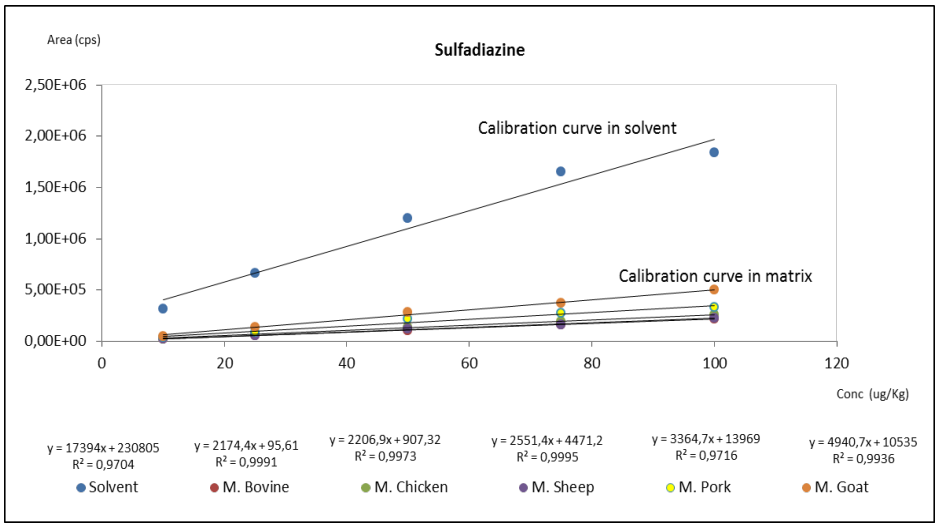

Figure 1. Matrix effect for sulfadiazine.

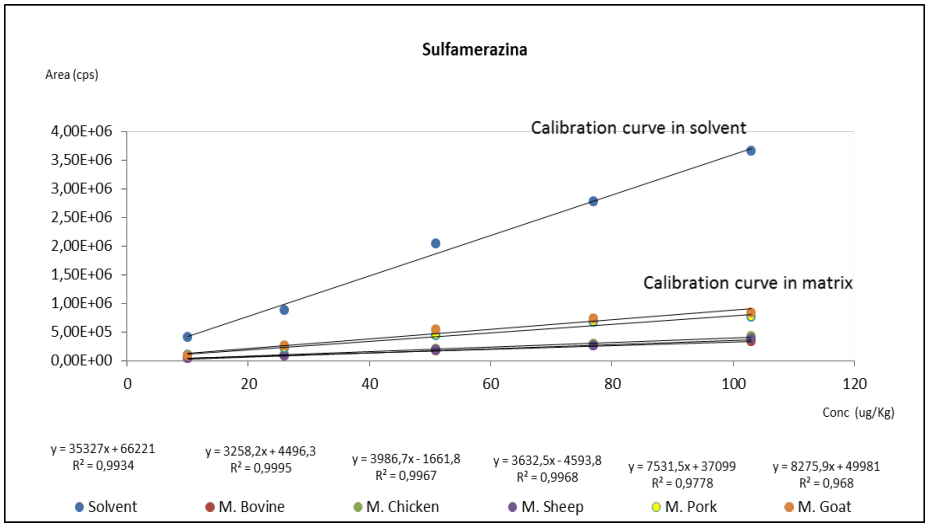

Figure 2. Matrix effect for sulfamerazine.

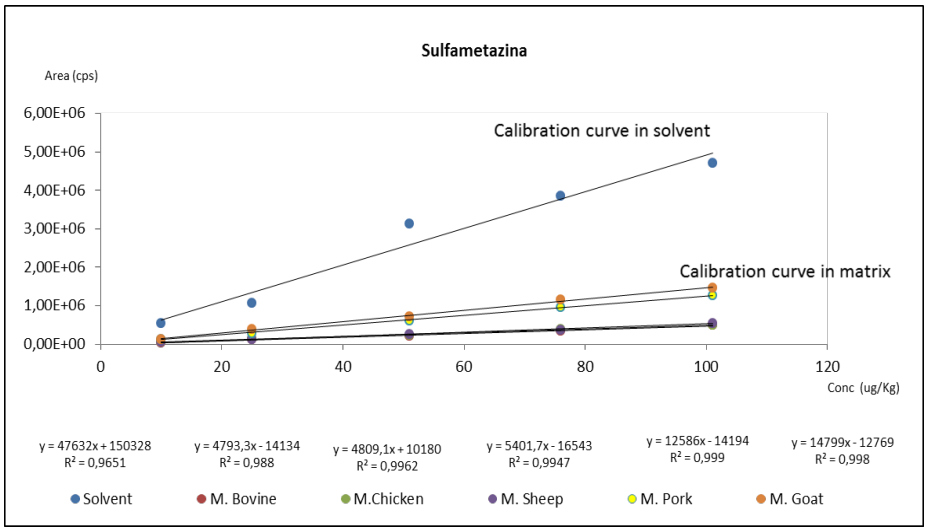

Figure 3. Matrix effect for sulfamethazine.

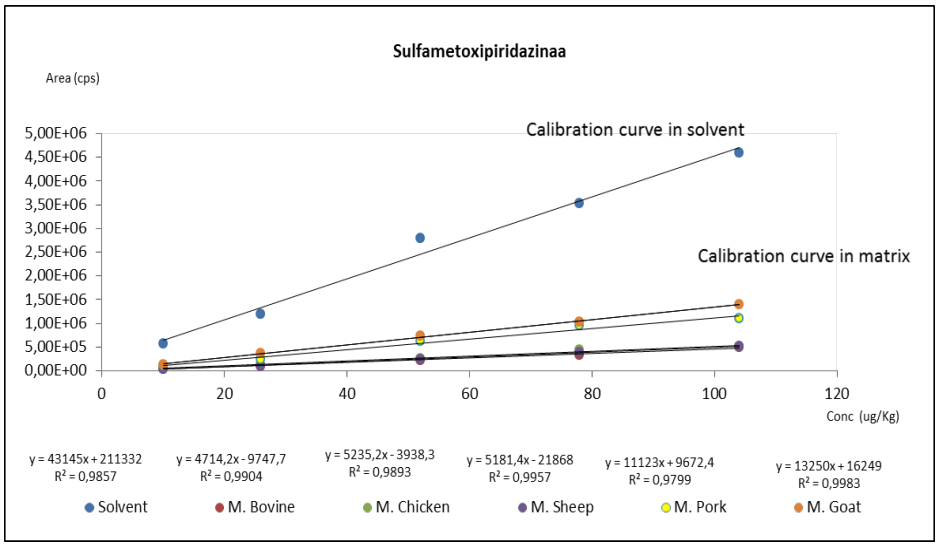

Figure 4. Matrix effect for sulfamethoxipiridazine.

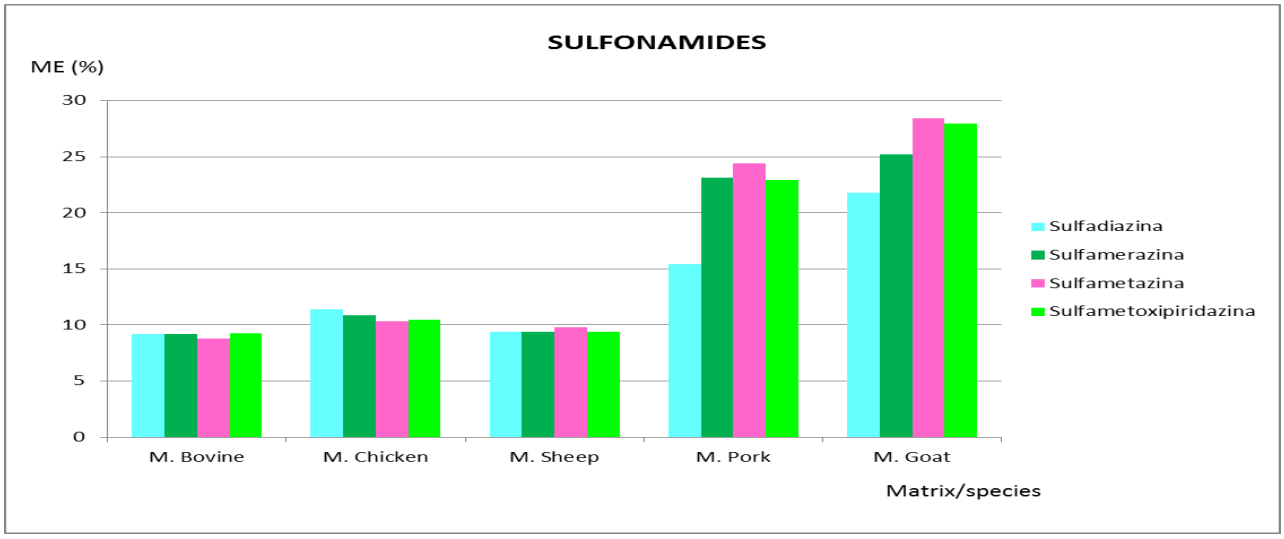

Figure 5. Matrix effect (%) for sulfadiazine, sulfamerazine, sulfamethazine and sulfamethoxipiridazine observed in bovine*, chicken, sheep,* porcine *and goat muscle.*

Discussion and Conclusions

In general, when matrix effect is between 70% and 110 %, the extraction or clean-up is considered to be adequate or sufficient to provide reproducible LC chromatography and consistent mass spectrometric responses. The results obtained in this study, showed values under 30% and the calibration curve in solvent is not accepted for the quantification in the matrixes studied. Therefore, it is necessary to create calibration curves in matrix in order to quantify the sulphonamides used in this study.

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DEGRADATION OF ANTIBIOTICS RESIDUES IN AQUEOUS MEDIUM BY ELECTROCHEMICAL AND PHOTOELECTROCHEMICAL PROCESSES

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Abstract

Over the last 15 years, residues of organic substances such as antibiotics, tranquilizers, steroid estrogens, and other veterinary pharmaceuticals, have been receiving an increasing attention as potential bioactive pollutants in soil and in the aquatic environment. Considering that these substances are not broken down or removed by normal water treatment, which include biological and physical methods, alternative procedures are studied. Such an alternative is electrochemical and photoelectrochemical degradation by advanced oxidation processes (AOP). In this work, the degradation of sulfathiazole (STZ) and sulfadiazine (SDZ) was studied by electrochemical (EC) and photoelectrochemical (PEC) processes in an aqueous medium using Ti-electrodes modified with titanium dioxide (TiO2). Drug concentrations were monitored by HPLC-DAD. For STZ, the degradation was more effective compared to SDZ which was degraded by 60% using the PEC process compared to 40% for the EC process. Furthermore, the results were similar in the case of SDZ degradation using PEC and EC processes giving 30% degradation for both processes. Adsorption and photochemical studies were performed without significant changes in concentration of the drugs studied.

Introduction

Pharmaceutical products used in the medical field for preserving human and animal health have received recently special attention and increasing concern. Antibiotics are largely consumed in human medicine for therapeutic purposes and widely used in the livestock and poultry industries for preventing diseases or improving growth (Kemper 2008). The annual production volume of drugs has been estimated in several hundreds of tons (Sarmah *et al*. 2006). These substances are excreted by the organism, disposed from the households and released into the aquatic environment. They were detected in influents of wastewater treatment plants, in surface water, in ground water and even in drinking water.

Sulphonamides are synthetic antibiotics derived from sulfanilic acid. They act as bacteriostatics by inhibiting the synthesis of dihydrofolic acid. They are widely prescribed to treat human and animal infections and are used as food additives in livestock production. Sulphonamides persist in the environment for a long time due to their low biodegradability, being reported in waters in the range of 0.13 to 1.9 ng L^{-1}, and can accumulate in various organisms. The physical removal methods such as adsorption, reverse osmosis or nanofiltration are able to separate them from water, but these methods only concentrate waste, without degradation, and induce another environmental problem.

Chemical oxidants like chlorine and chlorine dioxide have been applied to oxidize residues, but they form potential carcinogenic chlorinated by-products. Considering that the traditional processes of water treatment do not achieve to tumble totally the antibiotics and other organic compounds that can contaminate the environment, alternative methods for the degradation of this kind of substances were studied. Among these alternatives are advanced oxidation processes, the so-called POAs.

These processes are characterized in the production of special oxidants like free radicals that degrade the organic matter, achieving their total mineralization. The POAs are chemical, photochemical, photocatalytic and electrochemical methods based on the *in situ* generation of mainly radical hydroxyl (●OH). It is a severely oxidising not selective species that has proved to be very efficient in the elimination of organic contaminants in waters, even reaching a total mineralization of the substances. The mechanism commonly accepted consists of the existence of redox reactions that involved oxygen molecules or adsorbed over the surface of the semiconductor. A photo-assisted process starts with the absorption of a photon, in which an e^- is promoted from the valence band (BV) to the conduction band (BC). BY doing so it leaves a "hollow (h+)" in the BV. In this zone, the reaction with the H_2O or ions OH- produces OH• radicals, which can oxidize organic matter (Daghrir *et al*. 2013; Palominos *et al*. 2009).

Materials and Methods

All chemicals and solvents were at least analytical reagents grade and were purchased from local analytical suppliers. The sulphonamides standard (sulfadiazine, sulfathiazole) were supplied from Sigma – Aldrich. An Agilent 1260 series high-performance liquid-chromatography system coupled to DAD detector was used for analysis of the sulphonamides. A potentiostat CH-Instrument and power supply PSA 305 Sanlex were used for the degradation process. Titanium thickness 0.25 mm, 99.7% trace (Aldrich) were used for prepared modified electrodes of 2.5 x 5 cm sheets.

Preparation of electrodes

The electrodes of TI/TiO2 were prepared from the Ti sheets. These sheets were degreased with a solution composed by acetone and water (50:50) for a period of 10 min in ultrasound bath. The process of the Ti-sheet modification by anodizing was done using two different types of electrolytic solutions: 0.5 M H_2SO_4 + 0.044 M HF (EM-1) and 0.5 M H_2SO_4 + 0.13 M NH_4F (EM-2) with the purpose to evaluate the efficiency of both modified electrodes according to a process of anodizing. The anodizing process was done at a constant potential of 25 V in 100 mL solution under constant agitation. After that, the sheets were washed generously with distilled water and dried with N_2 stream. The anodized sheets were calcined in muffle at a temperature of 460°C for 4 h in order to obtain the crystalline anatasa structure, of major catalytic activity, which is characteristic under these conditions of preparation. The resulting electrodes were characterized through cyclic voltammetry and X-ray diffraction.

Electrolysis and sulfonamides:

The electrolysis of both sulfonamides SDZ and STZ was accomplished in an electrochemical cell containing 100 mL of a half electrolyte of 0.5 M Na_2SO_4 at room temperature, with constant agitation and bubbling of O_2. The initial concentration of each sulfonamide in the solution was 0.1 ppm. Aliquots of 1 mL solution over a period of 3 h were analysed by HPLC in order to determine the concentration of each sulfonamide. The electrolysis was carried out under controlled potential of -1 V. For the studies, modified Ti/TiO_2 was used as anode work electrode and a sheet of Ti with the same size (2.5 x 5 cm) as the cathode counter-electrode. As reference electrode, a saturated calomel electrode (+0.242 V) was used. To obtain photoelectrochemical reactions, direct radiation with a lamp of Hg located to 30 cm from the electrode of Ti/TiO_2 was applied. Additionally, adsorption (AD) and photochemical (FQ) assays were done in order to evaluate potential effects or incidences of these processes in the variation of concentration of the sulfonamides in the assay solutions.

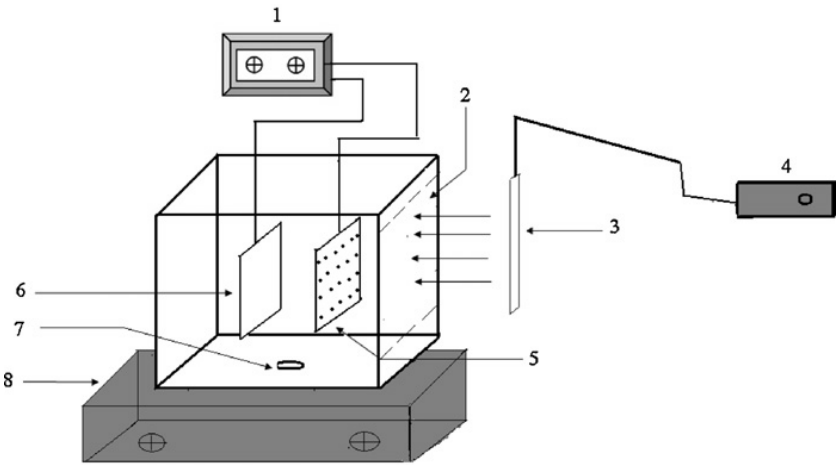

Figure 1. Schematic diagram of the photoelectrocatalytic reactor: (1) DC power supply, (2) quartz window, (3) UV lamp, (4) photometer, (5) photoanode, (6) cathode, (7) stirring bar and (8) magnetic stirrer.

Chromatographic analyses

The chromatographic analyses were carried out using an HPLC system (Agilent 1200 series) equipped with a 250 x 4.6 mm, 5 µm C18 column (phenomenex/gemini) and a pre-column with an equal fill maintained at 30°C in a oven.The chromatographic conditions were optimized with standards. Mobile phase was ACN: 50 mM NaH_2PO_4 at 17:83 and flowed isocratically at 0.8 mL min^{-1}. Injected sample volume was 50 µL and total run time 15 min. The retention time for SDZ and STZ were 7.1 min, and for Sulfatiazol 7.7 min. Each experiment corresponded to three hours of electrolysis during which 13 samples were collected and analysed.

Results

Electrolysis of SDZ and SDZ using modified electrodes of Ti / TiO2 (0.5 M H_2SO_4 + 0.044 M HF)

With electrodes (0.5M H_2SO_4 + 0.044 M HF), a greater effect of degradation of STZ compared to SDZ is observed. Degradation of STZ using the FEQ process was more efficient compared to the EQ process, reaching 60% conversion compared to 40% using EQ. No concentration changes were observed by adsorption. A conversion of 10% is observed by FQ effects for both sulfas. In the degradation of SDZ, similar conversion behaviours with the FEQ and EQ processes were observed, reaching 25% for both cases respect to adsorption processes no change is observed in the concentration of the analyte and a degradation of about 10% by FQ conversion.

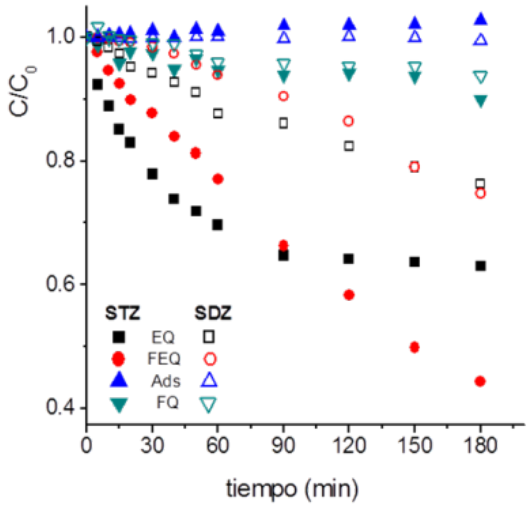

Figure 2. Degradation curve of SDZ and SDZ using modified electrodes TI/TiO2 (0.5 M H_2SO_4 + 0.044 M HF).

Electrolysis of SDZ and SDZ using modified electrodes of Ti / TiO2 (0.5 M H_2SO_4 + 0.13 M NH_4F)
The results for degradation and SDZ and SDZ with electrodes modified in middle electrolytic (0.5 M H_2SO_4 + 0.13 M NH_4F), a greater efficiency is observed with respect to modified electrodes using electrolytic medium (0.5 M H_2SO_4 + 0.044M HF), obtaining conversions between 40% and 50% for both SDZ and STZ. The STZ conversion reached approximately 50% values for both FEQ and EQ process, as in the previous case no adsorption effects are observed during the test time. Degradations near to 10% is observed by FQ effects for both sulfas. In the case of SDZ, we observed by FEQ effect a conversion close to 60% higher compared to 50% conversion by EQ effect. As in the analysis of STZ, no effects of AD are observed and only 10% for FQ conversion effect.

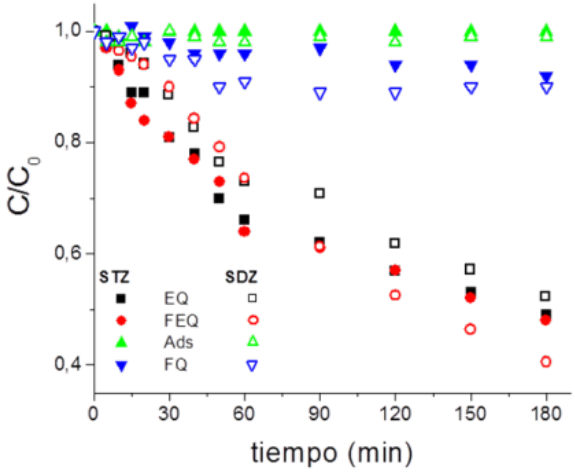

Figure 3. Degradation curve of SDZ and SDZ using modified electrodes TI/TiO2 (0.5 M H_2SO_4 + 0.13 M NH_4F).

Discussion and Conclusion
The results obtained in this work show that electrochemical and photelectrochemical are an excellent alternative for degradation of organic substances in solution. The use of modified electrode of Ti/TiO_2 were efficient in the degradation of sulphonamides. It is necessary and possible to optimise the parameters of reaction to improve the level of degradation of the substances.

Acknowledgements
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A RELIABLE METHOD FOR THE DETECTION OF RACTOPAMINE IN *BOVINE* MUSCLE BY LC-MS/MS ATTENDING REGULATORY SAFETY LEVELS ADOPTED BY CERTAIN MARKETS

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Abstract
Ractopamine is a β-adrenergic agonist increasingly used in the swine and cattle industry. Ractopamine redirects nutrients in favour of leanness rather than fat deposition, improves growth and carcass traits gaining higher economic benefit to producers. Countries around the world are divided whether to allow the use of ractopamine in meat production. In order to control it, the ability to detect residues in a zero-tolerance policy for ractopamine has become an important issue. Regularly, muscle is the tissue analysed by laboratories responsible to inspect the meat on the destination markets. Therefore, a LC-MS/MS for the determination of residual ractopamine in cattle muscle with a quantification limit of 0.1 µg kg^{-1}, using ractopamine-D5 as an internal standard has been developed and validated. The validation was performed according to the 2002/657 European Union Decision. The method meets the Brazilian regulatory requirement that establishes criteria and procedures for determination of parameters such as decision limit (CCα), detection capability (CCβ), precision, and recovery. CCα and CCβ were determined to be 0.02 and 0.03 µg kg^{-1}, respectively. Average recoveries from cattle muscle samples fortified with 0.05 to 0.5 µg kg^{-1} leads around 100%. A complete statistical analysis was performed on the results obtained.

Introduction
β-Adrenergic agonists (β-agonists) are widely used as bronchodilators, tocolytics and heart tonics in clinical and veterinary medicine (Antignac *et al*., 2002). Ractopamine is classified as a β-adrenergic agonist that acts as a repartitioning agent, redirecting nutrients away from adipose tissue towards muscle deposition, resulting in substantial improvements in average daily gain, feed conversion efficiency, dressing percent and carcass lean content (Gu *et al*., 1991; Rikard-Bell *et al*., 2009). It is licensed for use as an animal growth-promoter in more than 20 countries worldwide; however, due to human health concern, over 150 countries banned the use of ractopamine. Thus, because of the dual requirements of providing quality assurance for the consumer and of satisfying legal testing obligations, the ability to detect residues at zero-tolerance policy for ractopamine has become a very important issue.

At present, a number of assays for the determination of ractopamine residues in animal tissues have been reported including immunoassays (Elliott *et al*., 1998; Pleadin *et al*., 2012; Shelver and Smith, 2000), gas chromatography/mass spectrometry (Bocca *et al*., 2003), liquid-chromatography (Burnett *et al*., 2012) and liquid-chromatography coupled to mass-spectroscopy (LC-MS, LC-MS/MS, UPLC-MS/MS) (Antignac *et al*., 2002; Kootstra *et al*., 2005; Moragues and Igualada, 2009). LC-MS/MS is an excellent tool for determination of such compounds and has been used for our objective. In the present work, we describe a simple, rapid, and reliable LC-MS/MS method for the determination of ractopamine in *bovine* muscle. Regularly, muscle is the tissue analysed by laboratories responsible to inspect the meat on the destination markets, as this is the sample more accessible to destination laboratories. The performance of the method was evaluated in accordance with international and zero tolerance requirements. The validation parameters were determined on that sense and are being considered for use for surveillance purposes in Brazil.

Material and Methods

Reagents and chemicals
Chemicals and solvents used were of analytical grade and provided by Merck (Darmstadt, Germany), Carlo Erba (Milano, Italy) and Synth (São Paulo, Brazil). Ractopamine-D5 was applied as internal standard. The solid phase extraction (SPE) cartridges were provided by Phenomenex - Strong Cation 60mg/3mL type.

Apparatus
The experiments were carried out using an HPLC Agilent 6460 coupled with a Micromass-Waters Quattro Micro tandem mass spectrometer using an ESI ionization and MassLynx 4.0 software. The LC-MS/MS operating conditions are summarized in Table 1.

Sample extraction and clean up

An amount of 5 g muscle was weighed into a 50-mL Falcon tube and then 10 mL methanol was added. A shaking process was conducted for 1 min. The sample solution was then centrifuged at 3,500 rpm for 10 min, 20°C. The supernatant was transferred to another Falcon tube (15mL) and was brought to dryness using water bath at 60°C under a gentle stream of nitrogen and the residue re-suspended in 1mL of sodium acetate 25 mM pH 5 plus 20 µL of enzyme β-glucuronidase. The system was incubated for 1h at 65°C. The samples were then shaken for 1 min and then centrifuged at 3,500 rpm for 5 min, 20°C. The samples then were applied to SPE Phenomenex Strong Cation exchange 60mg/3mL pre-conditioned with 3.5 mL of methanol and eluted from the cartridge with ammonium hydroxide:methanol mixture. The eluate was brought to dryness using water bath at 60°C under a gentle stream of nitrogen and the residue was re-suspended with 250 µL methanol. The extract was filtered prior to LC-MS/MS analysis.

Table 1. Operating LC-MS/MS conditions for ractopamine analysis.

Parameters	HPLC conditions	Parameters	MS conditions
Column	Agilent Zorbax SB-Phenyl (4.6 x 50 mm, 3.5 µm)	Ionization	ESI, positive
Eluent	Ammonium acetate + Formic acid 0.1%: Acetonitrile + Formic acid 0.1% (70:30)	Drying gas	Ar (520 L h^{-1}, 250°C)
Flow rate	0.5 mL min^{-1}	V-cap	3,000 V
Oven temp.	30°C	Collision gas	N_2, 30L h^{-1}
Injec. vol.	4 µL	SIM ion	Ractopamine - 302.2>164.2, 302.2>284.2, 302>107 Ractopamine-D5 - 307.2>167.3
		Dwell time	0.2 s

Results and discussion.

Validation parameters such as matrix calibration curve, decision limit (CCα), detection capability (CCβ), precision and accuracy were determined. Linearity was demonstrated in the range of 0.05-0.5 µg kg^{-1}. The correlation coefficients (R^2) of the standard curves were at least 0.9923 for the analyte considered. Muscle *bovine* samples (n=6) were fortified at 0.05, 0.1 and 0.15 µg kg^{-1} levels with ractopamine and the results are shown in Table 2.

The CCα was determined by the intercept plus 2.33 times the standard error of the within laboratory reproducibility of the intercept. The CCβ was determined using the signal at CCα plus 1.64 times the standard deviation of the spike samples of the within laboratory reproducibility. The CCα parameter was determined to be 0.02 µg kg^{-1} and CCβ parameter was determined to be 0.03 µg kg^{-1} to ractopamine in *bovine* muscle. Typical chromatograms are shown (Figures 1 to 4).

Table 2. Precision and accuracy for ractopamine.

	Spike level (µg kg^{-1})	Precision (%)	Accuracy (%)
Ractopamine	0.05	4.20	96.0
	0.1	5.48	99.5
	0.15	5.38	99.5

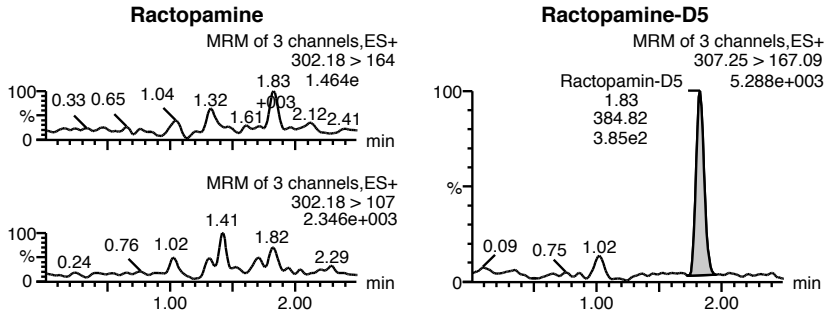

Figure 1. Chromatogram of a blank bovine *muscle.*

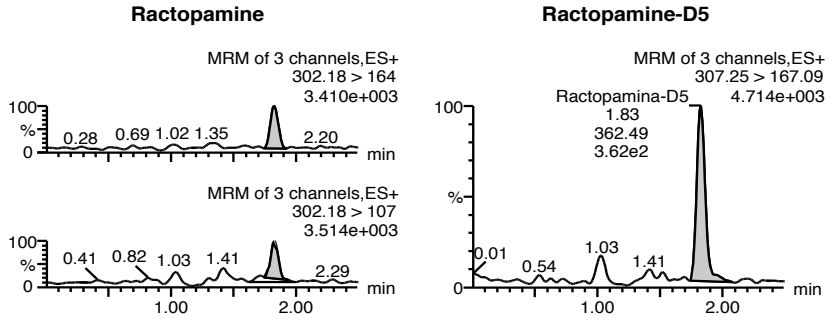

Figure 2. Chromatogram of ractopamine at 0.05 µg kg^{-1}.

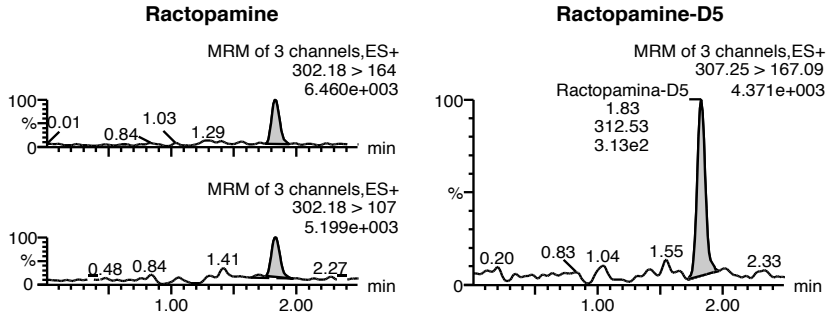

Figure 3. Chromatogram of ractopamine at 0.1 µg kg^{-1}.

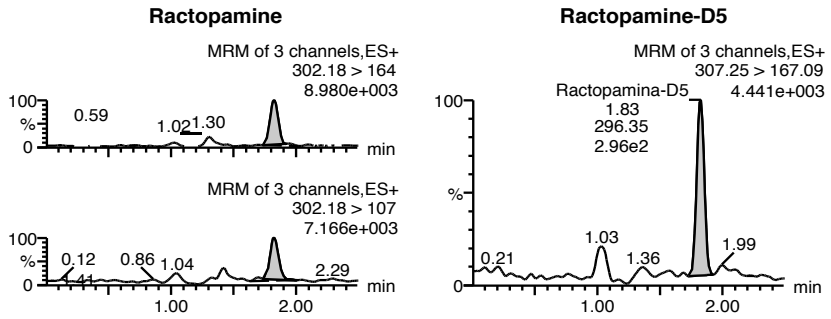

Figure 4. Chromatogram of ractopamine at 0.15 µg kg^{-1}.

Conclusion

On the basis of the results obtained for precision, decision limit, detection capability, and accuracy, it was concluded that the described method is suitable for analysis of ractopamine in *bovine* muscles fulfils the method performance criteria for zero tolerance substances, adopted by certain meat importers, and is suitable for Official Monitoring proposes in Brazil. The method is fast simple, accurate and confirmatory and can be applied to the control of meat consignments which destination to countries with a zero tolerance for ractopamine residues in foods. The method is able to quantify samples with a 0.05 µg kg^{-1} content of ractopamine qualifying the methods as very sensitive and provides assurances to the meat industry with respect of very low levels of ractopamine residues eventually present in their meat production.

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ANALYTICAL TECHNIQUES FOR MONITORING VETERINARY DRUG RESIDUES IN POULTRY IN SINGAPORE

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Abstract
Poultry meat, typically chicken and duck, are commonly consumed in Singapore. These products are imported from AVA-accredited farms and establishments. Monitoring of veterinary drug residues in poultry products in Singapore was initially implemented with Microbial Inhibition Assays (MIA) and Enzyme-Linked Immunosorbent Assays (ELISA), and was later enhanced with HPLC-MS/MS based multiple residue monitoring techniques. To achieve high throughputs in the laboratory to handle the increasing number of poultry samples under import surveillance, the multiple residue test method has been optimized by using single step solid-liquid extraction, doing away with tedious Solid Phase Extraction (SPE) clean-ups and analysing sample extracts with highly sensitive tandem mass spectrometry. The multi-residue method is validated in poultry meat and has been used for surveillance of poultry imports and local establishments. This paper presents the analytical techniques developed for monitoring veterinary drug residues in poultry products in Singapore.

Introduction
Veterinary drugs, especially antibiotics, nowadays have been widely used in the intensive farming of livestock, either for disease prevention or treatment, pest management, or most of the time growth promotion. Overdose, inappropriate usage or non-compliance with withdrawal period could result in residues above safety levels remained in animal tissues and products. Public health would therefore be placed on fringe of risk and antibiotic resistance would widely spread and become a global threat.

Under the Integrated Food Safety Programme of AVA, Singapore, poultry meat, typically chicken and duck, are only imported from AVA-accredited farms and establishments. The health certificates issued by the authority of exporting country are required, at the same time, receiving-end inspection and residues testing in laboratory are conducted to ensure the safety of the imported food commodities.

The analytical techniques for veterinary drug residue testing evolved fast with the development of modern analytical industry. Microbial Inhibition Assays (MIA) and the enzyme-linked immunosorbent assay (ELISA) were the dominant screening techniques in AVA before 2000. High performance liquid chromatography (HPLC) coupled to UV detector, on the other hand, was used for confirmation and quantitation of the suspected cases from preliminary screening. HPLC coupled to tandem mass spectrometer (MS/MS) was gradually introduced for drug residues analysis due to the excellent sensitivity and specificity. Starting with single drug group, *e.g*. detection of nitrofuran metabolites in 2002, now the multi-residue testing approach with HPLC-MS/MS is the major technique, which has already replaced MIA and ELISA, for screening and quantitative analysis of drug residues in AVA, Singapore.

This paper described a quantitative multi-residue method with HPLC-MS/MS for simultaneous detection of more than 70 veterinary drugs in poultry meat.

Materials and Methods

Instrument and analytical conditions
The HPLC-MS/MS system consisted of an Agilent 1290 HPLC and AB Sciex 5500 QTRQP mass spectrometer. The HPLC column used for the analysis was Zorbax C18 with a particle size of 1.8 µm and dimension of 50 x 2.1 mm. The guard column was Phenomenex C18 with a dimension of 4.0 x 2.0 mm. Column oven temperature was set at 40°C. The mobile phases were H_2O with 2 mM ammonium formate and 0.1% formic acid as A and MeOH with 0.1% formic acid as B. The flow rate was 0.5 mL min^{-1} and the injection volume was 10 µL. The detailed HPLC gradient was shown in Table 1.

Acquisition was conducted with scheduled multiple reaction monitoring (MRM) under ESI+ or ESI– mode. Direct infusion of reference standards was performed to tune the MS to obtain compound dependent parameters (DP, CE, CXP, etc.) for every analyte. Optimization of ion source and gas parameters was achieved by using the least sensitive compound to manually tune these parameters (IS, TEM, CUR, GS 1 and 2) to obtain the most intensive signals (Table 2).

Table 1. HPLC Gradient.

Time (min)	A (%)	B (%)
0.0	90	10
2.0	10	90
3.0	10	90
3.1	90	10
5.0	90	10

Table 2. Ion source and gas parameters.

Parameters	Settings
Curtain Gas (CUR)	20
Collision Gas (CAD)	medium
Ion Spray Voltage (IS)	4500 volts
Temperature (TEM)	550°C
GS1	50
GS2	50

Sample preparation

An amount of 5 g of homogenised poultry meat was weighed into 50 mL centrifuge tube. After spiking the internal standards, the tube was covered with aluminium foil and left stand at room temperature for 30 min. Then 10 mL of acetonitrile (ACN) acidified with 0.1% formic acid and a ceramic homogeniser (Agilent) were added. The mixture was vigorously shaken at 400 rpm for 10 min. After centrifugation at 4,000 rpm for 10 min, 5 mL aliquot of the supernatant was blow-dried with nitrogen gas at 40°C and further reconstituted (RS) with 0.5 mL of ACN:H_2O (75:25, v/v, 0.1% formic acid). The RS extract was filtered and injected in HPLC-MS/MS for drug residue analysis. The flowing drugs are analysed under positive mode: nitroimidazoles (*e.g*. ipronidazole, ronidazole, metronidazole), β-agonists, quinolones, fluoroquinolones, macrolides, sulfonamides, coccidiostats, and penicillins. The following drugs are analysed under negative mode: amphenicols (*e.g*. chloramphenicol, florfenicol, thiamphenicol), and coccidiostats (e.g. nicarbazin). A schematic flow chart of the sample preparation procedure was illustrated with Figure 1.

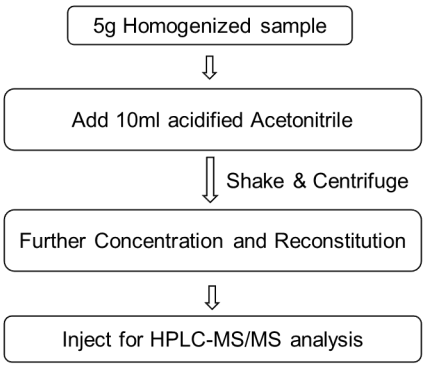

Figure 1. Flow chart of sample preparation

Results and Discussion

Selection and evaluation of internal standards

Stable-isotope-labelled (SIL) internal standards (IS), such as multiple deuterium or carbon-13 labelled analogues, are recommended for quantitative HPLC-MS/MS analysis when feasible. The SIL IS can help to correct the variability in the extraction and sample preparation steps, as well as the matrix effects encountered by mass spectrometers. We had encountered IS issues when analysing a proficiency test (PT) sample for oxolinic acid, a quinolone antibiotic. Two SIL ISs were used and two quantitation results were compared (Table 3). Oxolinic acid-D5, which has 5 deuterium replacements in the molecule, yielded more accurate result than the structural analogue nalidixic acid-D5, although the latter is also an SIL IS. The structures of oxolinic acid and nalidixic acid were show in Figure 2.

Table 3. SIL IS vs Structural Analogue SIL IS.

Test compound	IS	Assigned value (ppb)	Results (ppb)
Oxolinic acid	Nalidixic acid-D5	64.1	190.3
Oxolinic acid	Oxolinic acid-D5	64.1	65.6

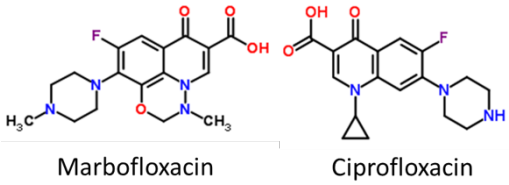

Figure 2. Structure of oxolinic acid, nalidixic acid, ciprofloxacin and marbofloxacin

It should be noticed that it is not possible to have commercially available SIL IS for every analyte, so sometimes a structural analogue as IS or external calibration may have to be used for quantitative analysis with HPLC-MS/MS. A typical example was the analysis of marbofloxacin with ciprofloxacin-D8 as internal standard in a PT sample (Table 4 and Figure 2). There was no SIL IS for marbofloxacin at the time of analysis, ciprofoxacin-D8, a structure analogue was then used and the obtained result was compared with the one from an external calibration experiment. External calibration rendered better result than internal calibration with structure analogue.

The above two cases have indicated that internal standard needs to be carefully evaluated no matter it is a SIL analogue or a structural analogue during method validation.

Table 4. Internal calibration vs External Calibration.

Test compound	IS	Assigned value (ppb)	Results (ppb)
Marbofloxacin	Ciprofloxacin-D8	89.7	137.3
Marbofloxacin	-	89.7	67.8

It has also been observed that the chromatographic behaviour of SIL IS may not be exactly the same as the analyte of interest, *e.g* slightly different retention time of clenbuterol (t_R=2.67 min) and its SIL IS clenbuterol-D9 (t_R=2.63 min). The ISs that have been evaluated and selected in this multi-residue screening method were shown in Table 5.

Table 5. Internal standards used in the analysis.

Drug group	IS
Amphenicols	Chloramphenicol-D5
Quinolones	Nalidixic acid-D5, oxolinic acid-D5
Fluoroquinolones	Ciprofloxacin-D3
Nitroimidazoles	Ronidazole-D3
Macrolides	Erythromycin-^{13}CD3
Beta-agonists	Clenbuterol-D9, Ractopamine-D3
Penicillins	Benzylpenicillin-D7
Sulfonamides	Sulfaphenazole
Coccidiostats	Robenidine-D8, decoquinate-D5

Matrix Matching Calibration

Ion suppression is very common when analysing drug residues in animal tissue samples with mass spectrometry. Matrix matching calibration is therefore critical for accurate quantitation. If standard solutions are used to construct calibration curve (standard calibration), the level of analyte in sample is likely to be under estimated (as shown in Table 6 and Figure 3). Due to the severe ion suppression from matrix, the MS detector may not be able to response sensitively with the increase of concentration of the analyte, resulting in a smaller slope of the matrix matching calibration curve than the one constructed with pure standard solutions (standard calibration) (Figure 3).

Table 6. Matrix matching calibration vs Standard calibration.

Test compound: sulfadiazine	Peak Area	Result of Matrix Matching Calibration (ppb)	Result of Standard Calibration (ppb)
Unknown sample X	448000	84.1	32.5

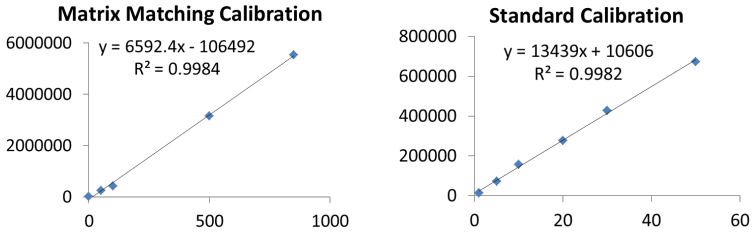

Figure 3. Matrix Matching Calibration curve vs Standard Calibration for quantitation of sulfadiazine in an unknown chicken sample.

Method Validation

Linearity. Validation on linearity in matrix was conducted by spiking reference standards into matrix blank at various concentrations (*e.g*. LOD, LOQ, 0.5 MRL, MRL, 1.5 MRL and 2 MRL). Six-point calibration curves were constructed using linear regression on three different days and the correlation coefficients (r^2) were expressed to demonstrate the good correlation between concentrations and MS signals within the linearity range (Table 7).

Table 7. Linearity range and r^2.

Drugs	Spiking range (ppb)	r^2
Amphenicols	0.3-1.5	>0.99
Beta-agonists	1.0-17	>0.98
Nitroimidazoles	2.0-34	>0.99
Quinolones	5.0-85	>0.98
Fluoroquinolones	10-170	>0.99
Sulfonamides	10-170	>0.99
Macrolides	10-150	>0.98
Penicillins	20-80	>0.98
Coccidiostats (monensin, narasin *et al*)	5.0-25	>0.99
Coccidiostats (nicarbazin *et al*)	10-100	>0.98

Sensitivity. Chloramphenicol was a prohibited drug due to its carcinogenic effect, so minimum required performance limit (MRPL) of the detection method was requested for its analysis by EU. This method was able to detect chloramphenicol at its MRPL of 0.3ppb. Similarly, nitroimidazoles were also prohibited substances and this method was able to sensitively detect nitroimidazoles at 1-2 ppb. As for the veterinary drugs with maximum residue limit (MRL), LODs far below the MRLs were achieved with this method. The details of method validation on sensitivity can be found in Table 8.

Precision. The precision was evaluated by the inter- and intra-day variations at various concentrations in matrix. In all cases the CV% was <15% for most of the drugs, except coccidiostats for which a slightly higher CV% (< 23%) was obtained.

Table 8. Method validation -sensitivity

Drugs	**MRL (EU) (ppb)*	*LOD (ppb)*	*LOQ (ppb)*	*CCα (ppb)*	*CCβ (ppb)*
Chloramphenicol	0.3 (RPA)	0.1	0.3	0.1	0.1
Thiamphenicol	50	0.1	0.4	0.1	0.2
Florfenicol	100	0.1	0.3	0.1	0.1
Beta-agonists	-	1	3	0.3	0.9
Tylosin	100	4.9	16.5	3.8	6.5
Erythromycin	200	9.5	31.6	7.4	12.5
Oleandomycin	-	6.1	20.5	4.8	8.1
Tiamulin	100	9.4	31.4	7.3	12.5
Josamycin	-	10.2	34.1	7.9	13.5
Tilmicosin	75	11.3	37.6	8.8	14.9
Spiramycin	200	10.5	34.8	8.1	13.8
Penicillin G	50	7.6	25.4	5.9	10.1
Ampicillin	50	4.8	16.1	3.2	5.8
Oxacillin	300	6.1	20.2	4.7	8.0
Nafcillin	300	6.9	22.9	5.1	8.9
Cloxacillin	300	8.7	29.0	4.9	9.7
Dicloxacillin	300	6.2	20.7	1.9	5.3
Amoxicillin	50	6.4	21.4	4.5	8.0
Norfloxacin	-	6.0	19.9	0.6	2.5
Ciprofloxacin	100 (Sum of Cipro & Enro)	6.9	23.0	4.2	7.9
Enrofloxacin	100 (Sum of Cipro & Enro)	8.4	27.9	3.8	6.5
Danofloxacin	200	19.5	65.0	9.0	15.4
Sarafloxacin	-	3.7	12.5	0.9	2.1
Difloxacin	300	10.4	34.7	4.7	7.9
Marbofloxacin	-	5.2	17.4	0.9	2.6
Sulfonamides	100 (all substances belonging to the sulphonamide group)	9-13	30-50	3-9	10-30
Oxolinic Acid	100	5.6	18.7	4.4	7.4
Flumequine	400	6.5	21.6	5.0	8.6
Nalidixic Acid	-	6.2	20.7	4.8	8.2
Dimetridazole	-	0.6	2.0	0.4	0.8
Hydroxy dimetridazole	-	0.6	2.0	0.5	0.8
Ipronidazole	-	0.6	1.9	0.4	0.8
Metronidazole	-	0.7	2.2	0.3	0.6
Hydroxy ipronidazole	-	0.6	1.9	0.4	0.7
Hydroxy metronidazole	-	0.6	1.9	0.1	0.3
Ronidazole	-	0.6	1.9	0.4	0.7
Nicarbazin	-	5.6	18.6	4.3	7.4
Narasin	-	1.9	6.4	0.5	1.6
Salinomycin	-	8.0	26.7	4.8	9.2
Monensin	-	2.5	8.2	0.6	1.6
Lasalocid	60	3.6	12.1	1.2	2.7
Diclazuril	500	4.2	13.9	3.2	5.5
Maduramycin	-	17.2	57.2	11.0	18.7
Robenidine	-	6.1	20.3	2.8	6.1
Clopidol	-	11.3	37.8	3.1	7.7
Amprolium	-	23.1	77.1	5.3	13.7
Decoquinate	-	2.8	9.5	0.5	1.5

**MRLs by EU are tabulated. Codex MRL may not be the same as EU MRL.*

HPLC-MS/MS vs HPLC-HRMS

High performance liquid chromatograph coupled with triple quadrupole mass spectrometer (HPLC-MS/MS) has good sensitivity and reproducibility which enable accurate quantitation of residues on a level of part per billion (ppb) or even part per trillion (ppt) and thus has become the choice of technology for drug residue analysis. However, reference standards are necessary for method development especially equipment tuning to obtain the desired sensitivity. As limited numbers of analytes can be analysed within one analytical run, a typical multiple residue method by HPLC-MS/MS can analyse up to maximum of about 300-400 compounds without compromising the sensitivity. If the desired coverage for residues is more than that limit, and/or extremely sensitive detection is required, more separate methods and analytical runs are unavoidable.

The advantages of high resolution mass spectrometry (HRMS) (*e.g*. time-of-flight (TOF) MS or orbitrap MS) are the incomparable mass resolution and accuracy, which enables the most accurate measurement of mono-isotopic mass signals for confident discrimination of co-eluting isobaric compounds from complex background. There is almost no limit on number of signals to be scanned and recorded, and the high definition data on molecule and fragment ions, isotopic patterns together with retention time enable the most confident confirmation for residue detection. The advanced technology also enables the fast switching between positive and negative monitoring modes which significantly shortens the analysis time by at least 50% comparing to two separate runs under either mode.

Based on the current understanding of HPLC-MS/MS and HPLC-HRMS (Table 9) and experience of multi-residue testing approaches, we would like to anticipate that HRMS may play an increasingly important role together with HPLC-MS/MS on fast screening of veterinary drug residues in complex sample matrices (Kaufmann *et al.*, 2008; Ortelli *et al.*, 2009). The potential new testing approach (HRMS for screening and MS/MS for quantitation) would provide the desired coverage and sensitivity, at the same time accurate and reproducible quantitation results. Laboratory may also be able to identify possible misuse/out-label use of human medicines or other contaminants in animal products if a well- established exact mass library is available.

Table 9. Comparison between MS/MS and HRMS.

Item for Comparison	MS/MS	HRMS
Sensitivity	+++	+
Reproducibility	+++	+++
Reference standards	+	+++
Numbers of analyte	+	+++
Mass Resolution & accuracy	+	+++
Specificity	++	++
Application	++	+++
Screening	+	+++
Quantitation	+++	+
Robustness	+	+++
Price	+++	+

+: acceptable; ++: good; +++: excellent

QuEChERS as a choice for sample clean-up

Sample preparation is always the bottle neck for fast screening techniques. The testing matrices for veterinary drugs are animal products, such as muscle, milk, egg, honey and edible organs (liver and kidney). Protein and phospholipid from matrix are the major sources for ion suppression and dirty sample extracts can easily bring problems to HPLC system and MS detector. Sample clean-up is therefore unavoidable for residues analysis and most of the time solid phase extraction (SPE) has to be applied before HPLC-MS/MS acquisition. In this multi-residue method, SPE was not introduced with the consideration that some drugs may be irreversibly retained in the SPE absorbing material leading to poor extraction recovery and tedious SPE cartridge preparation and elution steps also sacrifice the throughput of the testing laboratory. A 3K-mass filtration unit was used instead as a clean-up step for sample preparation to remove interfering big biomolecules and physical debris/particles from the sample extract. However, soluble interfering molecules may still remain (carbohydrates, small proteins, peptides, and lipids etc.) and cause problems on analysis (*e.g*. ion suppression) and result in frequent maintenance of equipment.

QuEChERS has been successfully used for pesticide residue analysis in vegetables and fruits (Anastassiades *et al.*, 2003). Studies on application of QuEChERS on veterinary drug residues are also increasing (Abdallah *et al.*, 2014; Martinex-Villalba *et al.*, 2013; Wei *et al.*, 2015). Our preliminary studies on QuEChERS as additional clean-up step in sample preparation have been encouraging, where 80% of tested drugs could be sensitively detected by both HPLC-MS/MS (ABSciex 5500) and HPLC-HRMS (Exactive from Thermo) at 50% MRL levels. QuEChERS or similar approach such as salting out supported liquid extraction

(SOSLE) may also be considered for drug residue analysis in aqueous matrices, such as liquid egg, milk and honey (Kaufmann *et al.*, 2014).

If instrument (either HPLC-MS/MS or HPLC-HRMS) with better sensitivity is to be used, further concentration and reconstitution may not be necessary and QuEChERS or similar technique still can be considered as a choice for sample clean-up.

Conclusions and recommendations

This method is fast, sensitive and robust, and is therefore suitable for routine screening of veterinary drug residues in poultry meat. This method can also be considered for detection of drug residues in other animal products such as edible organs (liver and kidney), eggs and milk.

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BEADYPLEX: A NOVEL MULTI-ANTIBIOTIC FLOW CYTOMETRIC SCREENING METHOD FOR FOOD COMMODITIES

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Abstract
Misuse or overuse of veterinary antibiotics may lead to the undesired presence of antibiotic residues in food commodities, thus representing a health risk for consumers or decreasing industrial processes yield. Efficient screening analytical methods for the early detection of antibiotic residues in food commodities are therefore essential in order to guarantee food safety. BEADYPLEX is a multi-residue screening method for the simultaneous analysis of antibiotics in meat. This flow cytometric-based competitive immunoassay is based on the use of fluorescent encoded microparticles as solid support to build the assays, which combined with their corresponding binders (antibodies and receptors) allow the multiple detection of more than 80 compounds belonging to the most relevant veterinary antibiotics, namely tetracyclines, sulfonamides, β-lactams, aminoglycosides, colistin, macrolides, lincosamides, (fluoro)quinolones, phenicols and pleuromutilins. This high throughput method provides the identification of the suspected antibiotic family in a single test, thus contributing to a significant reduction of global costs of analysis, as confirmatory methods by instrumental techniques can be rapidly selected.
The results of the validation of BEADYPLEX in *porcine*, *bovine* and poultry muscle following CRL Guidelines for screening methods are presented. Furthermore, a preliminary study shows the broad-applicability of the method, which can be used in diverse food matrices, including meat, seafood, milk and eggs.

Introduction
Antibiotics are extensively used in modern agricultural farming with prophylactic and therapeutic purposes against diverse infections. In the European Union their utilisation as growth-promoters is prohibited since 2006. The presence of antibiotic residues in final animal products represent a potential threat to consumer health. Adverse effects to antibiotics include allergies, toxic or gastro-intestinal reactions (Burke and Cunha, 2001). Furthermore, antibiotic misuse has also been correlated to the development of antibiotic resistance, an issue affecting human and veterinary medicine worldwide (McDermott *et al*., 2002). On the other hand, antibiotic residues in starting materials may as well hamper industrial fermentation processes involving dairy or meat derivative products (Kjeldgaard *et al*., 2012). European Regulation 2010/37/UE sets up maximum residue limits (MRL) for authorized antibiotics in different food commodities. For the banned substance chloramphenicol a minimum required performance limit (MRPL) is established instead (Commission Decision 2003/181/EC).

Efficient screening analytical methods providing an early detection of antibiotic residues in food are needed to guarantee food safety, and ultimately to ensure consumer protection. Inhibition microbiological tests are inexpensive, require minimal sample preparation, and allow the analysis of multiple samples in parallel. Nevertheless, the identification of the antibiotic family in a single analysis per sample is not possible. Immunological methods, such as ELISA or immunodipstick, have been as well extensively used for the monitoring of antibiotic residues in food commodities. Despite their multiple advantages (rapidity, cost-effectiveness, high-throughput capability, high sensitivity and specificity, on-site application), classical immunoassays are usually contaminant or family-specific, or are limited to the simultaneous analysis of few contaminants or families in the same sample. As a consequence, in order to detect multiple targets it is required to conduct different assays in parallel. Flow Cytometry-based ImmunoAssays (FCIA), by exploiting the multi-parametric characterization of microparticles, allow the simultaneous analysis of multiple targets in a single analysis per sample, thus efficiently contributing to increase analysis volume, while reducing global analysis time and costs.

Essentially, in FCIA tests, a set of different microparticles individually encoded by their specific size and internal fluorescence intensity are used as solid support for the development of different immunoassays. By using a combination in the same assay of several microparticles and receptors/ antibodies, multiple immunoassays are performed simultaneously in a 96-well microplate format, and the detection of the complete range of compounds targeted by the individual immunoassays can be achieved in one single analysis. FCIA constitute an alternative technology enabling simultaneous multi-analyte, rapid, efficient and cost-effective screening of potentially harmful contaminants in large number of food samples. Furthermore, FCIA is a very versatile and flexible technique which allows the implementation of other immunoassays to previously existing ones, thus increasing the multiplexicity of the system.

Herein we present the results of the validation of BEADYPLEX, a multi-residue screening FCIA method for the simultaneous analysis of antibiotics, in *porcine*, *bovine* and poultry muscle following CRL guidelines for the validation of screening methods

for residues of veterinary medicines, based on European Decision 2002/657/EC. The detection capabilities (CCβ) for all the antibiotics recognized by the method have been determined.

Materials and Methods

BEADYPLEX kits
FCIA BEADYPLEX kits (Unisensor SA) are constituted by a 12-plex mixture of fluorescent conjugated particles dedicated to the detection of tetracyclines, sulfonamides, β-lactams, aminoglycosides, colistin, macrolides, lincosamides, (fluoro)quinolones, phenicols and pleuromutilins; a mixture of primary binders (antibodies and receptors) directed to the previously mentioned antibiotic families; and a mixture of fluorescence-labelled reporter molecules. Extraction and assay buffers, as well as 96-microwell plates, are also provided in the kits.

Sample fortification
An amount of 1 g of homogenised muscle was fortified with 25 µL of a 40-fold concentrated standard solution containing one representative antibiotic per family. At least 20 samples per matrix were fortified with every antibiotic mixture, and used for the determination of the CCβ (β error ≤ 5%). Samples were incubated 1 h prior to extraction.

Sample extraction
A portion of 1 g of homogenised muscle (either blank or fortified) was extracted with 1 mL of extraction buffer upon vigorous shaking for 10 min. The supernatant obtained upon centrifugation (4,500 *g*, 15 min) was filtered using a 96-well filterplate.

BEADYPLEX protocol
A volume of 50 µL of the filtered extracts were mixed in a 96-well filterplate with 50 µL of the 12-plex mixture of particles and 50 µL of the primary binders reconstituted solution. After 30 min of incubation (RT, shaking, darkness), plates were washed (3x) with assay buffer. A second incubation step was performed with 150 µL well^{-1} of reconstituted solution of fluorescent reporters for 15 min (same conditions). Particles were finally re-suspended in 150 µL assay buffer after final washing of the plate. The plates are immediately analysed with the flow cytometer (Accuri C6, BD Biosciences; or Novocyte 2000, ACEA Biosciences).

Upon automatic identification of each particle (according to their size and internal fluorescent intensity), the external fluorescent signal provided by the reporters for every particle was then compared to the signal provided by the blank samples. These normalized values (Fluorescence of the fortified sample*100/Fluorescence of the blank sample) were compared to the cut-off associated to every particle, and used to identify the samples as screen positive or screen negative for each antibiotic family. CCβ values were determined as the lowest concentration of antibiotic for which at least 95% of the samples fortified at this level were identified as screen positive by BEADYPLEX.

Results
Initially, antibiotics from the ten families of interest (tetracyclines, sulfonamides, β-lactams, aminoglycosides, colistin, macrolides, lincosamides, (fluoro)quinolones, phenicols and pleuromutilins), altogether with other veterinary drugs not included in the scope (banned substances, growth-promoters, endocrine disruptors, anaesthesics, β-agonists, AINS, steroids/corticosteroids, antithyroids, coccidiostatic, anthelmintics), were tested in order to establish the detection profile of BEADYPLEX. No interference was observed from any compound other than those belonging to the ten mentioned families, thus demonstrating the specificity of the method. Furthermore, each antibiotic only provided a positive response for its corresponding particle.

Then, CCβ values were determined for all of the detected antibiotics following CRL Guidelines for *porcine*, *bovine* and poultry muscle. For most compounds, the detection capabilities are at or below the corresponding European MRL (Table 1).

The application of BEADYPLEX for the determination of residues in other food matrices than meat was further investigated. Figure 1 shows the results obtained from a fortification study conducted with muscle (*porcine*, *bovine*, poultry), fish (salmon and tuna), shrimps, milk and eggs, using the positive control included in the kit, which contains a mixture of one antibiotic from each family at the MRL value established for *porcine* muscle.

These results clearly indicate that BEADYPLEX is potentially suited for the screening of multiple antibiotics in diverse food commodities, as for all the evaluated antibiotics a signal below the corresponding assay cut-off (dark bars) is observed. The only exceptions were oxytetracycline in milk, for which an increase of the tetracycline assay cut-off is required, and tiamphenicol in tuna and egg, for which an improvement on assay sensitivity is necessary in order to fulfil regulatory limits.

Table 1. Detection capabilities (µg kg^{-1}) for BEADYPLEX in muscle

		Porcine muscle		*Bovine* muscle		Poultry muscle	
Family	Antibiotic	MRL	CCβ	MRL	CCβ	MRL	CCβ
Aminoglyco-sides	Streptomycin	500	250	500	250	-	250
	Dihydrostreptomycin	500	125	500	125	-	125
	Gentamicin (mix C1, C1a, C2, C2a, C2b)	50	50	50	50	-	50
	Neomycin B	500	100	500	100	500	100
	Kanamycin	100	100	100	100	100	100
	Paromomycine	500	250	500	500	500	500
	Apramycin	-	50	1000	2500	-	2500
β-Lactams	Cefquinome	50	50	50	50	-	50
	Ceftiofur	1000	50	1000	50	1000	50
	Desfuroylceftiofur (DCF)	1000	2000	1000	2000	1000	2000
	Cefoperazone	-	3	-	3	-	3
	Cefalexin	-	3500	200	3500	-	3500
	Cefalonium	-	5	-	5	-	5
	Cefapirin	-	75	50	50	-	50
	Desacetylcefapirin	-	100	-	100	-	100
	Cefazolin	-	100	-	100	-	100
	Cefacetrile	-	50	-	50	-	50
	Penicilline V	25	12,5	-	12,5	25	12,5
	Penicilline G	50	5	50	5	50	5
	Ampicillin	50	25	50	25	50	25
	Amoxycillin	50	25	50	25	50	25
	Oxacillin	300	50	300	50	300	50
	Cloxacillin	300	50	300	50	300	50
	Dicloxacillin	300	30	300	30	300	30
	Nafcillin	-	300	300	300	300	300
	Piperacillin	-	5	-	5	-	5
(F)Quinolones	Marbofloxacin	150	20	150	20	-	20
	Flumequine	200	800	200	2000	400	2000
	Enrofloxacin	Sum = 100	5	Sum = 100	5	Sum = 100	5
	Ciprofloxacin		10		20		20
	Danofloxacin	100	250	200	200	200	200
	Oxolinic acid	100	800	100	1000	100	1000
	Difloxacin	400	50	400	50	300	50
	Norfloxacin	-	2,5	-	10	-	10
	Sarafloxacin	-	125	-	125	-	125
	Pefloxacin	-	10	-	20	-	20
	Enoxacin	-	50	-	50	-	50
	Lomefloxacin	-	15	-	15	-	15
	Ofloxacin	-	15	-	15	-	15
	Cinoxacin	-	800	-	800	-	800
	Nalidixic acid	-	2000	-	2000	-	2000
Lincosamides	Lincomycin	100	25	100	25	100	25
	Clindamycin	-	150		500	-	300
Macrolides	Tilmycosin	50	50	50	50	75	50
	Tylosin A	100	20	100	10	100	10
	Tylvalosin	50	125	50	125	-	125
	Tildipirosin	1200	1200	400	1200	-	1200
	Spiramycin	250	250	200b	200	200b	200
Polymyxin	Colistin	150	150	150	150	150	150

Table 1. (continued).

Phenicols	Chloramphenicol	0,3	0,45	0,3	0,45	0,3	0,45
	Florfenicol	300	2100	200	2100	100	2100
	Thiamphenicol	50	100	50	100	50	100
Tetracyclines	Chlortetracycline	100	25	100	100	100	100
	Doxycycline	100	25	100	25	100	25
	Oxytetracycline	100	50	100	100	100	100
	Tetracycline	100	50	100	50	100	50
	Demeclocycline	-	50	-	100	-	100
	Methacycline	-	25	-	50	-	50
Sulfonamides	Sulfadimidine = sulfamethazine	Combined sul-fonamides = 100	100	Combined sul-fonamides = 100	100	Combined sul-fonamides = 100	100
	Sulfadiazine		50		50		50
	Sulfamethoxazole		10		10		10
	Sulfadimethoxine		50		50		50
	Sulfadoxine		10		10		10
	Sulfachloropyridazine		1		1		1
	Sulfapyridine		0,5		0,5		0,5
	Sulfaguanidine		1		1		1
	Sulfathiazole		0,5		0,5		0,5
	Sulfamethoxypyridazine		0,25		0,25		0,25
	Sulfamerazine		20		20		20
	Sulfaclozine		500		500		1000
	Sulfamethizole		1		1		1
	Sulfamonomethoxine		10		20		10
	Sulfaquinoxaline		50		50		50
	Sulfameter = sulfamethoxydi-azine		5		5		5
	Sulfanilamide		50		50		50
	Dapsone		0,2		0,2		0,2
Pleuromu-tilins	Valnemulin	50	50	-	50	-	50
	Tiamulin	100	25	-	25	100	25

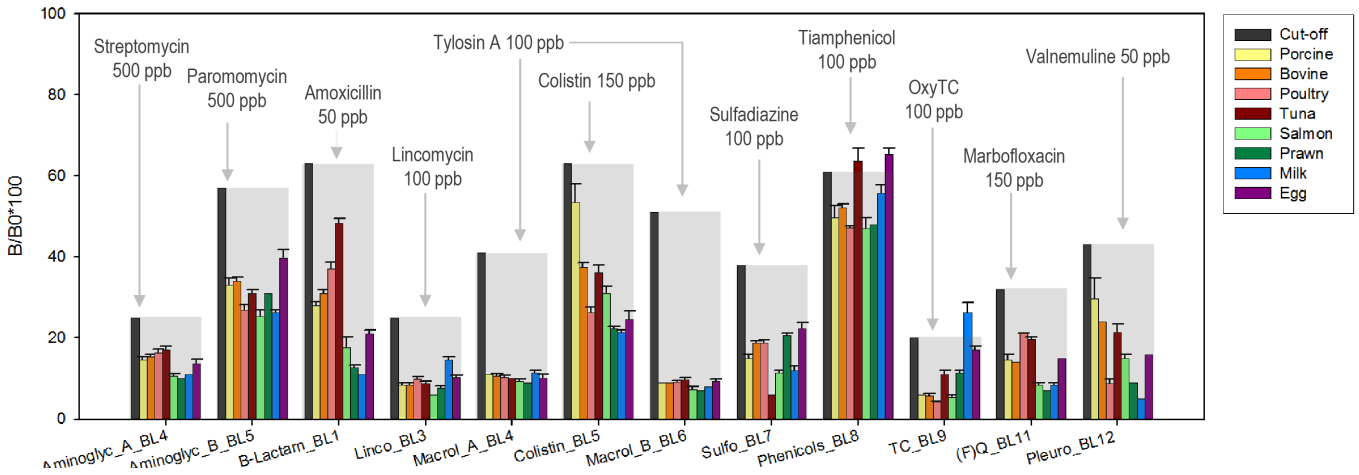

*Figure 1. Fortification study with a multi-antibiotic mixture in different food commodities. The normalized response (B*100/B0) calculated with respect to the corresponding blank matrix is represented for every individual bead included in the 12-plex mixture of BEADYPLEX particles (BL = Bead Level).*

Discussion and conclusions

We present a unique flow cytometric-based immunoassay, BEADYPLEX, for the simultaneous screening in food commodities of more than 80 compounds from the most relevant antibiotic families (tetracyclines, sulfonamides, β-lactams, aminoglyco-

sides, colistin, macrolides, lincosamides, (fluoro)quinolones, phenicols and pleuromutilins). BEADYPLEX present multiple advantages over other screening methods, mainly broad-range detection profile, broad applicability, the identification of the antimicrobial family in one single test per sample and a high-throughput capability.

The implementation of this method for the monitoring of antibiotics may significantly reduce both analysis time and global costs of analysis, by the rapid identification of the antibiotic suspected family, facilitating the choice of the confirmatory technique. Furthermore, the flexibility and versatility of the technique is another remarkable advantage, as the same assay may be customized, either by adapting the required levels of sensitivity or by the addition of new contaminants of interest. New kits based upon the same technology for the screening of other relevant food contaminants are under development.

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CHLORAMPHENICOL AND NITROFURAN METABOLITES – A COMBINED METHOD FOR FAST AND RELIABLE MONITORING OF SHRIMPS

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Abstract

The antibiotics chloramphenicol (CAP) as well as nitrofuran (NF) metabolites are still relevant to investigate within monitoring programs for shrimps and other seafood. Especially CAP is a cheap and effective antibiotic veterinary drug. Concentrations found in seafood are very low, but permanent exposure to them can induce antibiotic resistance. However, there are potential health risks to humans. Therefore, these substances have been prohibited in the European Union for more than 20 years. Low minimum required performance levels (MRPL) of 0.3 µg kg^{-1} for CAP and 1 µg kg^{-1} for each NF metabolite in aquaculture products were established by the European Commission (Decision 2003/181/EC.).

Within the industry, self-control of, in particular, raw materials has to be performed just-in-time to avoid delay at import. To meet all requirements, a method was developed for the simultaneous analysis of chloramphenicol and nitrofuran metabolites in seafood. The usual two-day sample preparation procedure for nitrofuran metabolites was optimized. The presented method showed that one-day-analyses with an LOQ of 0.1 µg kg^{-1} for CAP and 0.5 to 1.0 µg kg^{-1} for the NF metabolites is feasible. The method was successfully validated in accordance with the requirements of Commission Decision 2002/657/EC.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic exhibiting activity against both gram-positive and gram-negative bacteria as well as other groups of micro-organisms. It exerts its action through protein inhibition and is effective in the treatment of several infectious diseases. This, together with its low cost and ready availability, has made it extensively used since the 1950's in the treatment of animals worldwide, including shrimp and seafood production. However, at certain susceptible individuals, CAP is associated with serious toxic effects in humans in the form of bone marrow depression, particularly severe in the form of fatal aplastic anaemia [3].

Nitrofurans (NF) comprise a group of antibiotic substances that have been used widely in intensive farming of pigs, poultry, fishes and shrimps. Studies in the late 1980's and early 1990's have proven that they are metabolised shortly after administration and form persistent residues that could be detected in animal tissues for weeks after treatment. Both, the NFs as well as their metabolites, have been classified as genotoxic compounds.

Since their toxicological data did not support the deduction of an ADI and due to a lack of data no maximum residue limit (MRL) could be fixed for CAP or the NF metabolites. Both are banned in many countries and the EU, and are included in Annex IV of Council Decision 2077/90 [4]. European Commission established low minimum required performance limits (MRPL) of 0.3 µg kg^{-1} for CAP and 1 µg kg^{-1} for each NF metabolite in aquaculture and other products (Decision 2003/181/EC) [1].

Within the industry, especially raw materials have to be analysed just-in-time to avoid delay at import. It was usual that the analyses of NF metabolites required two days for hydrolyses, derivatization, extraction and measurement. To meet all requirements of sensitivity and speed, the procedure for the NF metabolites was optimized. A one-day analysis with one LC MS/MS run is presented here. The method was successfully validated with an LOQ of 0.1 µg kg^{-1} for CAP and an LOQ of 0.5 to 1.0 µg kg^{-1} for the NF metabolites in accordance with the requirements of the Commission Decision 2002/657/EC [2].

Materials and Methods

Chemicals

All chemicals were at least analytical grade unless stated otherwise. Acetonitrile was of HPLC grade and the ultrapure water was obtained from a Millipore Synergy UV system (Milipore, Bedford, MA, USA). Formic acid and sodium chloride were also of analytical grade. The solution for derivatization was prepared fresh daily out of 2-nitrobenzaldehyde (2-NBA, Merck, Darmstadt, Germany) and dimethyl sulfoxide. The weighed substances with the corresponding analyte and application are given in Table 1. The analytical standards 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), Semicarbazide (SEM) and 1-Amino-hydantoin (AHD, SEM-^{13}C3, AOZ-D4, AMOZ-D5, AHD-^{13}C3 were purchased from Witega (Berlin, Germany). The corresponding 2-NBA derivatives were purchased from the same supplier. Chloramphenicol and chloramphenicol-D5 were purchased from Dr. Ehrenstorfer /LGC (Augsburg, Germany).

Stock solutions for the nitrofuran metabolites were prepared in methanol and in acetonitrile for CAP. From these solutions the measurement standards, the spiking solution and the internal standard solution were prepared.

Table 1. Weighed substances with corresponding analyte and application solution for the nitrofuran metabolites.

weight substance	application solution	analyte
SEM-hydro chloride	spiking standard	SEM
AHD-hydro chloride	spiking standard	AHD
AOZ	spiking standard	AOZ
AMOZ	spiking standard	AMOZ
1,2-15N2-13C-SEM-hydro chloride	internal standard	1,2-15N2-13C-SEM
AOZ-D4	internal standard	AOZ-D4
AMOZ-D5	internal standard	AMOZ-D5
AHD-13C3-hydro chloride	internal standard	AHD-13C3
2-NBA-1,2-15N2-13C-SEM-hydro chloride	measurement standard	2-NBA-SEM-15N2-13C
2-NBA-AOZ-D4	measurement standard	2-NBA-AOZ-D4
2-NBA-AMOZ-D5	measurement standard	2-NBA-AMOZ-D5
2-NBA-AHD-13C3-hydro chloride	measurement standard	2-NBA-AHD-13C3
2-NBA-SEM-hydro chloride	measurement standard	2-NBA-SEM
2-NBA-AHD-hydro chloride	measurement standard	2-NBA-AHD
2-NBA-AOZ	measurement standard	2- NBA-AOZ
2-NBA-AMOZ	measurement standard	2-NBA-AMOZ

Sample preparation

Homogenisation is crucial for reliable analytical results. The ground sample was weighed into plastic tubes. Hydrochloric acid was added and the sample was spiked with internal standard (containing CAP-D5, SEM-^{13}C3, AOZ-D4, AMOZ-D5, AHD-^{13}C3). After that, the derivatization reagent for the nitrofuran metabolites was added to the mixture and homogenized. The derivatization took place at 50°C for 2 h (water bath). Phosphoric buffer was added to the cooled extract and the pH was adjusted to 7.4. After addition of ethyl acetate, the extract was mixed and centrifuged. An aliquot of the upper phase was reduced to dryness. After reconstitution in methanol/water, the extract was filtered and measured by LC-MS/MS.

LC-MS/MS

Samples were measured with a HPLC 1200 series (Agilent, Waldbronn, Germany) and an AB Sciex API 4000 Triple Quad mass spectrometer (Concord, Ontario, Canada). The eluents were 0.5 mM ammoniumacetat and methanol. The separation was achieved on a Raptor Biphenyl column (150 x 4.6 mm, 5 µm; Restek, Bad Homburg, Germany). The flow rate was 0.7 mL min^{-1} and the column temperature was 50°C. The total runtime was 13 min.

The mass spectrometer operated in positive and negative mode at different time intervals. Mass transitions are given in Table 2.

Table 2. Mass transitions of nitrofuran metabolites and CAP.

measured analytes	parent ion	transitions			
positive mode	[m/z]	[m/z]			
2-NBA-SEM	208.9	191.9	166.3	149.1	134.0
2-NBA-SEM-15N2-13C	212	195.1	168.3		
2-NBA-AOZ	236.2	134.0	104.0		
2-NBA-AOZ-D4	240.2	134.0	104.0		
2-NBA-AMOZ	335.3	291.3	262.1		
2-NBA-AMOZ-D5	340.2	296.2	265.1		
2-NBA-AHD	249.1	178.1	134.1		
2-NBA-AHD-13C3	252.3	179.2	134.0		
negative mode					
Chloramphenicol-35	321.0	152.0	194.0	257.0	
Chloramphenicol-37	323.0	152.0			
Chloramphenicol-35 -D5	326.0	157.0	262.0		

Results and Discussion

Validation data

The validation was carried out according to the commission decision (2002/657/EC) [1]. To determine CCα and CCβ at ~~a~~ low level, validation was done with shrimps spiked at 0.1 µg kg^{-1} CAP and 1 µg kg^{-1} of each NF metabolite The obtained data are listed in Table 3. The method showed recovery rates between 90 and 107% and very good reproducibility (see also Table 3).

Table 3. Validation data of CAP and nitrofuran metabolites

Analyte	CCα	CCβ	LOD	LOQ	MRPL [1]	recovery	repeatability (CV)	spiking level
	µg kg^{-1}	µg kg^{-1}	µg kg^{-1}	µg kg^{-1}	µg kg^{-1} // aquaculture products	%	%	µg kg^{-1}
SEM	0.6	0.8	0.2	0.6	1	98	17	1
AHD	0.6	0.7	0.1	0.4	1	107	14	1
AOZ	0.7	0.9	0.1	0.4	1	103	14	1
AMOZ	0.6	0.7	0.2	0.5	1	90	29	1
CAP	0.1	0.1	0.01	0.03	0.3	103	7.2	0.1

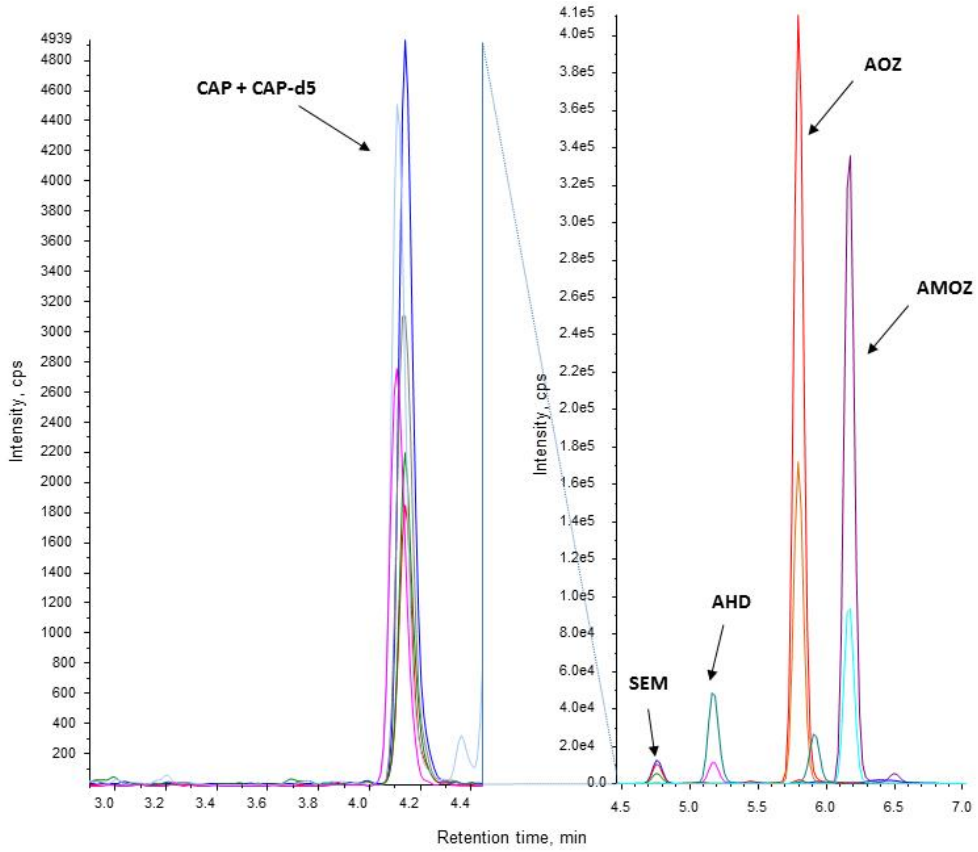

Figure 1. Chromatogram of a fish sample, spiked with 5 µg kg^{-1} for each nitrofuran metabolite and 0.5 µg kg^{-1} for chloramphenicol.

Sample preparation and measurement

The sample preparation includes a fast and easy extraction and allows the preparation of a high number of samples in a short time. The LC-MS/MS measurement was done with two transitions for each compound. Calculation of the results was carried out by external calibration and correction with the recovery of the internal standard. Every batch was additionally checked by a control sample. Altogether this procedure is a fast and quality controlled method to obtain reliable results. The chromatogram of a spiked fish sample is shown in Figure 1.

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DEVELOPMENT AND STRENGTHENING OF RADIO-ANALYTICAL AND COMPLEMENTARY TECHNIQUES TO CONTROL RESIDUES OF VETERINARY DRUGS AND RELATED CHEMICALS IN AQUACULTURE PRODUCTS

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Abstract
Aquaculture is the world's fastest growing source of animal protein. Intensive aquaculture practices and fish diseases are associated with use of pharmacologically active substances that may expose consumers to residues and impact trade. There is need to research suitable methods to support chemical hazard monitoring and control programs. A six-year FAO/IAEA coordinated research project (CRP) initiated in 2015 to develop reliable and tailored radiometric and complementary analytical techniques as well as transferable protocols that will facilitate residue testing in aquaculture products and production systems, is ongoing and some results are presented here. Fifteen, mainly developing, countries are involved. Participants in Singapore, China, Chile and Brazil have developed multi-residue high performance liquid chromatography (HPLC)-high resolution mass spectrometry (HRMS), Liquid Chromatography–tandem mass spectrometry (LC-MS/MS) as well as radioimmunoassay (RIA) techniques for a number of veterinary drug residues including amphenicols, nitrofuran metabolites and hormones in fish and shrimps.

Introduction
Antimicrobials and hormonal growth promoters are used in aquaculture to control diseases or improve yields (FAO, 2005). Nitrofurans (NFs), for example, are widely used to treat farmed animals with gastrointestinal bacterial and protozoal diseases (Vass *et al*., 2008), the most commonly used being furaltadone (FTD), furazolidone (FZD), nitrofurantoin (NFT) and nitrofurazone (NFZ). In the animal, NFs are rapidly metabolized to their respective protein-bound metabolites (NFMs), namely 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) from FTD, 3-amino-2-oxazolidinone (AOZ) from FZD, 1-aminohydantoin (AHD) from NFT, and semicarbazide (SEM) from NFZ (Barbosa *et al*., 2007). Due to toxic effects, many countries have banned the use of NFs in food-producing animals since the 1990s (Leitner *et al*., 2001).

Commonly used hormonal growth promoters, another class of synthetic chemicals widely used in animal husbandry, include methyltestosterone, medroxyprogesterone, trenbolone and diethylstilbestrol. However, the use of hormonal growth promoters was prohibited (Council Directive 96/22/EC, 1996), because of their possible toxic effects on public health (Waltner-Toews and McEwen, 1994).

Under the FAO/IAEA CRP, several individual projects have been initiated to develop methods and mechanisms to control residues of the compounds mentioned above and similar classes of drugs in aquaculture production. For example, in Singapore work is ongoing on novel analytical approaches to harness the strength of LC-MS/MS (sensitivity and reproducibility) and HRMS (specificity, coverage and screening speed) to support residue monitoring. In China, a project on "Development of Multi-Residue Methods for Veterinary Drugs and Growth Promoters by LC-MS/MS to Strengthen the Analytical Capability in Aquaculture Products in South China" is under way. In Chile, related work on the development and validation under the title "Multi-residue Method for the Detection of Veterinary Drug Residues and Other Organic Contaminants in Aquaculture Products and Feed by LC-MS/MS and Gas Chromatography-MS/MS Spectrometry Techniques" is also ongoing. Similar work on "Development and Validation of a Radioimmunoassay Kit for the Screening of Chloramphenicol in Fish and Shrimp Tissues and Related Feeds" is also underway in Brazil.

Materials and Methods

Singapore Study

MS and Chromatographic Conditions. A hybrid LC-MS system (Exactive, Thermo Scientific) was used to develop a screening method for a number of veterinary drugs. The MS parameters included: dual polarity scan; maximum injection time: 50 ms; resolution: high; AGC target: balanced; sheath gas flow rate: 40 L min^{-1}; aux gas flow rate:10 L min^{-1}; capillary temperature: 350°C; heater temperature: 200°C.

The mobile phase consisted of: solvent A (H_2O, 10 mM ammonium formate, 0.1% formic acid) and; solvent B (methanol, 0.1% formic acid). Gradient elution was used; the gradient profile started with 98% A to 80% B after 7 min, increasing to 98% B by the 10th min. Solvent A was then maintained at 98% until the 15th min.

Exact-mass database. Reference standards between 100 ppb and 1 ppm were injected into the Exactive LC-MS/MS to set up an exact-mass database which included retention times and associated MS parameters.

Sample preparation and analysis. Homogenized samples (5 g) containing 10 mL of 1% acetonitrile were extracted by centrifugation and either injected directly into the LC-MS/MS or diluted/concentrated or further cleaned-up using dispersive solid phase extraction before LC-MS/MS analysis.

China Study

MS Conditions. An LC-QTRAP (AB 4500, Applied Biosystems/MDS Sciex, Toronto, Canada), equipped with a turbo ionspray source and a syringe pump was used in the positive mode electrospray ionization (ESI). The MS conditions were optimized by infusing each derivative compound (1 mg L^{-1}) separately at a flow rate of 10 µL min^{-1}. The dwell time for all analytes was 50 ms. The MS ion source parameters including curtain gas, ion source gas 1, ion source gas 2, collision gas, ionspray voltage and ion source temperature were 40 psi, 55 psi, 55 psi, medium, 5,500 V and 550°C, respectively.

Sample preparation. Minced fish sample (2 g) was weighed into a 50 mL polypropylene centrifuge tube, fortified with the working mixed standard solution and 10 mL of methanol/water (50:50, v/v) added. The content was shaken vigorously (10 min), centrifuged (5,000 rpm, 5 min), and 10 mL 0.2 mol L^{-1} hydrochloride added to the separated liquid layer. The mixture was then homogenized (1 min), 100 µL 100 µg L^{-1} mixed internal standard and 100 µL 0.1 mol L^{-1} 2-nitrobenzaldehyde solutions added before mixing the content (30 s) using a vortex. The mixture was then kept for 16 h (overnight) in a water bath at 37°C, samples cooled to room temperature, 1 mL of 0.3 mol L^{-1} aqueous sodium phosphate added. After mixing (1 min), the pH was adjusted to 7.4 using 2 mol L^{-1} aqueous sodium hydroxide. Ethyl acetate (10 mL) was then added, content mixed (350 rpm, 10 min) and centrifuged at (10,000 rpm,10 min), before the upper layer was collected into another centrifuge tube. The extraction was repeated and the ethyl acetate supernatants combined before drying (nitrogen at 40°C). The residue was reconstituted with 1 mL of the initial mobile phase and excess fat removed from the content by centrifugation twice using 3 mL of acetonitrile saturated n-hexane. The fat free material was pressed through a 0.22 µm membrane filter before LC–ESI–MS/MS analysis. Direct exposure of the sample solutions to light was avoided.

Linearity, recovery, precision, limit of detection (LOD) and limit of quantification (LOQ). Matrix matched calibration curves were prepared at 0, 0.5, 2.5, 5.0, 25, 50 µg kg^{-1}. Isotope-labelled internal standards were maintained at 5.0 µg kg^{-1}.

Recoveries and precision (intra-day) were calculated using 7 replicates of each sample fortified at three levels (0.5, 2.0, 10 µg kg^{-1}). The LOD and LOQ, defined as three and ten times the signal-to-noise ratio for each compound, respectively, were calculated using results from the replicates of spiked negative fish samples.

Brazil Study

Sample preparation and analysis. Sample preparation was conducted as follows; fish muscle (0.8 g) was homogenized and hydrolysed using *Helix pomatia* from *E. coli,* centrifuged and extracted further using ethyl acetate. After clean-up on a C-18 cartridge, the concentrated sample was dissolved in 1 mL of PBS-gelatine buffer. The samples were analysed by RIA using tritium-labelled chloramphenicol (~2 µCi), chloramphenicol standard (Sigma), and anti-chloramphenicol antibodies. The following working standards were used for calibration: 10 µg L^{-1}, 5 µg L^{-1}, 2.5 µg L^{-1}, 1.25 µg L^{-1}, 0.625 µg L^{-1}, 0.132 µg L^{-1} and 0.156 µg L^{-1} in PBS buffer.

Comparison was made between six replicates of blank fish samples (tilapia, catfish and shrimp) and five replicates of samples spiked at 0.25 µg kg^{-1}.

Chile Study

All reagents used were analytical grade. Also 25 high purity standards, including deuterated materials for internal standards (*e.g*. chloramphenicol-d5) were used (HPC and Witega).

MS and chromatographic conditions. Chromatographic separation and detection of the 25 analytes was achieved using a UHPLC (Agilent 1290) coupled to QTRAP 5500 (AB Sciex) in both positive and negative electrospray (ESI) and multiple reaction monitoring mode (MRM). Data was processed using Multiquant 3.0. Software. A Thermo Scientific, Accucore® C18 column (2.6 µm, 2.1 x 100 mm) and Accucore TM C18 guard column (10 x 2.1 mm, 2.6 µm) were used in the separation process.

The gradient mobile phases (flow of 0.4 mL min^{-1}) were solvent A) 0.1% formic acid + 5 mM ammonium formate in water and solvent B) 0.1% formic acid in 50:50 methanol:acetonitrile. Solvent B was increased from 2% to 100 % over 8 min and maintained at 100 % for 3 min before reducing to 30% in one minute and then gradually to 2 % from 11.5 min to 20th min.

The MS conditions for both positive and negative modes included: collision gas: high; curtain gas:15 psi; ion source gas 1: 45 psi; ion source gas 2: 50 psi; source temperature: 500°C; ion spray voltage: 5,000 V (positive) and -4,500 V (negative).

Sample preparation. Samples were extracted using a modified QuEChERS method involving Agilent Bond Elut Enhanced Matrix Removal-Lipid (EMR-Lipids). Samples (5 g) were weighed into a 50 mL polypropylene tube, 10 mL of formic acid (5 %) in acetonitrile added and the content homogenized by shaking vigorously for 2 min, followed by centrifugation at 4,000 rpm for 5 min. Ammonium acetate (5 mM, 5 mL) was transferred into a 15 mL EMR-Lipid dSPE tube and 5 mL of the extract added followed by shaking and centrifugation. The supernatant (5 mL) was transferred into the 15 mL EMR-Lipid Polish tube, 200 µL of the subsequent extract and 800 µL of water were transferred into a 2 mL vial.

The LOQs were established for each substance (as applicable) by spiking blank matrix with the analytes at maximum residue limits.

Results and Discussion

Singapore Study

Exact masses for 53 drugs were obtained and entered into a veterinary drug database. An Acquity BEH C18 column (Waters) was used to separate several veterinary drugs including stilbenes, amphenicols, nitroimidazoles, β-lactams, quinolones, fluoroquinolones, diaminopyridines, lincosamides, macrolides, polypeptides, sulphonamides, anthelmintics and antiseptics. The method was optimised to detect these analyses with LODs ranging from 1 to 100 ppm. The method harnessed the strengths of LC-MS/MS (sensitivity and reproducibility) and high resolution mass spectrometry (specificity, coverage and screening speed) to address mass accuracy and resolution issues that have a bearing on confirmatory criteria (Stolker *et al*., 2007).

China Study

The MS parameters were successfully optimized. Characteristic fragment ions were determined and separation of analytes achieved over a 15 min run time (Figure 1).

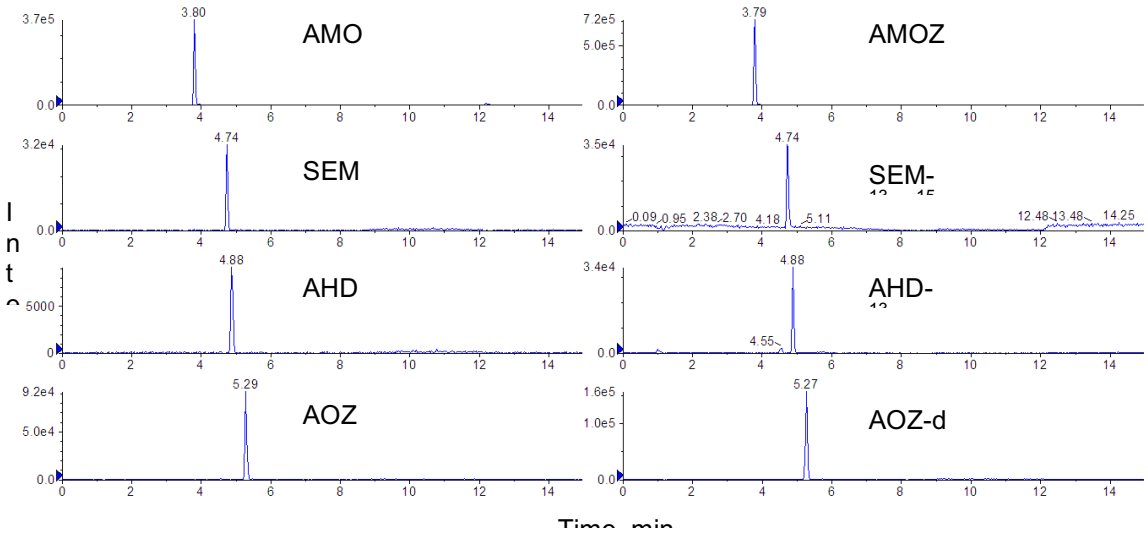

Figure 1. Total Ion chromatograms of fish sample spiked with 4 NFMs at 5 µg kg^{-1} obtained using LC-ESI-MS/MS.

Recoveries and precision (intra-day) were determined from 7 replicates of each sample fortified at three levels (0.5, 2.0, 10 µg kg^{-1}). The recoveries and precision were 87.2-104% and 2.7–10.4%, respectively. The LOQs for AMOZ, SEM, AHD and AOZ were determined as 0.25, 0.5, 0.5 and 0.25 µg kg^{-1}, respectively. The inclusion of suitable internal standards improved precision and the method is consistent with work done elsewhere (Hossain *et al*., 2013), although the inclusion of more internal standards gives this method a greater advantage in terms of performance.

Brazil Study

The RIA method was able to detect chloramphenicol in tilapia, catfish and shrimp samples. The method was optimized to ensure sensitivity and specificity based on counts per min. The mean counts per min for blank tilapia (n = 6) = 1,394 (4.2%, CV); spiked tilapia - 0.25 µg kg^{-1} = 555 (4.3%, CV); blank catfish (n = 6) = 1,268 (4.3%, CV); spiked catfish - 0.25 µg kg^{-1} = 467 (4.8%, CV), blank shrimp = 1,297 (4.1%, CV), spiked shrimp - 0.25 µg kg^{-1} = 452 (4.7%, CV) were reported.

The method was tested on 30 shrimp samples from the state of Rio Grande do Norte, north-east of Brazil. No residues were found in these samples. The RIAs provide suitable and robust alternatives for national residue monitoring programmes (Granja *et al*., 2008).

Chile Study

In Chile, 25 analytes belonging to tetracyclines, quinolones, macrolides and amphenicols, among others were identified using the QTRAP. Suitable fragmentation patterns were established and used in determining LOQs and recoveries.

Basic green, basic violet, malachite green, leucomalachite green and leucocrystalviolet had LOQs of 0.4 µg kg^{-1} with recoveries ranging from 89.9 to 117%, while ciprofloxacin, enrofloxacin, flumequine, ivermectin, oxolinic, sarafloxacin, florfenicol, diflubenzuron, teflubenzuron, abamectin, trimethoprim, tylosin and erythromycin gave LOQs of 20 µg kg^{-1} with recoveries ranging from 75.2 to 118 %. Doxycycline, oxytetracycline and tetracycline had LOQs of 40 µg kg^{-1} with recoveries ranging from 79.7 to 110%. Emamectin had an LOQ of 2 µg kg^{-1} and recovery of 94.3% while chloramphenicol had corresponding values of 3 µg kg^{-1} and 103%.

Improving analytical methods for food hazards such as triphenylmethane dyes is necessary for testing laboratories (Verdon *et al*., 2015) and a combination of the analytes with other pharmacologically active substances in the same method will greatly enhance Chile's export of fish products such as salmon.

Conclusions

In China, an LC–ESI-MS/MS method has been developed for the simultaneous determination of four nitrofuran metabolites, AMOZ, AOZ, AHD and SEM using multiple deuterated internal standards. In Singapore, a multi-residue screening method involving HPLC-MS/MS—HRMS has been developed. An exact mass database has been established with information on the retention time, exact masses of precursor and major fragment ions of the target chemicals. A generic sample preparation method has also been set up while method improvement is ongoing.

In Brazil, a RIA method has been developed for analysis of chloramphenicol in tilapia, catfish and shrimp samples below known minimum required performance limit and tested using 30 shrimp field samples. Further development and the validation of the method is ongoing. In Chile, a multi-residue LC-MS/MS method has been developed for analysis of 25 veterinary drug residues in salmon and preliminary validation done. The use of EMR-lipid QuEChERS resulted in cleaner extracts and no background interference in the chromatograms.

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EFFECT OF HEAT TREATMENTS ON SULFADIAZINE AND TRIMETHOPRIM RESIDUES IN COW AND GOAT MILK WITH A NEW LC-UV METHOD

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Abstract

A simple and rapid liquid chromatographic method for the determination of sulfadiazine (SDZ) and trimethoprim (TMP) residues in cow milk was developed and applied in heat treatment experiments of cow and goat milk. Milk samples were extracted with dichloromethane and following addition of hexane the extracts were concentrated into diluted phosphoric acid. Separation was carried out isocratically on a Nucleosil C18, 5-µm column using an acetonitrile:5 mM phosphoric acid (20:80) mobile phase. Detection was performed at 275 nm. The fully validated method, according to Commission Decision 2002/657/EC, gave good analytical characteristics for both analytes. The method was successfully applied to investigate the stability of SDZ and TMP residues in spiked cow and goat milk samples, under various heat treatments (pasteurization, boiling and sterilization). The results showed that under pasteurization conditions no significant changes were noted, whereas in boiling some decrease of the concentration of both analytes was observed. In sterilization conditions both SDZ and TMP residues concentrations in milk of both species, were decreased significantly. In conclusion, SDZ and TMP seem to be relatively stable up to boiling conditions which is of great importance for the protection of Public Health.

Introduction

Sulphonamides are a group of synthetic organic compounds that have played an important role as effective chemotherapeutics in bacterial and protozoal infections in veterinary medicine. Trimethoprim, a structural analogue of the pteridine portion of dihydrofolic acid, is a diaminopyrimidine derivative used extensively in food animal production for treatment of respiratory and alimentary tract infections (Botsoglou and Fletouris, 2001). The combination of sulfonamides and diaminopyrimidines (potentiated sulfonamides) are widely used antibacterials in the veterinary practice in a number of animal species, including cows and goats. Sulphadiazine (SDZ) and Trimethoprim (TMP) act together in a synergistic manner and possess a wide-spectrum of antibacterial action. The combination of SDZ with TMP acts against a large number of both Gram-positive and Gram-negative bacteria. The existing EU Maximum Residue Limits (MRLs) for all substances belonging to the sulphonamide group and for TMP in milk are 100 µg kg^{-1} and 50 µg kg^{-1}, respectively (Commission Regulation (EU) No 37/2010).

Various analytical methods that can be employed in the analysis of sulphonamides have been published in a review (Wang *et al*., 2006). Papapanagiotou *et al*. (2000) developed a method for the simultaneous determination of SDZ and TMP in cultured sea bream tissues. The same group (2005) reported the effect of heat-treatments on sulphamethazine residues in cow milk. Another study (Roca *et al*., 2013) investigated the thermal stability of eight sulphonamides spiked in skimmed milk powder using an LC-MS/MS method, and kinetic models and thermodynamic parameters to estimate degradation percentages.

Other studies (Javadi *et al*., 2011; Xu *et al*., 1996) have reported on the effects of cooking methods on sulphadiazine and trimethoprim residues in edible tissues of broiler chicken using microbiological methods, and on ormetoprim and sulphadimethoxine in muscle of channel catfish. Rose *et al*. (1995) have reported on the effect of cooking on sulphamethazine. As can be concluded, the influence of heat treatments on the residues of sulphadiazine and trimethoprim in milk has not been examined thoroughly apart from the study of Roca *et al*. (2013) on sulphonamides only.

The aims of the study were to develop a reliable and robust, fully in-house validated LC-UV method for the simultaneous quantification of SDZ and TMP in cow milk, which could be used in heat stability studies in cow and goat milk.

Materials and Methods

Chemicals and Equipment

Analytical grade *ortho*-phosphoric acid, hexane and dichloromethane, and LC grade acetonitrile and methanol were from Merck (Darmstadt, Germany). SDZ and TMP were obtained from Sigma-Aldrich (St. Louis, MO, USA).

A modular LC system (Gilson Medical Electronics, Villiers-le-Bel, France) consisting of a Model 305 piston pump, a Model 805 manometer and a Model TC 831 column oven was used in this study. Injections were made through a Model 7125 Rheodyne valve (Cotati, CA) equipped with 100-µL loop. A Model 119, variable wavelength UV-Vis detector (Gilson, Middleton, WI, USA) was used to monitor the signal. The detector was linked to a Model BD 111 Kipp & Zonen (Delft, The Netherlands) pen recorder. The chart speed of the recorder was set at 5 mm min^{-1}.

A FT74P miniature-scale UHT/HTST processing system (Armfield, Ringwood, United Kingdom), a HA-300MII sterilization system (Hirayama, Tokyo, Japan) and a water-bath (Memmert, Schwabach, Germany) were used for thermal processing of milk, a MDF-U7386S deep-freezer (Sanyo Electric Co., Osaka, Japan), a freezer, a refrigerator and a controlled temperature incubator (JP Selecta S.A., Barcelona, Spain) were used for sample storage, a Milli-Q purification system (Millipore, Bedford, MA, USA) was used for purification of tap water, a G-560E vortex mixer (Scientific Industries, Bohemia, NY, USA), a Centra-MP4 centrifuge (IEC, Needman Heights, MA, USA), and an Ultra-Turrax (Janke & Kunkel, GmbH, Germany) high-speed blender were used for sample preparation.

Standard solutions

Stock standard solutions were prepared in individual 25-mL volumetric flasks by dissolving 10 mg of each reference standard (SDZ and TMP) and diluting to volume with methanol. Mixed intermediate standard solution, containing both analytes, was prepared by combining appropriate aliquots of each of the stock solutions in 10 mL volumetric flask and diluting to volume with methanol. Mixed working standard solutions were prepared by appropriate dilutions of the mixed intermediate standard solution, using 0.1 M *ortho*-phosphoric acid.

Mixed spiking solutions were prepared by appropriate dilutions, with methanol, of the mixed intermediate standard solution. Fortified milk samples were prepared by spiking control samples with the appropriate mixed spiking solutions. Quantification was done on calibration curves generated during chromatographic runs.

LC-UV analysis

The mobile phase contained 20% acetonitrile and 80% 5 mM *ortho*-phosphoric acid (v/v). Following its preparation, the mobile phase was passed through 0.2 µm Nylon-66 filter (Anachem, Luton, UK) and degassed using helium. The mobile phase was delivered in the system at a rate of 1 mL min^{-1}. The stationary phase was Nucleosil 100-5 C_{18}, 5-µm material in a Mackerey Nagel LC column 250 x 4.6 mm id. The stationary phase was kept equilibrated at 40^{o}C. The injection volume was 100 µL. The UV detector was set at 275 nm with a sensitivity of 0.01 aufs. Under the established conditions, SDZ eluted at 5.6 min and TMP at 7.6 min.

Sample extraction and clean-up

An amount of 1.5 g cow milk sample was accurately weighed in a 20-mL centrifuge tube. The sample was extracted with 10 mL of dichloromethane using a vortex mixer for 2 min at high speed. The sample was centrifuged at 4,000 *g* for 3 min. Eight mL of the organic phase were transferred to a 20-mL centrifuge tube and 3 mL *n*-hexane and 1 mL 0.1 M phosphoric acid were added. The mixture was vortexed for 1 min and following centrifugation at 4,000 *g* for 3 min, the upper aqueous layer was transferred to another tube and finally 100 µL were used for high-performance liquid-chromatography.

Calculations

The calibration curves were generated by running mixed standard working solutions, plotting the recorded peak heights versus the corresponding mass of the analytes injected, and computing slope, intercept and least squared fit of standard curves. The calibration curve slope and intercept data were used to compute the mass of analytes in injected extracts. The concentration C of each analyte in milk samples (ng g^{-1}) was calculated according to the equation C = 8.34 x M x V, where M is the mass (ng) of the analyte determined in the injected extract (100 µL), and V is the dilution factor, if any, applied.

Method validation

Validation was performed according to the Commission of the European Communities guidelines (European Commission, 2002) using cow milk. The specificity, linearity, sensitivity, accuracy, precision, applicability, and stability of the analytes were the criteria used to evaluate the developed method.

Effect of heat treatment on SDZ and TMP

Effect of heat treatment on water solutions of SDZ and TMP. Standard solutions containing 137.6 ng mL^{-1} SDZ and 444.0 ng mL^{-1} TMP were prepared in water. These solutions were dispensed into capped glass centrifuge tubes, which were subjected to pasteurization (65°C and 72°C for 30, 45 and 60 min, and for 15 s, 2 and 10 min, respectively), boiling (100°C for 2, 5 and 10 min) and sterilization (121°C for 10, 15 and 20 min). Six replicates were carried out for all the above mentioned heat treatments.

Effect of heat treatments on cow and goat milk samples fortified with SDZ and TMP. Milk samples fortified with 412.8 ng g^{-1} SDZ and 1028 ng g^{-1} TMP were dispensed into capped glass centrifuge tubes, which were subjected to pasteurization (65°C and 72°C for 30, 45 and 60 min, and for 15 s, 2 and 10 min, respectively), boiling (100°C for 2, 5 and 10 min) and sterilization (121°C for 10, 15 and 20 min). Six replicates were performed for all the above mentioned treatments.

Results and Discussion

Method validation

The calibration curves and the corresponding correlation coefficients (r^2) were y=9.4052x + 0.3615 and r^2=0.9993 for SDZ, and y=2.6139x -0.7906 and r^2=0.9998 for TMP. The overall recovery for SDZ and TMP in cow milk was 74.1 ± 4.8% with an overall RSD (%) of 6.4, whereas for TMP in cow milk the overall recovery was 92 ± 14% with an overall RSD (%) of 14.8. The limits of determination (LOD) and limits of quantification (LOQ) for SDZ and TMP were 3.5 and 11.4 ng g^{-1} and 8.6 and 25.7 ng g^{-1}, respectively. Method characteristics for goat milk were similar to those for cow milk.

Injection system precision was evaluated for both compounds by injecting a mixed calibration solution ten times on a single occasion. The results showed an adequately precise injection with RSD of the peak heights better than 2.9 % for both analytes.

Storage stability experiments of the analytes in solution (0.1 M phosphoric acid) indicated that the test compounds were stable for at least 7 days when stored at 20°C, and for at least 30 days when stored at 4°C. The test compounds were stable during the storage of spiked milk samples at -28°C and -80°C for at least 4 months. As regards to the stability of the final milk extracts (solution ready for LC analysis), the experiments indicated that no significant change in the concentration of both analytes during storage for 2 days at 4°C in the dark, was observed.

Effect of heat treatments on SDZ and TMP

Effect of heat treatments on water solutions of SDZ and TMP. The concentrations of SDZ and TMP in standard solutions in water were quite stable in all heat treatments examined in this study. This is important when assessing the stability of both analytes after heating fortified milk samples (see below). The results concerning SDZ are in agreement with those by Rose *et al*. (1995) for sulphadimidine.

Effect of heat treatments on cow and goat milk samples fortified with SDZ and TMP. The concentrations of SDZ and TMP in fortified cow and goat milk samples in both pasteurization treatments (at 65°C and 72°C) were stable, whereas in boiling treatments a moderate degree of decrease (for both substances) was noted. However, sterilization was the heat treatment by which the observed decrease was even higher (Table 1). The fate of SDZ after pasteurisation of cow milk are in agreement with the estimates for similar heat treatments by Roca *et al*. (2013), but are not consistent with the sterilization treatment (120°C for 20 min) in which they estimated a much greater loss for SDZ (47.9%). The latter finding could be explained, since they used skimmed milk powder spiked with sulphadiazine and mathematical equations (kinetic models and thermodynamic parameters) to estimate the degradation percentages.

Table 1. Effect of heating on the stability of sulphadiazine (413 ng g^{-1}) and trimethoprim (1028 ng g^{-1}) in bovine *and* caprine *milk.*

Heating treatment	Temperature/ time	Concentration change (%) ± SD*			
		Cow milk		Goat milk	
		SDZ	TMP	SDZ	TMP
Pasteurization	65°C/30 min	-2.45 ± 7.57	-1.56 ± 8.35	3.88 ± 4.27	-2.08 ± 0.59
	65°C/45 min	1.76 ± 7.34	2.68 ± 4.19	1.19 ± 0.61	-2.17 ± 2.35
	65°C/60 min	4.09 ± 3.85	-3.57 ± 5.05	2.08 ± 1.82	2.51 ± 2.33
	72°C/15 s	-3.19 ± 0.61	-0.39 ± 6.70	2.46 ± 2.90	0.98 ± 7.07
	72°C/2 min	-1.03 ± 3.67	3.29 ± 1.67	-2.76 ± 0.58	-0.41 ± 0.58
	72°C/10 min	2.16 ± 2.44	-1.25 ± 1.11	-1.23 ± 1.74	2.08 ± 1.76
Boiling	100°C/2 min	-5.26 ± 3.07	-7.90 ± 3.91	-6.58 ± 0.58	-5.33 ± 3.53
	100°C/5 min	-8.17 ± 5.22	-7.46 ± 7.11	-11.87 ± 10.47	-6.05 ± 2.99
	100°C/10 min	-10.26 ± 6.76	-9.34 ± 6.70	-10.28 ± 4.65	-8.58 ± 10.01
Sterilization	121°C/10 min	-15.90 ± 3.21	-15.47 ± 6.17	-14.22 ± 1.82	-13.83 ± 2.35
	121°C/15 min	-18.00 ± 6.92	-18.73 ± 10.10	-12.93 ± 1.21	-17.75 ± 5.30
	121°C/20 min	-18.63 ± 7.64	-20.23 ± 3.92	-18.96 ± 6.21	-19.16 ± 4.35

** Six replicates.*

Bearing in mind that the standards of both analytes in aqueous solutions remained unchanged at all of the above-mentioned heat treatment methods, it is logical to suggest that the decrease in concentration in milk samples is a result of binding to milk proteins. This is in agreement with the results by Papapanagiotou *et al* (2005) for sulphamethazine when subjected to various heat treatments of spiked cow milk. This suggestion could prove quite important in dietary exposure assessment studies of residues of veterinary medicinal products in milk. Thus, sulphadiazine and trimethoprim residues in cow and goat

milk could potentially pose a health risk to consumers and should be denied entry in the food-chain with the proper adhesion to their withdrawal times, when the drug formulations are administered to the animals.

Conclusions

The results of the present study clearly indicate that SDZ residues in both types of fortified milk (cow and goat) samples are stable when exposed to temperature/time combinations equivalent to both low temperature long time (LTLT) and high temperature short time (HTST) pasteurization. A moderate decrease of the concentration of SDZ residues is noted in boiling of fortified samples but a more significant decrease occurs under sterilization conditions. A similar trend is noted for TMP where sterilization of fortified milk samples (cow and goat) leads to a substantial decrease of its concentration. What becomes clear through this particular research is that cooking does not necessarily protect human health from the presence of non-compliant residues in milk. This conclusion is critical, since cooking methods should probably not be relied upon for the significant reduction of existing levels of non-compliant residue levels in milk. The results of this study could possibly assist in a more detailed understanding stability of veterinary drug residues during cooking and thus public health protection.

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THE OCCURRENCE OF CHLORAMPHENICOL ISOMERS RESIDUES IN HONEY SAMPLES

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Abstract
Chloramphenicol (CAP) was first isolated from *Streptomyces venezuelae* in the 1940's. It is a broad-spectrum antibiotic, effective against a wide variety of Gram-positive and Gram-negative bacteria. Due to a potential carcinogenicity of its residues and association with the development of aplastic anaemia in humans, CAP is banned for use in food-producing animals in the USA, Canada and the EU. In Ukraine, CAP is banned since 2002, but despite the prohibition its residues are occasionally found in animal products such as honey. Exporting manufacturers carry out control of incoming honey using ELISA. The efficiency of ELISA has been proved over the years of its use due to a good correlation with results of confirmatory LC-MS/MS analysis. In 2013, we observed the first cases of non-corresponding ELISA and LC-MS/MS results. From 2013 to 2015, we found eleven cases in which the value of chloramphenicol content in samples detected by LC-MS/MS did not coincide with ELISA results. Analysis of the chloramphenicol isomers in non-compliant honey samples showed the presence of the non-immuno-bound *SS-p*-CAP isomer known as dextramycin in all samples.

Introduction
Chloramphenicol (2,2-dichloro-*N*-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl] acetamide) was first isolated by David Gottlieb from *Streptomyces venezuelae* in 1947. It is a broad-spectrum antibiotic effective against a wide variety of Gram-positive and Gram-negative bacteria [1]. CAP is biosynthesized by several soil actinomycetes and produced by chemical synthesis. The chemical structure of chloramphenicol was studied for the first time in the USA. In 1949, chemical synthesis was made possible.

Chloramphenicol (CAP) has two asymmetric carbon atoms and therefore can exist as four *meta*-stereoisomers and four *para*-stereoisomers. The most important isomers are *RR-p*-CAP and *SS-p*-CAP, known as chloramphenicol and dextramycin, respectively [2]. As a result of the chemical synthesis performed in the Soviet Union in 1953, a racemic preparation was obtained, *viz*. a substance comprising L-(+) *threo* and D-(-) *threo* forms of chloramphenicol. This drug is called synthomycin. Synthomycin's biological activity is 50% compared with chloramphenicol. Its laevorotatory isomer was isolated in 1968 and this drug was called levomycetin [3].

Due to potentially carcinogenic chloramphenicol residues and the association with the development of aplastic anaemia in humans, CAP is banned for use in food-producing animals in the United States, Canada and the European Union. In Ukraine, CAP is banned since 2002, but despite the prohibition its residues are occasionally found in animal products such as honey. Exporting manufacturers carry out a control of incoming honey using ELISA before the homogenization. The efficiency of ELISA has been demonstrated over the years of its use as reflected by its good correlation with the results obtained by confirmatory LC-MS/MS analysis and other immuno-chemical methods.

In 2013, we have observed the first cases of non-corresponding ELISA and LC-MS/MS results. The laboratory of SCIVP received honey samples, which were non-compliant with respect to CAP residues according to Intertek laboratory (Bremen, Germany). Incoming control of the exporter had not shown the presence of chloramphenicol above the values obtained for pure honey samples. We have verified the results of Intertek laboratory using LC-MS/MS. At the same time the testing of the obtained sample by ELISA did not show the presence of CAP residues. We therefore considered the hypothesis of contamination of honey with a synthomycine residue, as this gives less cross-reaction with anti-CAP antibodies. The test of our hypothesis was by analysing honey samples to which a known quantity of synthomycine was added. The ELISA method identified only 48% of the added amount of racemic mixture of CAP isomers. The main aim of our study was to establish the cause of the discrepancy between the analysis results for the same honey samples obtained by different methods.

Methods and Materials

MS/MS analysis
Methanol, acetonitrile, ethyl acetate and absolute ethanol were obtained from Merck (Darmstadt, Germany). Water was prepared using a Millipore Direct-Q system at a resistance of at least 18.4 MΩ cm^{-1} (Millipore, Billerica, MA, USA). The reference standard *RR-p*-CAP was obtained from Sigma–Aldrich (St. Louis, MO, USA). *SS-p*-CAP, a mixture of *RR-m*-CAP and *SS-m*-CAP were obtained from Witega (Berlin, Germany). Stock solutions were prepared in methanol at 100 mg L^{-1} and all dilutions were prepared fresh daily in Milli-Q water.

For the first analysis, a system consisting of a Waters e2695 separation module, quadrupole MS/MS Quattro Premier XE equipped with a SunFire™ C18 analytical column (2.1 mm × 100 mm, 3.5 µm; Waters) and SunFire™ C18 guard column (2.1 mm × 10 mm, 3.5 µm; Waters) was used. The gradient using solvent A (water) and solvent B (methanol) was: 0–0.5 min, 10% B; 0.5–4.0 min, linear increase to 95% B; final hold of 0.5 min. The flow rate was 0.3 mL min^{-1}. The injection volume was 40 µL.

The subsequent analysis was performed with a high-resolution RP-LC system consisting of a vacuum degasser, autosampler, and a binary pump (Acquity Waters, Milford, MA, USA) equipped with a Waters Acquity UPLC BEH C18 analytical column (2.1 mm. 100 mm × 1.7 µm) placed in a column oven at 50 °C. The gradient (solvent A, water; solvent B, methanol) was 0-0.5 min at 10% B, 0.5–4.0 min linear increase to 100% B and then a hold of 0.5 min, all steps at a flow rate of 0.4 mL min^{-1}. The injection volume was 35 µL.

The chiral LC system consisted of the same equipment but combined with a Chromtech Chiral AGP (α1-acid glycoprotein) analytical column of 2.0 mm ×150 mm, 5 µm (Chromtech Inc, Apple Valley, MN, USA) placed in a column oven at 20 °C. The gradient (solvent A, water; solvent B, methanol/acetonitrile (1:1, v/v)) was 0–4.0 min at 9% B, 4.0–4.1 min linear increase to 12% B with a final hold of 4.9 min, operating at a flow rate of 0.5 mL min^{-1}. The injection volume of standard solutions was 35 µL and of extracted plant material 5 µL. The latter was to extend the column life time.

Detection was carried out using a Waters Quattro Ultima mass-spectrometer in the negative electrospray ionization (ESI) mode. The operating parameters were: capillary voltage, 2.7 kV; cone voltage, 25 V; source temperature, 120 °C; desolvation temperature, 300 °C; cone gas flow, 200 L h^{-1}; and desolvation gas, 500 L h^{-1}. The CAP isomers were fragmented using collision-induced dissociation and the selected reaction monitoring (SRM) transitions *m/z* = 321.0 > 257.1, *m/z* = 321.0 > 152.0 and *m/z* = 321.0 > 207.0 were monitored. For *RS*- and *SR*-*p*-CAP-d5 the SRM transitions *m/z* = 326.0 > 262.1 and *m/z* = 326.0 > 157.0 were monitored. Data were acquired and processed using MassLynx 4.1 software (Waters).

Element compositions of product ions were derived from exact masses determined using a MicrOTOF-Q (Bruker Daltonics, Billerica, MA, USA) operating in negative ESI mode using the integrated syringe pump to continuously infuse 10 mg L^{-1} solutions of *RR*-*p*-CAP, *SS*-*p*-CAP (DEX). Using this instrumentation, a resolution of at least 10,000 was obtained. Proposed fragmentations of the isomers were supported by MSn measurements using an LCQ Deca XP (Thermo Scientific, Waltham, MA, USA) ion trap mass spectrometer operating in negative ESI mode also by continuous infusion.

ELISA analysis

RIDASCREEN® Chloramphenicol ELISA (R-Biopharm) is a commercially available competitive enzyme immunoassay for the quantitative analysis of CAP in milk, milk powder, honey, shrimp, meat, fish meal and eggs. The method is highly specific for the detection of CAP in the range of 0.05 to 0.75 ppb. It is based on monoclonal antibodies against protein-bound CAP.

Samples

Honey samples were obtained from exporting companies which were confronted with non-compliant results by LC-MS/MS analyses (CAP>0.3 µg kg^{-1}) by Intertek (Bremen, Germany). All samples were taken from the homogenized honey lots which were composed mainly of sunflower honey (70% or more). Before and after homogenization, these honey samples were tested for CAP residues by immune methods, including RIDASCREEN Chloramphenicol, R-biopharm, ELISA, Antimicrobal Arry III CAP (EV3738) Randox Food Diagnostics. In all these samples, CAP residues were not detected at a level > 0.3 ppb.

Preparation of honey samples for ELISA analysis was performed in two ways: by ethylacetate extraction and IAC purification. Ethylacetate extraction procedure was similar for the ELISA and LC-MS/MS methods. Honey sample was diluted with distilled water, ethylacetate was added and after shaking the extract was centrifuged for the separation of liquid layers. Ethylacetate supernatant (corresponding to 1.0 g of honey) was evaporated to dryness at 60°C by nitrogen. The dried residue was dissolved in 1.0 mL buffer for ELISA kit or in LC mobile phase and vortexed before the solution was used in the assay. Before LC-MS/MS analysis, sample solutions were additionally purified with a hexane / tetrachloromethane mixture (1:1 v/v) and centrifuged. IAC purification was performed on RIDA Chloramphenicol columns (R1508) according to the manufacturer's instructions.

Results and Discussion

The first case of CAP residues in honey which did not interact with the specific anti-CAP antibodies was in September 2013. The concentration of CAP in this sample found by Intertek laboratory using LC-MS/MS was 1.3 ppb. Our LC-MS/MS test confirmed this value, *viz*. 1.24 ppb. ELISA analysis did not show the presence of chloramphenicol residues in this sample. After receiving the first honey sample (sample No 79), of which analysis revealed a discrepancy between the results of screening and confirmation methods, it was tested for the presence of inhibitory substances. ELISA analysis of this sample was implemented in parallel in several laboratories, *viz*. the laboratory of Intertek, laboratories of exporting companies, control laboratories SCIVP and the laboratory of R-biopharm AG. In the last three laboratories, the kit from one lot was used for analysis. Results of ELISA from all laboratories except the laboratory of R-biopharm were less than 0.1 ppb. R-biopharm laboratory

results are presented in Table 1. Molecules of CAP which was found in the sample No. 79 by LS-MS/MS did not interact with antibodies specific to CAP. This was demonstrated by means of immuno-affinity columns (Table 1).

Table 1 Immuno-chemical detection of CAP: inhibitory effect of honey matrix (mean, n=3).

Sample	Sample preparation A (Etyl acetate extraction)		Sample preparation B (IAC purification)	
	Conc. (ppb)	Recovery (%)	Conc. (ppb)	Recovery (%)
Blank honey	0.05	-	<0.03	-
Blank honey + 0.3 ppb	0.32	92	0.28	94
Sample No 79/1	0.25	-	<0.03	-
Sample No 79/1 + 0.05 ppb	0.34	167	0.05	107
Sample No 79/1 + 0.10 ppb	0.41	156	0.09	88
Sample No 79/1 + 0.30 ppb	0.61	120	0.25	84

The possibility that substances present in the sample were able to inhibit the immune response was ruled out in the study in which the sample was fortified with CAP. The recoveries of different quantities of chloramphenicol added to the tested honey were 107%, 88% and 84% for the concentrations of 0.05 ppb, 0.1 ppb and 0.3 ppb, respectively. Also we have rejected the assumption of sample contamination with synthomycin, which is a racemic mixture of chloramphenicol isomers. In the case of sample contamination with a racemic mixture, ELISA should give approximately 50% recovery of the added concentration, which was confirmed by adding 1.0 ppb of synthomycin and obtaining 0.49 ppb by ELISA (Table 2).

During 2014 and 2015, SCIVP lab has received ten comparable samples. Table 2 contains selected samples which revealed a complete mismatch of the results of the ELISA and LC-MS/MS analyses. To investigate whether the honey was contaminated with isomers, a chromatographic method able to separate isomers was necessary. Chiral LC was used to detect specifically the administration of the antimicrobial active isomer of CAP [4]. A chiral LC-MS/MS system is able to separate *RR-p*-CAP from other isomers. Table 2 contains the results of the comparison of immuno-chemical data with LC-MS/MS, which however do not directly indicate the presence of CAP isomers. Therefore, the method of chiral LC-MS/MS was used and clearly showed the presence of dextramycin, *SS-p*-CAP.

Table 2 Concentrations of chloramphenicol and its isomer dextramycin in honey samples detected by LC–MS/MS and ELISA methods.

Honey Sample No.	Chloramphenicol (*RR-p*-CAP) ELISA (ppb)	Chloramphenicol (*RR-p*-CAP) LC-MS/MS (ppb)	LC-MS/MS isomers	
			Chloramphenicol (*RR-p*-CAP) (ppb)	Dextramycin (*SS-p*-CAP), (ppb)
79/2	0.059	0.290	0.04	0.32
113/2975	0.062	0.590	ND*	0.73
141/3259	0.032	0.57	ND*	0.65
113/488	0.070	0.200	ND*	0.33
113/529	0.051	2.2	ND*	3.12
Honey blank + 1.0 ppb of syntomycine	0.49	0.92	0.41	0.43

**- ND- not detected < 0.04*

The large number of cases forces to recognize the presence of a mixture of chloramphenicol isomers as a very likely contamination of honey. The chain of biochemical reactions by which actinomycetes produces bioactive CAP, delivers only the active *RR-p* isomer. In view of the above, we can assume only that anthropogenic factors were the cause of the problem with the contaminated honey. As shown in Table 2, the content of chloramphenicol isomer in certain samples of honey reaches sufficiently high values of over 2 mg, which suggests the use of drugs with a high portion of isomers.

The study of biological properties of synthesized isomers of chloramphenicol was performed in the 70's of last century. It revealed that the antimicrobial effect of the isomers is lower and the toxicity is higher than for chloramphenicol [5,6].

There were no objective reasons for the assumption of unauthorized use of chemically synthesized isomers of chloramphenicol in beekeeping. In addition to dextramycin, however, traces of chloramphenicol were detected in one of the samples as well. This may be evidence for the use of the wastes of the chemical synthesis of CAP in beekeeping. In the Ukraine no antibiotics are registered for apiculture. So, the contaminated samples may be the result of illegal production of medical prepara-

tions from low-quality raw-materials. The next step in our research is to focus on the traceability of all non-compliant samples from exporting companies. Non-compliant samples should lead to the individual beekeeper producing the contaminated product.

Conclusions

By means of chiral LC-MS/MS analysis we have detected the presence of the chloramphenicol isomer dextramycin in honey samples, for which non-compliant results by immuno-chemical screening and confirmatory LC-MS/MS analysis concerning chloramphenicol were obtained. On the basis of the level of determined concentrations, we can assume that anthropogenic factor has caused exclusively this problem, *viz*. illegal use of the wastes from CAP chemical synthesis in beekeeping.

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RESIDUES IN POULTRY FED WITH HIGH CONTENT OF POLYPHENOLS

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Abstract

The production of olive oil yields a considerable amount of olive mill wastes, which is a powerful pollutant and is currently discarded, creating costs for their disposal. On the other hand, these wastes contain valuable resources such as a large proportion of interesting phytochemicals that could be recovered. Polyphenols are a group of secondary metabolites widely distributed in plants and found in olive mill waste. They are bioactive compounds with interesting properties. Among the well-known antioxidant and anticancer activity, they could control pathogenic bacteria. In this work, the presence of residues of four polyphenols (tyrosol, hydroxytyrosol, verbascoside and pinoresinol) has been investigated in poultry meat after an farm experiment: four poultry groups received feed enriched with polyphenols using olive oil by-products (treated) and a fifth group was kept as control. The quantitative analysis of poultry meat samples has been carried out by LC-MS/MS. For structural confirmations, a high-resolution hybrid mass analyser was used too (LC-Q-Exactive). The results showed sporadic low concentrations of tyrosol and hydroxytyrosol in meat of animals belonging to the treated groups. Investigating the metabolite presence (glucuronates and sulphates), hydroxytyrosol-3-sulphate was found in meat of all the treated animals.

Introduction

Numerous investigators have quantified hydroxytyrosol and other bioactive compounds in fruits and vegetables. Interest in hydroxytyrosol and other phenolic compounds is associated with their many biological benefits, especially antioxidative capacity and other effects associated with low incidences of coronary heart disease and cancers in the population consuming the Mediterranean diet (Leopoldini *et al.,* 2011). Produced from olives (*Olea europaea*), the oil contains an unsaponifiable fraction (about 1.5%) that contains antioxidants. Twenty percent of the yield of olive processing is in oil, the remaining 80% is made up of pulp, nut, skin and water. These olive mill wastes are a powerful pollutant and are currently discarded, creating costs for their disposal. On the other hand, they have good lipid content and high concentration of antioxidant compounds such as tocopherols and polyphenols. For these characteristics this by-product can be used, after drying, in feed industry (Terramoccia *et al*., 2013). Most of the research conducted had focused on feeding olive by-products to ruminants. Studies concerning feeding olive by-products to monogastrics are limited.

An *in vivo* study was performed in poultry to assess the possible beneficial effects of polyphenols on animal health and meat characteristics. A farm experiment was designed as follows: four poultry groups received feed enriched with polyphenols using olive oil by-products (paste and concentrate at two different concentrations) and a fifth group kept as control. Several parameters were evaluated on samples of *Pectoralis major* muscle at the end of the study. Animal performance was checked for the whole study period, including thermotolerant *Campylobacter* prevalence. This work focuses on the analytical methods developed and validated to determine polyphenols in the feed and poultry meat. For the first time, residues of tyrosol, hydroxytyrosol, pinoresinol and verbascoside in control and treated chicken muscle samples are reported.

Materials and Methods

Animal experiment

Broilers were bred in an experimental farm with environmental conditions similar to those commonly found in a conventional industrial structure. A population of 495 "ROSS308" female chicks of one day of age were placed in 5 fences of 99 subjects each, for 5 different dietary treatments. Each group was divided into three sub-groups of 33 subjects each in order to make a valid statistical survey. The study lasted 49 days, during which the health status and production performance of the animals were monitored. In order to verify the possible dose-dependent effect we studied four different dietary treatments according to the following scheme with cornmeal diets containing: 16.5% olive paste (Group 2); 16% phenolic concentrate (Group 3); 33% olive paste (Group 4), and 32% phenolic concentrate (Group 5). At the same time, a control group was fed with non-supplemented feed (group 1).

After slaughter, muscles of 15 animals belonging to the fifteen subgroups were pooled (5 x 3 pooled samples). A further division was carried out: the pooled samples of the three sub-groups were analysed immediately after the slaughter and only two sub-groups were analysed after 6 and 11 days of storage at refrigeration temperature (ca 4°C). Therefore, seven samples were analysed for each group for a total of thirty-five determinations. Each determination was repeated twice.

To test the effects of supplemented feed on the slaughter performance, carcasses were weighed and the individual percentage yield was calculated on all trial subjects. Chemical-physical, chemical-bromatological and nutritional characteristics were evaluated on samples of *Pectoralis major* muscle. The detection of thermotolerant *Campylobacter* from cloacal swabs was

performed by sampling 15 chickens for each group at 21 days of age (before starting the experimental diets), at 35 and at 49 days of age.

Standards

Tyrosol and pinoresinol were purchased from Sigma Aldrich (St. Louis, MO, USA), hydroxytyrosol and verbascoside from Extrasynthese (Genay, France). Hydroxytyrosol-d4 and hydroxytyrosol-3-sulphate were obtained from TRC (Toronto, Canada).

Sample preparation.

Poultry feed. After the addition of 250 µL of the stock solution of hydroxytirosol-d4 (100 µg mL^{-1}), 5 g feed were extracted twice with a mixture methanol/water 80/20 (v/v) containing 20 mg L^{-1} BHT (2 x 25 mL). Two aliquots of the extract, 1 mL and 100 µL were diluted to 5 mL with the mixture 0.1 M Na_2EDTA/methanol 90/10 (v/v). After filtration, the two aliquots were injected in LC-MS/MS.

Poultry meat. After the addition of 25 µL of a solution of hydroxytirosol-d4 (1 µg mL^{-1}), 5 g poultry muscle were extracted twice with a mixture methanol/water 80/20 (v/v) containing 20 mg L^{-1} of BHT (2 x 10 mL). The extract volume was reduced under nitrogen stream and purified using SPE OASIS HLB (200 mg/ 6 mL). The eluate was evaporated and suspended in 1 mL of a mixture of 0.1 M Na_2EDTA /methanol 90/10 (v/v). After filtration, the sample was injected in LC-MS/MS.

LC-MS/MS conditions

Liquid-chromatographic separation and mass-spectrometric detection and quantification were performed on an LC–MS/MS system consisting of a Finnigan Surveyor LC pump combined with a triple quadrupole TSQ Quantum Ultra mass spectrometer via electrospray ionization (ESI) interface (Thermo Scientific, San Jose, CA, USA). The chromatographic separation was done by a Gemini analytical column (100 mm x 2.0 mm, 3 µm) connected with a guard cartridge Gemini (10 mm x 2.1 mm, 5 µm) obtained from Thermo Electron Corporation (Waltham, MA, USA). HPLC eluent A was water and eluent B was methanol. The gradient was initiated with 5% eluent B for 1 min, continued with linear increase to 43% B in 8 min, followed by a linear increase to 95% B in 6 min. After 7 min, the system returned to 5 % B in 1 min and was re-equilibrated for 12 min. The column temperature was 30°C and the sample temperature was kept at 16°C. The flow rate was 0.25 mL min^{-1} and the injection volume 10 µL. For MS detection, the ESI source was operated in negative ion mode and run with Xcalibur 2.0 software (Thermo Fisher). High-purity nitrogen was used as the sheath (30 arbitrary units) and auxiliary (20 arbitrary units) gas and high-purity argon was used as the collision gas (1.5 mTorr). The parameters were as follows: spray voltage, -2.5 kV; capillary temperature, 200°C. The SRM transitions and collision energy levels of analytes and IS are listed in Table 1. Additional measures were carried out using Q-Exactive high resolution mass analyser equipped with an Ultimate 3000 LC system (Thermo Scientific).

Table 1. MS/MS conditions.

Analyte	Parent (*m/z*)	Product (*m/z*)	Collision Energy (V)	Tube Lens (V)
Hydroxytyrosol	153.1	93.1	35	90
		95.1	28	
		123.1	17	
Hydroxytyrosol-d4	157.1	124.1	24	70
Tyrosol	137.1	106.1	18	70
		107.1	17	
		119.1	18	
		137.1	10	
Verbascoside	623.2	135.1	48	110
		161.0	29	
		342.1	37	
Pinoresinol	357.1	136.0	37	106
		151.0	21	
		342.1	21	

Method validation

The instrument linearity range was studied from 1.00 to 1,000 ng mL^{-1} preparing fourteen solutions in 0.1 M Na_2EDTA /methanol 9/1 (v/v): 1, 5, 10, 25, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1,000 ng mL^{-1}. The spiking experiments for poultry feed were performed at four concentrations: 0.1, 0.5, 5 and 50 mg kg^{-1} (six replicates per level in two different occasions: 48 experiments). For poultry meat, three different spiking levels were prepared: 0.5, 1 and 5 µg kg^{-1} (six replicates per level in two different occasions: 36 experiments). Operative method limits (limit of detection, LOD, and limit of quantification, LOQ) were fixed taken into the account the accuracies obtained at the lowest validation levels.

Results and Discussion

Method accuracy was satisfactory for feed and meat. For feed, recoveries ranged from 79% (hydroxytyrosol at 0.5 mg kg^{-1}) to 106% (verbascoside at 0.1 mg kg^{-1}). The coefficients of variation (CV) were in the range of 2.5-12% and 2.6-12% for repeatability and intra-lab reproducibility, respectively. At first validation level, tyrosol was not always detectable and therefore for this analyte the method LOD/LOQ was fixed at 0.5 mg kg^{-1}, whereas for hydroxytyrosol, verbascoside and pinoresinol the LOD/LOQ were equal to 0.1 mg kg^{-1}.

The feed used in the animal experiment was analysed (Table 2). In the linearity study the following ranges were established: 5-800 ng mL^{-1} for tyrosol, 1-800 ng mL^{-1} for hydroxytyrosol and 1-600 ng mL^{-1} for pinoresinol and verbascoside. Therefore, two different dilutions of the extract were injected to have a reliable quantification of the variable concentrations found in enriched feed (Table 2). The results show that the two feeds supplemented with the phenolic concentrate had much higher levels of polyphenols than those with olive paste. The feed "1° period" was used before the start of experiment for all the five animal groups.

Table 2. Polyphenols levels in feed used in the animal experiment.

Feed	Tyrosol (mg kg^{-1})	Hydroxytyrosol (mg kg^{-1})	Verbascoside (mg kg^{-1})	Pinoresinol (mg kg^{-1})	Polyphenol sum (mg kg^{-1})
1° period	7.8	0.26	ND[a]	ND[a]	8.1
Group 1 (Control)	3.7	0.28	ND[a]	ND[a]	4.0
Group 2 (16.5% olive paste)	8.7	4.6	0.22	0.28	14
Group 3 (16% phenolic concentrate)	28	190	45	0.18	263
Group 4 (33% olive paste)	13	9.5	0.75	0.47	24
Group 5 (32% phenolic concentrate)	59	402	105	0.54	567

[a] *Not detected (<0.1 mg kg^{-1})*

Recoveries in poultry meat ranged from 66% (hydroxytyrosol at 0.5 µg kg^{-1}) to 90% (tyrosol at 5 µg kg^{-1}). The coefficients of variation (CV) were in the range of 6-16% and 8-17% for repeatability and intra-lab reproducibility, respectively. The first validation level of tyrosol and verbascoside demonstrated low precision (about 22-23%) and therefore method LOD and LOQ were fixed at 0.5 and 1 µg kg^{-1}, respectively, for these two analytes. For hydroxytyrosol and pinoresinol, LOD and LOQ were considered equal to 0.5 µg kg^{-1}.

Analytical methods developed and validated to determine polyphenols in tissues are scarce. To the best of our knowledge, the first procedure was published by Rodríguez-Gutiérrez *et al.* (2011) in rat muscle. The authors demonstrated good method accuracy both for tyrosol and hydroxytyrosol, but applying HPLC-UV-Vis technique, the LODs were relatively high (10 µg kg^{-1}). Previously Suárez *et al.* (2009) developed an LC-MS/MS method in plasma and in 2012 they applied this procedure with some modifications to tissues (liver, kidney, testicle, brain and spleen) (Serra *et al.*, 2012). The authors did not report method limits for tissues, but considering the amount of processed sample (ca 7 mg) and the limits listed for plasma (Suárez *et al.*, 2009), LOD could be about 150 µg kg^{-1} (1 nmol g^{-1}) for hydroxytyrosol and ten times higher for tyrosol (about 1,400 µg kg^{-1} or 10 nmol g^{-1}). Therefore, at present, our method seems to have the lowest LOD and LOQ.

As mentioned in the "Material and Methods" Section, for each group seven determinations on pooled muscles were carried out. Pinoresinol and verbascoside were never detected. Tyrosol was detected in four samples: one belonging to group 3, two to group 4 and one to group 5. The measured concentrations ranged from 8 to 47 µg kg^{-1}. Hydroxytyrosol was present only in two samples belonging to group 5 (0.65 and 0.74 µg kg^{-1}) in which animals were fed with the highest level of this polyphenol (402 mg kg^{-1}). No relationship between the found residues and the refrigeration times was observed. Unfortunately, to the best of our knowledge, levels of polyphenols in real muscle samples were not published. However, "heart" could be the organ with the more or less similar characteristics. Comparing the values of some polyphenols and their metabolites in different rat organs (liver, kidney, brain and heart) after administration of a refined olive oil enriched with hydroxytyrosol, Lopez *et al*. (2015) found the lowest concentrations in heart. Although at high administered doses, hydroxytyrosol was never detected. Probably, to have chance to detect some traces of polyphenols in muscle tissues the applied analytical methods should have limits about 0.5-1 µg kg^{-1} as demonstrated in this study.

The presence of the metabolites in biological samples, mainly glucuronides and sulphates, was well documented in literature both in biological fluids and tissues (Serra *et al.* 2012; Serra *et al*., 2013; Lopez *et al.,* 2015). Therefore, specific SRM transitions were acquired along all the chromatographic run: 217>137 (tyrosol sulphate), 313>137 (tyrosol glucuronide), 233>153 and 233>123 (hydroxytyrosol sulphate), 329>153 and 329>123 (hydroxytyrosol glucuronide).) Only the possible presence of hydroxytyrosol sulphate was detected in all muscles belonging to the four supplemented groups (2, 3, 4 and 5). Figure 1 shows the LC-MS/MS chromatograms of three different samples for each group. Actually, also in the control group (1) traces of hydroxytyrosol sulphates were detected, but in most of cases the identification criteria (ion ratio) were not respected due

to the low signals. As shown in Figure 1, two almost co-eluting peaks were present: their supposed identities were the two isomers of hydroxytyrosol-sulphate, *i.e*. 3- and 4-sulphate.

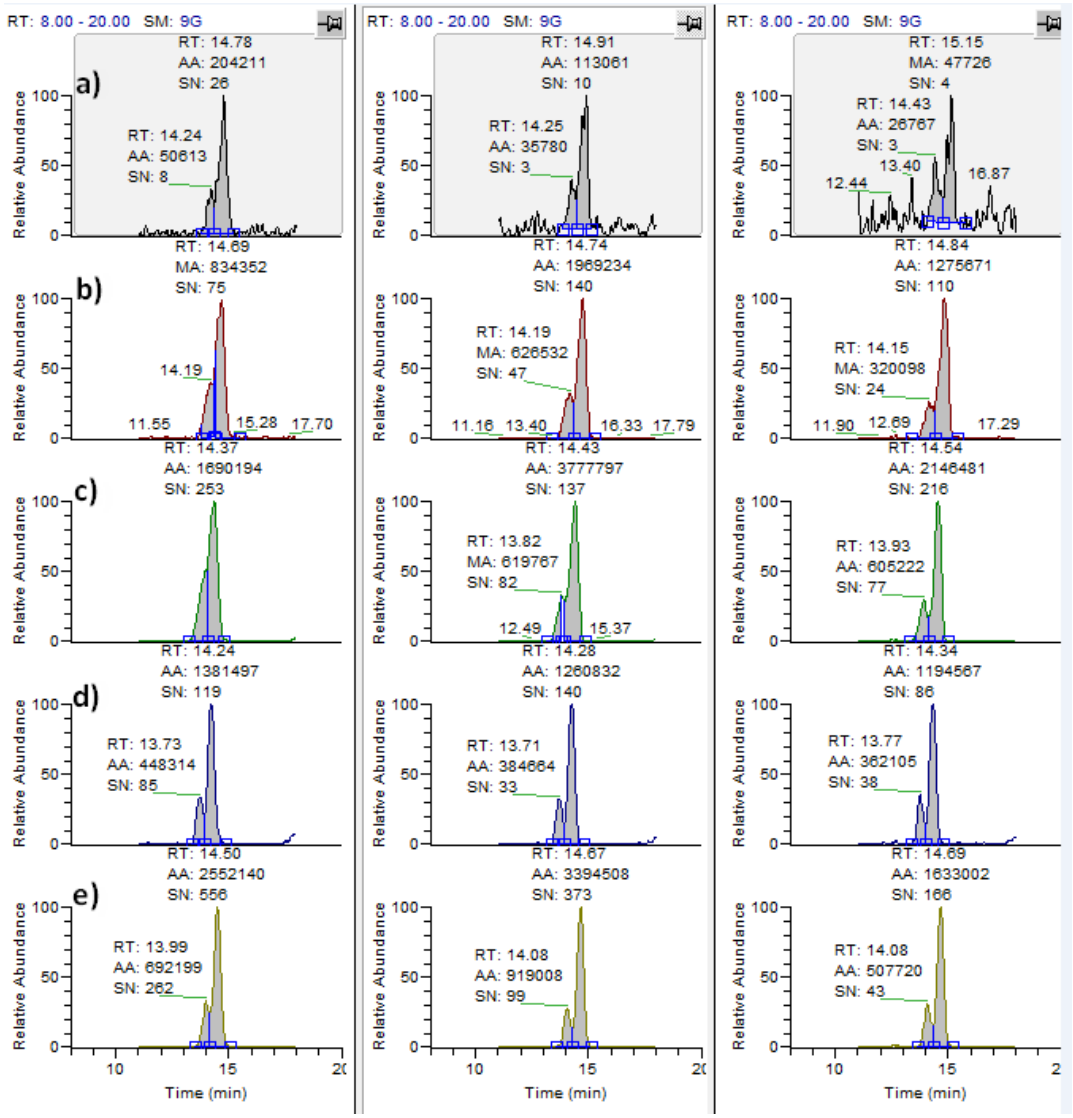

Figure 1. LC-MS/MS chromatograms of three samples of pooled poultry muscle belonging to the five groups. The set transitions were those of hydroxytyrosol-sulphate.

Later, the pure standard of hydroxytyrosol-3-sulphate was purchased. It was not possible to obtain hydroxytyrosol-4-sulphate. The peak with the highest retention time was definitely assigned to hydroxytyrosol-3-sulphate (Figure 1). In Figure 2 the co-chromatography experiment adding increasing amounts of pure standard to an incurred extract was shown (LC-Q-Exactive). The estimated levels in the samples of supplemented groups ranged from 1 to 5 µg kg^{-1}. The identity of peak immediately before the hydroxytyrosol-3-sulphate was better investigated. For this purpose, HR-MS/MS spectrum was acquired, too, and compared to that of hydroxytyrosol-3-sulphate. The same characteristic parent and fragment ions were detected: 233.0126 *m/z*, 153.0549 *m/z* (hydroxytyrosol) and 123.0442 *m/z* with mass accuracies of 0.4, -5 and -7 ppm, respectively (Qi *et al*., 2013). Therefore, the presence of hydroxytyrosol-4-sulphate was highly likely.

Finally, the results obtained from the prevalence study of thermotolerant *Campylobacter* in the five groups of chickens showed that groups 3 and 5 have a lower possibility of infection compared to the control group (*P*-value ≤0.05) after 49 days of age, *i.e*. after 28 days of feeding supplemented diets. The same result was not found for the groups fed with paste supplemented diet (*P*-value > 0.05). This may depend on the higher contents of polyphenols (mainly hydroxytyrosol in diets 3 and 5). On the other hand, the measured live weights at the end of the experiment were significantly higher for groups 2, 3 and 4 and the feed conversion efficiencies were significantly lower in the same three groups.

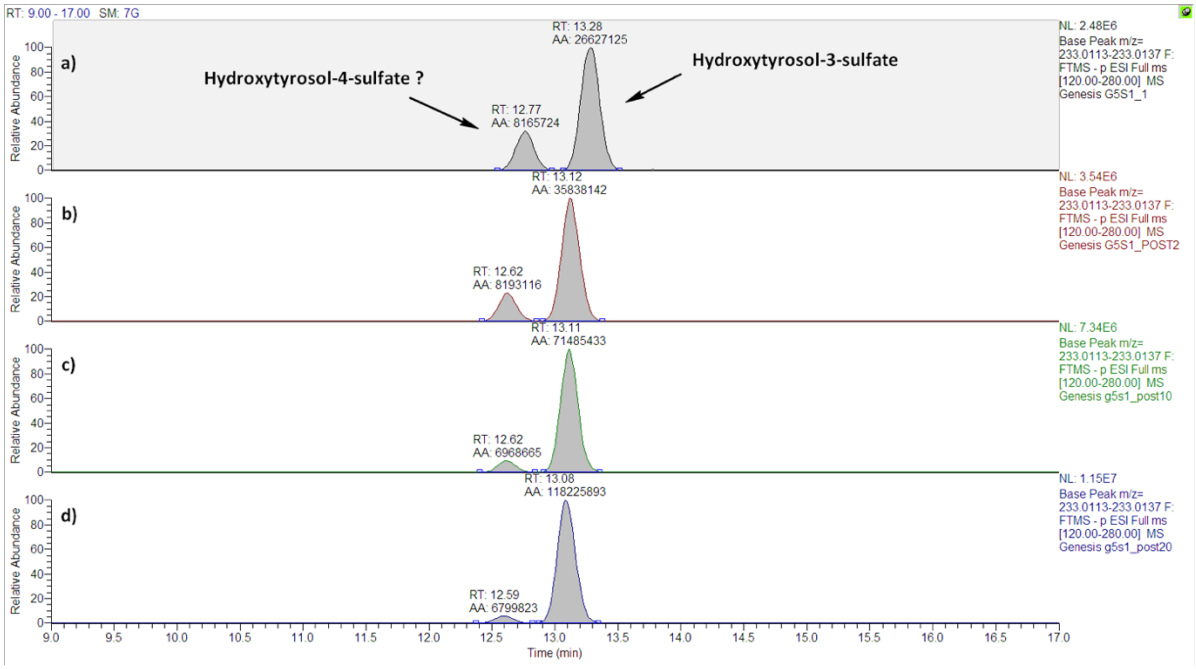

Figure 2. Full MS ion chromatograms of a poultry muscle belonging to group 5 (a), the same sample added with 2 (b), 10 (c) and 20 µg kg^{-1} (d) of pure hydroxytyrosol-3-sulphate, respectively.

Conclusions

For the first time residues of four polyphenols were measured in poultry meat after an *in vivo* experiment. The found levels of native compounds were sporadic and generally low, whereas sulphate metabolites of hydroxytyrosol were detected in all the supplemented groups (1-5 µg kg^{-1}). Analysing the supplemented feed, the phenolic concentrate (groups 3 and 5) gives a significant higher content of polyphenols than olive paste (groups 2 and 4). This is probably the cause of the beneficial effects on thermotolerant *Campylobacter* prevalence, but, at the same time, also the cause of the worst animal performances observed mainly for group 5. Therefore, after these results, a next *in vivo* experiment focusing on the more suitable concentrations of polyphenols and supplementation type is in progress. To conclude, the incorporation of olive mill wastes in animal feed could help to obtain two important goals in the era of sustainability: the recycling of agricultural wastes and decreasing of the use of antibiotics in animal husbandry.

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ULTRA-HIGH PERFORMANCE LIQUID-CHROMATOGRAPHY AND ULTRA-HIGH PERFORMANCE SUPERCRITICAL FLUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY TO CHARACTERIZE B-AGONISTS IN FEED

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Abstract

β-Agonistic drugs are forbidden as growth-promoters in rearing animals in Europe since the late 80s (Dir 96/22/EC). Specific and sensitive analytical methods based on UPLC-MS/MS allow monitoring a large set of such substances. However, optimal performances are not observed for all the target analytes, especially for those exhibiting the most extreme polarities. We developed an SFC-MS/MS approach to overcome the challenge of the detection of the most polar β-agonists which are usually eluted in the void volume when using reversed-phase chromatography in conventional HPLC. The present analytical development focused on the detection of a large set of β-agonists in feeding stuff with the objective to reach performances in accordance with, or better than, the current recommended concentrations as proposed by the European Union Reference Laboratory (50 µg kg^{-1} in feed). Comparison of performances reached with LC/MS and SFC/MS approaches will be presented.

Introduction

The use of anabolic compounds in livestock has been prohibited in the European Union since 1988 (Directive 96/22/EC). However, some growth promoters such as β-agonists are still supposed to be misused and the control of their use has to be maintained at a high level of performance. Indeed, and for example, ractopamine is authorized in pigs in several countries but remains strictly forbidden in the EU. Nowadays, LC-MS/MS is the consensual analytical strategy for β-agonists monitoring in feed, food and fluids collected from animals, since their secondary amine allows them to be easily protonated in electrospray ionisation. Thanks to the development of sub-2 µm and core-shell chromatographic technologies, their separation was recently improved. However, the most polar compounds remain eluting close to the void volume with conventional reversed phases and suspicious samples have to be re-analysed with an alternative chromatographic strategy such as HILIC. The introduction of a new reliable SFC system (UPC², Waters) led us assessing the separation efficiency for a set of 28 β-agonists of interest, within the same analytical run. In this study, we have compared LC and SFC performances (*i.e.* selectivity and sensitivity) in using, on the basis of our knowledge, the most adapted column for each strategy. The developed methods were then assessed for the detection of the β-agonists in different feed samples like pellets, milk powder and silage.

Materials and Methods

Materials

All the experiments were performed on the same triple quadripole instrument Xevo TQS (Waters,Milford, US). Source and desolvation temperatures were 150°C and 450°C, respectively. Desolvation gas flow was 650 L h^{-1}, nebuliser gas pressure was around 7 bar, capillary voltage was 2,400 V, sampling cone was optimised for each molecule from 10 to 30 V and the ionisation mode used was ESI+.

All the LC analyses were achieved on ACQUITY UPLC system (Waters) using PFP Waters column (100 x 2.1 mm, 1.9 µm). Elution solvents were water (A) and acetonitrile (B) both containing 0.1% of formic acid. The mobile phase composition (A:B, v/v) was 95:5 from 0 to 2 min, 10:90 from 7 to 8 min, 95:5 from 8.1 to 10 min. The flow rate was 0.45 mL min^{-1} and the injected volume was 1 µL. The column temperature was 40°C.

All SFC analyses were carried out on a SFC ACQUITY UPC2 system from Waters (Milford, US) using a Torus 2-PIC column (100 x 3 mm, 1.7 µm) from the same provider. Elution solvents were CO_2 (A) and methanol containing 0.1% of formic acid and 20 mM ammonium acetate (B). The mobile phase composition (A:B, v/v) was 95:5 from 0 to 2 min, 70:30 at 12 min, 95:5 from 12.1 to 15 min. The flow rate was 1.5 mL min^{-1} and the injected volume was 1 µL. The ABPR was 2000 psi and column temperature was 50°C.

Method

Feed samples were homogenized and 5 g were weighed and spiked with 28 β-agonists at 25 µg kg^{-1} each. The extraction was carried out by adding 15 mL methanol and 2 mL 0.2M acetate buffer (pH 5.2). The mixture was then shaken and centrifuged. The supernatant was evaporated to dryness. The residue was reconstituted in aqueous phase and adjusted to pH between 6 and 7 using a solution of sodium hydroxide (0.1 N). Pentane purification was achieved followed by 0.45 µm filtration before

clean-up using on a molecular imprinted polymer column (Biotage, MIP β-agonists). The residue was evaporated to dryness and finally reconstituted in a mixture of H_2O/MeOH (80/20) containing 0.1% formic acid.

In order to evaluate advantages and drawbacks of both chromatographic strategies, the same sample extracts have been injected in UPLC-MS/MS and UPC²-MS/MS.

Table 1 indicates the SRM transitions for a selection of six β-agonists used to compare LC and SFC performances. All compounds exhibit a transition based on the water loss (*m/z* 18), usually the most sensitive transition, and another more selective one for identification purposes according to Dec 2002/657/EC requirements.

Table 1. SRM transitions of 6 β-agonists

Compounds	Transition 1	Transition 2
Zilpaterol	262.2>244.0	262.2>184.9
Salbutamol	240.2>147.9	240.2>165.9
Ractopamine	302.2>163.9	302.2>284.0
Clenbuterol	277.1>202.8	277.1>149.9
Bromobuterol	365.0>290.8	365.0>258.9
Isoxuprine	302.2>284.0	302.2>346.8

Results and Discussion

Separation efficiency and elution order

The main advantage of the SFC relies in its ability to retain and separate both polar and apolar compounds, while using RP- LC polar compounds such as isoproterenol and metaproterenol (compounds 1 and 2) are eluted close to the void volume. Figure 1 enables comparing the selectivity between LC and SFC β-agonists separations in spiked pellets sample.

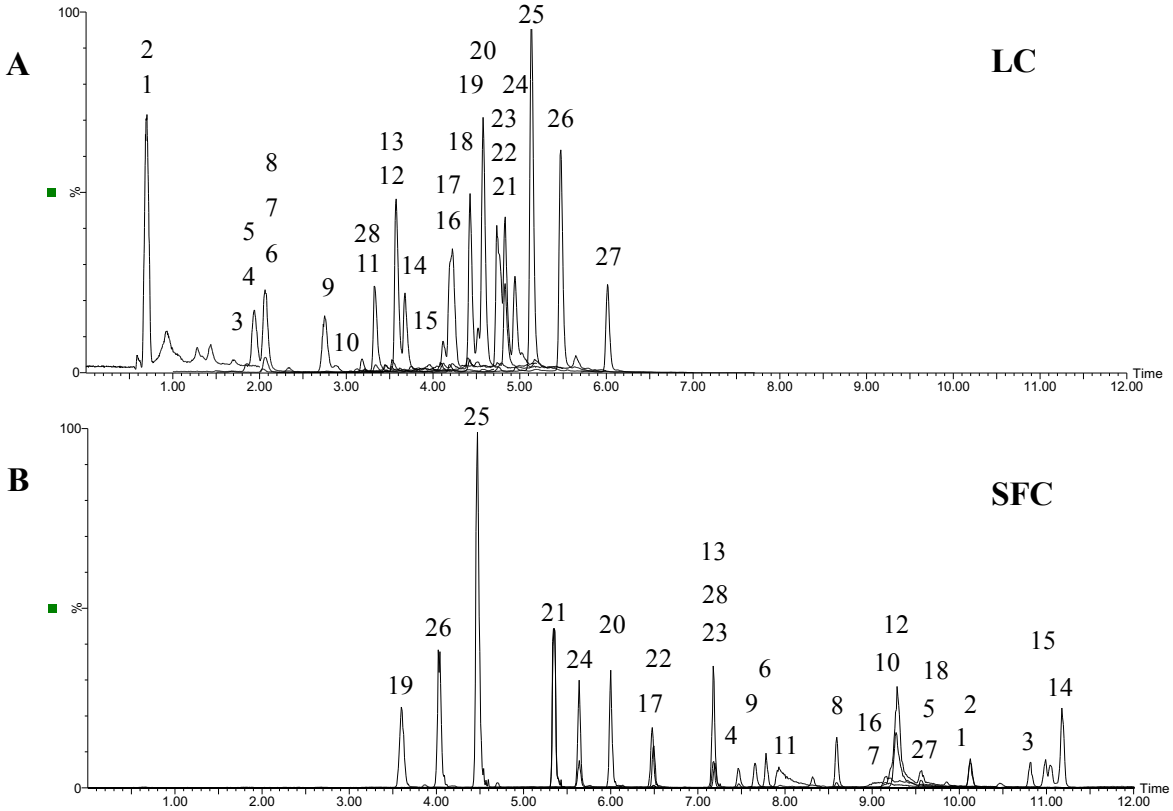

Figure 1. (A) LC SRM chromatograms using PFP column and (B) SFC SRM chromatograms using Torus 2-PIC column, for pellets sample spiked with 28 β-agonists at 25 µg kg^{-1} each.

SFC selectivity was observed as more efficient than the LC because the CO_2 mobile phase in the former case actually increases the interaction with the stationary phase compared to aqueous based mixtures used in LC. The SFC separation even enabled resolving ractopamine isomers which was not observed in LC.

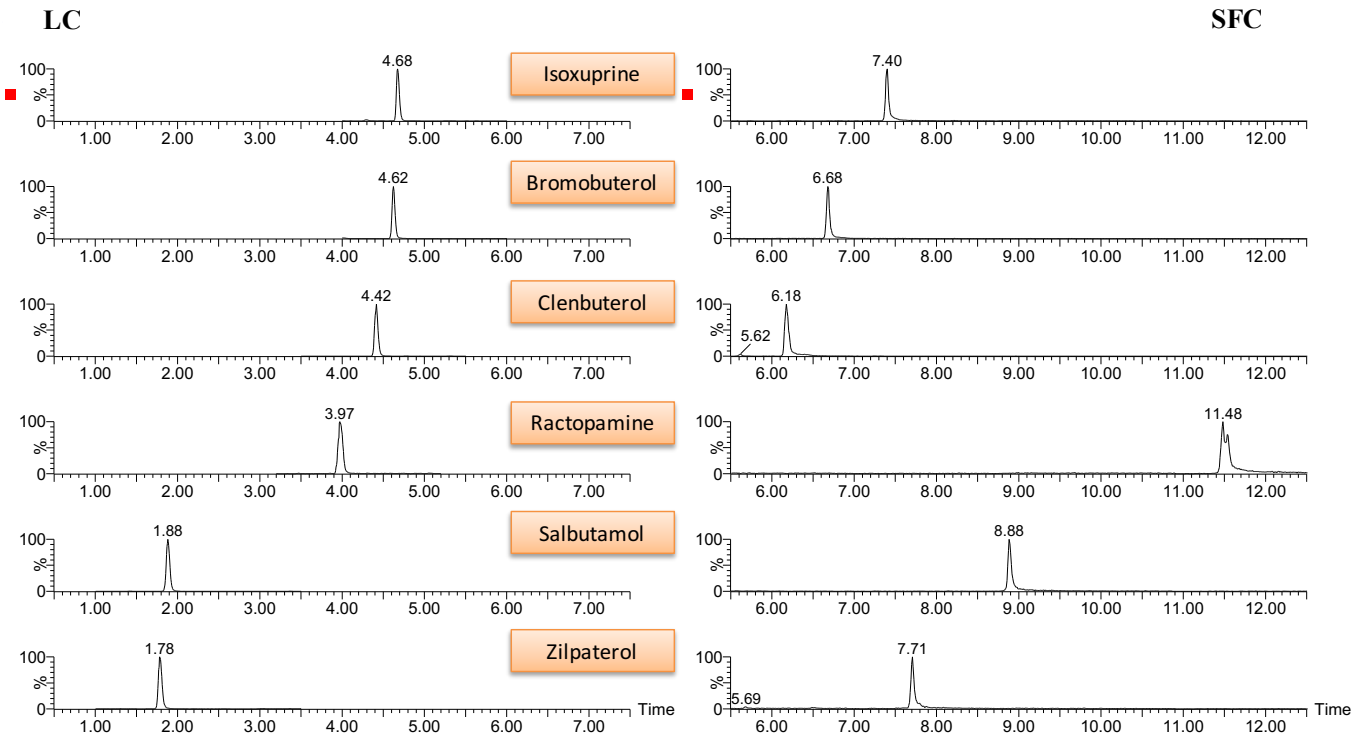

Figure 2. Zilpaterol, salbutamol, ractopamine, clenbuterol, bromobuterol and isoxuprine LC (left) and SFC (right) SRM chromatograms for pellets sample spiked at 25 µg kg^{-1}.

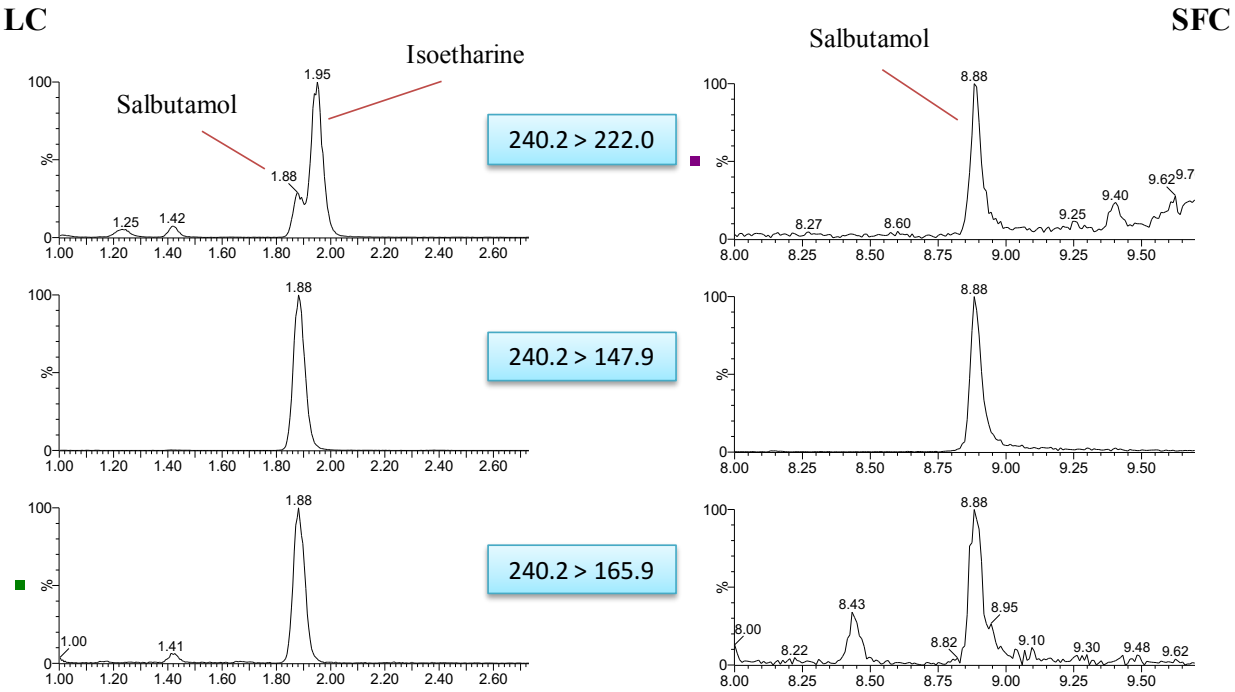

Figure 3. LC (left) and SFC (right) SRM salbutamol chromatograms for pellets sample spiked at 25 µg kg^{-1}.

Moreover, the elution order appeared totally different between LC and SFC chromatograms. Figure 2 shows individually the improvement of selectivity using SFC compared to LC for six β-agonists and the modification of the elution order with extracted chromatograms from pellets sample spiked at 25 µg kg^{-1}. The same chromatogram profile was obtained for each matrices studied (pellets, milk powder and silage). The sensitivity performances were observed as similar with the two technologies and easily fulfilled for all compounds the expected performances as required by the EURL.

Furthermore, such modification in the elution order leads to an orthogonal separation of the β-agonists mixture which may be considered as an advantage of the SFC technology especially in case of co-elution using LC as is the case for salbutamol and isoetharine eluted at 1.88 min and 1.95 min in LC, respectively, whereas they are respectively eluted at 8.88 min and around 10 min in SFC. Figures 3 and 4 further illustrate such efficiency in the separation.

While the separation of salbutamol and isoetharine was better using SFC, the elution of isoetharine was disappointing with SFC compared to LC (Figure 3). The isoetharine chromatographic peak is broader in SFC (about 1 min) than LC (0.2 min only).

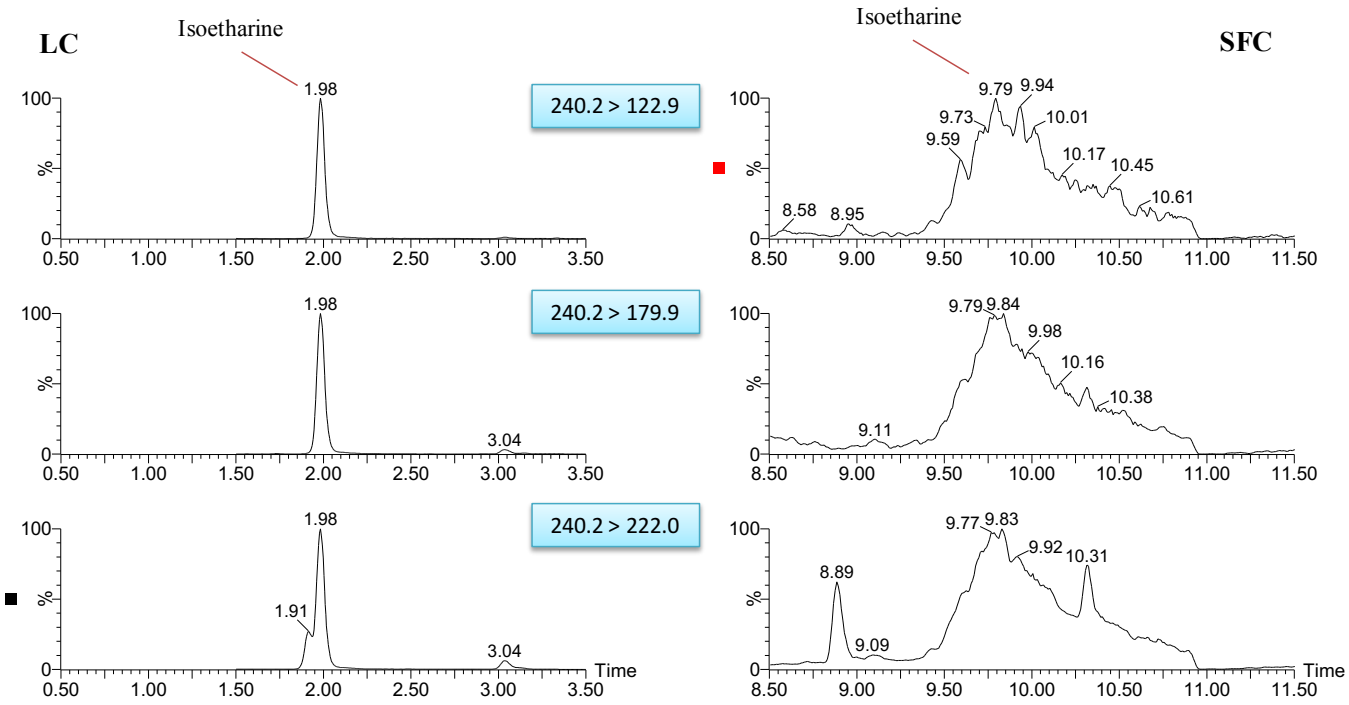

Figure 4. LC (left) and SFC (right) SRM isoetharine chromatograms for pellets sample spiked at 25 µg kg^{-1}.

Impact on subsequent detection efficiency.

It appeared during the study, when comparing LC-MS/MS and SFC-MS/MS strategies that diagnostic signal intensities differed. Indeed, the selection of relevant transitions may be different between LC and SFC when considering the couple selectivity/sensitivity. For salbutamol (*cf.* Figure 3), 240.2>147.9 was selected as the quantitative transition and 240.2>165.9 as the qualitative one using the LC protocol, whereas 240.2>147.9 was observed as the quantitative transition and 240.2>222.0 the qualitative one (more sensitive and specific than 240.2>165.9) when applying the SFC strategy.

Table 2. Comparison between LC and SFC of area responses for six targeted β-agonists in pellets, milk powder and silage matrices.

Matrices	Technologies	Zilpaterol	Salbutamol	Ractopamine	Clenbuterol	Bromobuterol	Isoxuprine
Pellets	LC	1230079	979433	219252	2255233	1133228	4158293
	SFC	219885	260498	347481	2783549	1828916	2616506
Milk powder	LC	1763613	1261125	3075494	4605669	2056802	6847370
	SFC	376894	306132	2263463	5926198	3205496	6390413
Silage	LC	1261819	551432	191104	1735135	1211463	4793711
	SFC	340450	192242	829460	4891360	2524983	5756761

Table 2 is a summary of the chromatographic peak areas of the six β-agonists obtained by LC or SFC for each studied matrix. Whatever the matrix, the signals for zilpaterol and salbutamol enabled more sensitive detection in LC., while, ractopamine, clenbuterol, bromobuterol and isoxuprine presented highest areas responses in SFC whatever the matrices.

Conclusions

The use of the same mass spectrometer and the injection of the same sample extract in LC and SFC allowed us to compare and to investigate deeply both separation strategies. As main advantages for β-agonists detection in feed matrices, SFC offers an enhanced retention on column for both polar, like metaproterenol and isoproterenol, and apolar compounds, like salmeterol, compared to an RP-LC approach. Moreover, selectivity was improved and some co-eluting issues could be overcome using SFC. While the detection in matrices such as pellets, milk powder or silage of some compounds like zilpaterol or salbutamol was observed more sensitive using LC, our results show that SFC enables more sensitive detection for other compounds such as ractopamine, clenbuterol, bromobuterol and isoxuprine. Finally, both SFC and LC techniques allowed reaching detection performances far below the recommended concentrations at 50 µg kg^{-1} for all β-agonists in feed. Indeed, the signal-to-noise ratio was always above 3 whatever the technology for samples spiked at 25 µg kg^{-1}.

Acknowledgements

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GC/APCI/MS/MS TO PROVIDE RELIABLE SIGNALS FOR THE CHALLENGING MEASUREMENT OF ANDROSTANEDIOLS AND ESTRANEDIOLS

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Abstract

5ξ-Androstane-3ξ,17ξ-diols (AAD) and 5ξ-estrane-3ξ,17ξ-diols (EAD) are known biomarkers of testosterone and 19-nortestosterone in mammals, respectively, which are monitored for scientific clinical studies and anti-doping control purposes. Their analysis is performed either by LC/MS or GC/MS with derivatization. The main drawback of GC/EI/MS remains the extensive fragmentation of labile diol steroids. A phenomenon that leads to non-specific mass spectra characterized by low *m/z* fragments. Thereby MS/MS methods do not deliver the expected gain of sensitivity that may be expected from such strategy as precursor ions are generally low *m/z* ions. As an alternative, we have investigated an analytical strategy combining high chromatographic resolution with weak energy ionization with main aim to provide specific and sensitive signals to be further used in MS/MS. GC/APCI/MS/MS offered a perfect combination for this purpose. Ionization (API source atmosphere, corona discharge parameters) and transfer line conditions (temperature, gas flow) were investigated together with various derivatization strategies. Chromatographic (resolution, peak shape, noise) and spectrometric (selectivity, sensitivity, repeatability) performances have been improved for optimized detection. Observed performances are critically discussed and compared with standard analytical approaches currently implemented by analysts for AAD and EAD detections in various scopes of application.

Introduction

Estranediols and androstanediols are still considered as relevant steroid phase I metabolites for scientific clinical studies and anti-doping control purposes. Indeed, estranediol is a reduced metabolite of nandrolone which consists in an estrane skeleton with three asymmetric carbons 3, 5 and 17 which correspond to eight different isomers, described as 5ξ-estrane-3ξ,17ξ-diol (EAD). For instance, two particular EAD isomers mark the nandrolone abuse in boar, horse, greyhound and cattle (1-3). Androstanediol is a reduced metabolite of testosterone, which consists of an androstane skeleton with three asymmetric carbons 3, 5 and 17. They give eight possible isomers denoted as 5ξ-androstane-3ξ,17ξ-diol. 5α-Androstane-3α,17β-diol (aab-AAD) is considered an endocrinological and a gonadal activity marker monitored by clinicians to reveal prostate disease (4), 21-hydroxylase deficiency (5) and as a receptor-modulating neurosteroid (6). aab-AAD is also monitored with additional AAD (bab, aaa, baa) to confirm intake of androgens for doping purposes (7).

The analytical approaches used in clinical studies differ from those used in anti-doping control. Indeed, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is generally used in clinical studies based on electrospray (ESI) (4,8,9) or atmospheric pressure chemical ionization (APCI) in positive mode (6). The compounds are then directly analysed or derivatised with FMP (4) in order to improve the sensitivity by at least a factor 10. Using this best condition of sensitivity, 3α- and 3β-AAD remain however co-eluting on a chromatographic reversed-phase. Their separation is possible using a chiral phase, but will lose a factor of 10 in sensitivity. In laboratories dealing with anti-doping control, gas chromatography coupled to (tandem) mass spectrometry is usually used in electron ionization (GC/EI/MS(/MS)) (1-3,7). Trimethylsylilation alone or coupled with oximination enable AAD and EAD analysis with GC. GC-separation provides enough resolution to separate each isomer from the others and to correctly quantify them separately. It is probably for this reason that GC is preferred to LC in anti-doping controls. Nevertheless, sensitivity of LC/MS/MS is not achieved with GC/MS/MS analysis. Indeed, EAD and AAD are rather unstable in electron ionization and their mass spectra are highly fragmented, leading to a loss of sensitivity in SRM analysis. The aim of our study was to improve the sensitivity in GC/MS/MS in assessing (1) a derivative which can be stable and compatible with GC analysis (2) a softer ionization, the APCI one, to improve the specificity and the sensitivity of the measurement in the same time.

Materials and Methods

Chemicals, reagents, solvents and standards

Chemicals, reagents and solvents were of high quality grade for trace analysis: ethanol was LC grade and purchased from LGC Promochem (Molsheim, France), *n*-nonane was from Merck (Fontenay-sous-bois, France). Ultrapure water was obtained using a Nanopure system from Barnstead (Waltham, MA, USA). *N,O*-Bistrimethylsilyltrifluoroacteamide (BSTFA), acetic anhydride (99.5%) pentafluoropropionic anhydride (PFPA) and trifluoroacetic anhydride (TFAA) were from Sigma-Aldrich (Saint Quentin Fallavier, France), pyridine on molecular sieves and toluene on molecular sieves were from Acros Organics (Geel, Belgium).

Aba-, abb-, baa-, aaa-, bba-AAD, abb-, aba-, bab-, aab and baa-EAD standards were provided by Steraloids (Newport, United States), bab- and bbb-AAD were from Sigma-Aldrich Chimie SARL (Lyon, France) and aab-AAD was purchased by AGAL NARL (Kensington, Australia). The purity of all standards was higher than 98%.

GC/EI/MS

The system used was a gas chromatograph A6890 coupled to a simple quadrupole mass spectrometer 7890 (Agilent Technologies, Palo Alto, CA, USA) using electron ionisation. The column was a DB5-MS (30 m x 0.25 mm, 0.25 µm). Injector temperature was 280°C. Splitless mode was used (2 µL, 2 min) as injection mode and dihydrogen was used as carrier gas (11 psi under constant pressure). Oven temperature was 120°C (2 min), 40°C min^{-1} until 250°C (4.5 min) and 40°C min^{-1} until 300°C (2 min). Analysis was performed in SCAN mode. Ionisation energy was moved between 30 and 90 eV to study the ionisation and fragmentation performances.

GC/APCI/MS/MS

The system used was a gas chromatograph A7890 (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole mass spectrometer Xevo TQS (Waters, Milford, US) using an atmospheric pressure interface. The column was a DB5-MS (30 m x 0.25 mm, 0.25 µm). Injector temperature was 280°C. Splitless mode was used (2 µL, 2 min) as injection mode and helium as carrier gas (at 22, 28 and 35 psi under constant pressure). Oven temperature was 120°C (2 min), 40°C min^{-1} until 250°C (4.5 min) and 40°C min^{-1} until 300°C (2 min). Analysis was performed in SCAN mode. Transfer line temperature was set at 380°C with a sheath nitrogen gas at 31 psi (constant pressure). Source temperature was 150°C. Auxiliary and cone gas flow rates were 300 and 0 L h^{-1}. Atmospheric pressure chemical ionisation (APCI) was achieved in positive dry mode. Corona discharge was generated at a current from 1 to 5 µA to study the ionisation and fragmentation performances. Cone voltage was set at 25 V.

Derivatization reactions

Trimethylsilylation. 20 µL BSTFA was added to the dried standards, vortexed and heated 40 min at 60°C before injection (10).

Acetylation. 50 µL of acetic anhydride was added to the standards dissolved in 50 µL pyridine. After homogenization, the mixture was heated at 60°C for 60 min. The reagent was then eliminated by evaporation before reconstitution of the dry residue in 20 µL nonane (11).

Pentafluoropropyl acylation. 100 µL of PFPA was added to the standards dissolved in 100 µL toluene. After homogenization, the mixture was heated at 80°C during 60 min. The reagent was then eliminated by evaporation before reconstitution of the dry residue in 20 µL nonane (12).

Trifluoromethyl acylation. 40 µL of TFAA was added to the standards dissolved in 100 µL toluene. After homogenization, the mixture was heated at 70°C during 30 min. The reagent was then eliminated by evaporation before reconstitution of the dry residue in 20 µL nonane (13).

Table 1. retention times on DB5MS column for each steroid of interest depending on the derivatives produced.

Steroids	TMS derivatives retention time (min)	Acetylation derivatives retention time (min)	PFPA derivatives retention time (min)	TFAA derivatives retention time (min)
5β-androstan-3α,17α-diol	7.74	10.22	7.48	7.88
5β-androstan-3β,17α-diol	7.86	10.24	7.55	7.91
5α-androstan-3α,17α-diol	7.96	10.52	7.64	8.19
5β-androstan-3β,17β-diol	8.53	10.55	8.78	8.78
5α-androstan-3α,17β-diol	8.70	10.69	8.83	8.98
5β-androstan-3α,17β-diol	8.78	10.71	9.07	9.22
5α-androstan-3β,17α-diol	9.03	10.83	9.09	9.40
5α-androstan-3β,17β-diol	9.52	10.94	10.03	9.97
5β-estran-3α,17α-diol	7.60	9.94	7.17	7.62
5α-estran-3α,17β-diol	7.96	9.95	7.90	7.95
5α-estran-3β,17α-diol	8.18	10.09	8.04	8.18
5β-estran-3α,17β-diol	8.47	10.26	8.63	8.62
5α-estran-3β,17β-diol	8.54	10.36	8.74	8.69

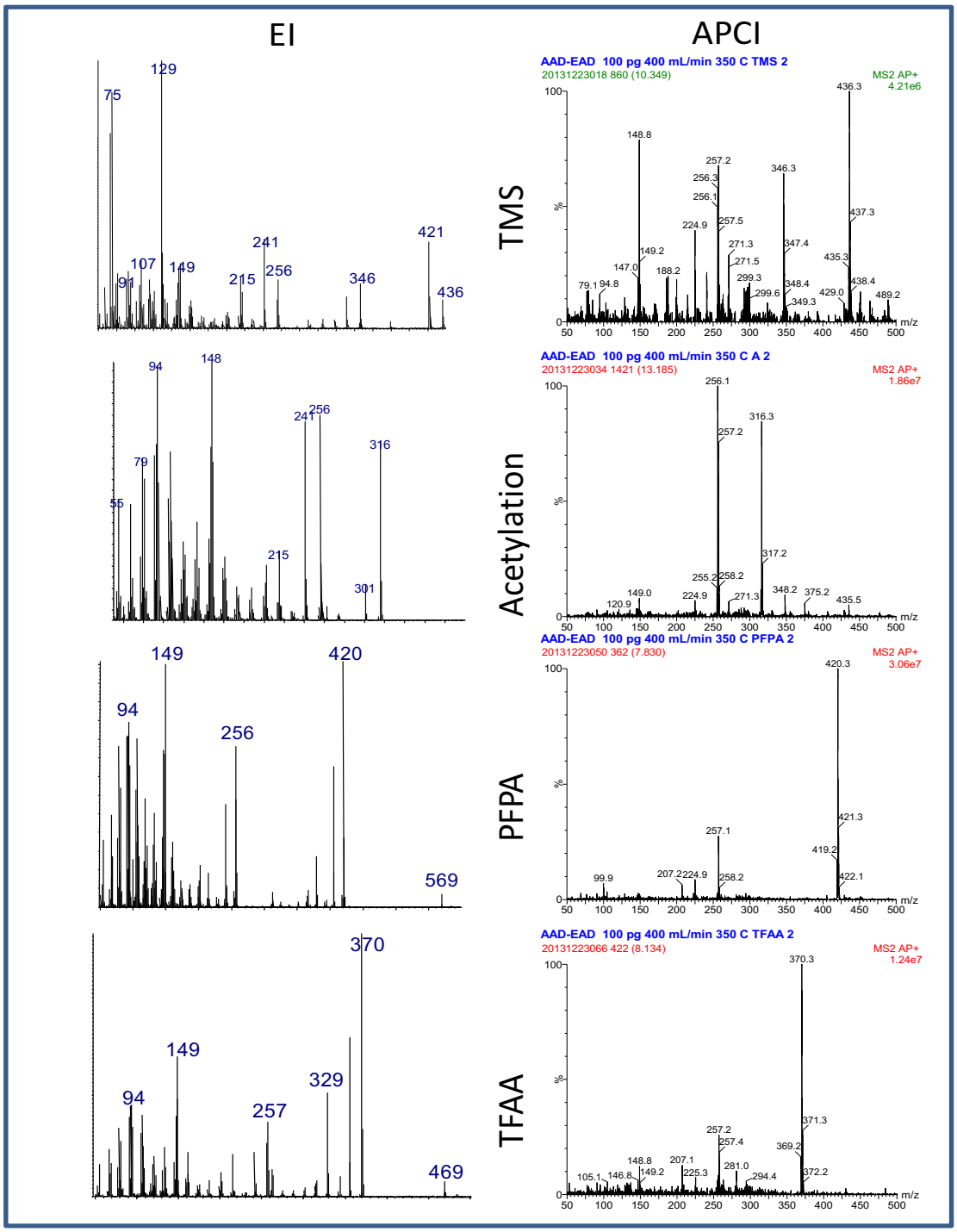

Figure 1. Mass spectra of baa-AAD (100 pg injected) obtained in electron ionisation (left column) and in atmospheric pressure chemical ionisation (right column) after TMS derivatization, acetylation, pentafluoropropyl and trifluoroacetyl acylations.

Results and Discussions

Chromatographic efficiency in using 4 derivatization strategies

Several derivatization procedures were compared in order to define the best compromise between selectivity (capability of isomer separation) and run duration. Table 1 presents the retention times of each steroid of interest on the DB5MS column (30x0.25 mm, 0.25 µm) for the four derivatization procedures after GC/MS analysis. Acetylation produced the less volatile products with retention times close to 10 min. Peak shapes were wider than with the other studied reactions because elution occurred at the end of the isotherm of 4.5 min (9.75 min on the total run). Isomers were less well separated with an elution window of 1 min and a bad separation of four couples of compounds, *i.e*. bbb/aaa-AAD, baa/bba-AAD, bab/aba-AAD and baa/aab-EAD. The best separation was achieved with PFPA derivatives where the retention window was extended to 3 min,

followed by TFAA and TMS derivatives with 2.4 and 1.9 min retention windows respectively. For these three last derivatives, the retention times were always below 10 min which is compatible with fast analysis in GC. Despite its widest range of retention times, PFPA derivatization did not allow an efficient separation of the couple bab/aba-AAD, whereas TFAA reaction gave a bad separation of the couple baa/bba-AAD. TMS derivatives were all well-separated (more than 0.07 min). However, these TMS-derivatives are well-known as water-sensitive and cannot be kept for a long time after derivatization. As TMS, TFAA and PFPA can be used, the sensitivity of response was also investigated in order to select the final derivatization procedure.

Sensitivity capability

Derivatization procedures were also compared in order to create the most sensitive MS/MS events. The strategy was to focus on the most intense and the highest *m/z* fragment after ionisation -the molecular ion when possible - to increase both sensitivity and selectivity at the same time. Different ionisation energies were also studied either in EI or in APCI to determine the most adapted parameters for all the 13 analytes of interest; 70 eV and 0.8 µA were thus respectively selected to enhance the ionization recoveries (results not shown here). For all the derivatives studied, APCI generated the best results with lower associated fragmentations, as illustrated in Figure 1. Acylation produced more informative spectra than silylation, with 2 major and relevant ions for MS/MS events. Finally, the GC/MS/MS method was found the most efficient using TFAA derivatives thanks to both the separation efficiency and the sensitivity. These performances are illustrated in Figure 2 with the separation of the eight AAD after 100 fg injected on-column.

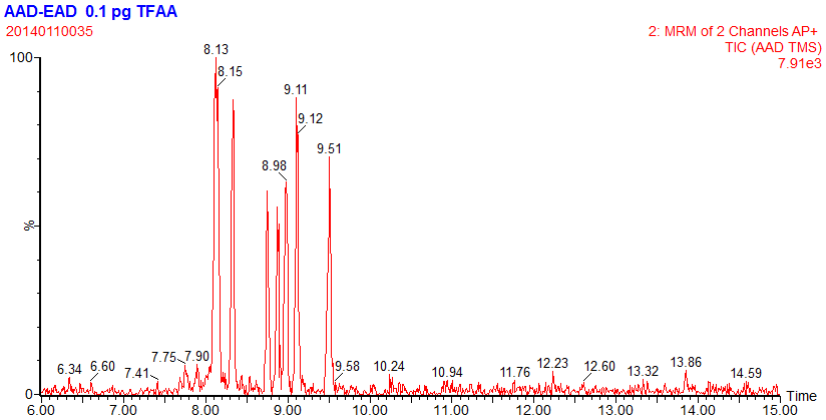

Figure 2. TIC of the 8 androstanediols after TFAA derivatization (100 fg injected) of the two transitions 370.2>241.2 @ 20 eV and 370.2>256.2 @ 10 eV performed on the GC/APCI/MS/MS instrument.

Conclusions

Our study allowed the improvement of GC/MS/MS performances for AED and AAD analysis, in combining speed, sensitivity and selectivity of the measurement. Derivatization is a necessary step for the analysis of hydroxylated steroids. The TFAA reagent was selected as the best reagent to achieve an efficient separation of isomers coupled with a specific MS/MS method. Compared to EI, APCI interface provides a smooth but efficient ionisation which leads to enhancement of the final sensitivity and selectivity of the mass spectrometry measurement. The proposed analytical workflow covers the specific analysis of 13 steroid stereoisomers within the same analytical run of 10 min, which is highly compatible with high throughput analysis as required for clinical routine purposes.

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DEVELOPMENT OF A MULTI-RESIDUE METHOD FOR *B*-LACTAM ANTIBIOTICS IN *BOVINE* MUSCLE USING UHPLC-MS/MS

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Abstract
A quantitative, multi-residue method was developed for measurement of 30 β-lactam antibiotic residues in *bovine* muscle. The method includes twelve penicillins (amoxicillin, ampicillin, cloxacillin, dicloxacillin, mecillinam, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, ticarcillin), twelve cephalosporins (cefacetrile, cefadroxil, cephalexin, cefalonium, cefazolin, cefoperazone, cefotaxime, cefquinome, cefuroxime, desacetyl cephapirin, desfuroylceftiofur cysteine disulfide, desfuroylceftiofur dimer), five carbapenems (biapenem, doripenem, ertapenem, imipenem, meropenem) and faropenem. Samples were extracted using a simple solvent extraction with acetonitrile:water (80:20, v/v) and C_{18} dispersive solid-phase extraction (dSPE) clean-up followed by ultra-high performance liquid chromatography coupled to tandem mass-spectrometry (UHPLC-MS/MS) detection. Validation was performed in accordance with Commission Decision 2002/657/EC. Calibration curve ranges were 2.5 - 20 µg kg^{-1} for mecillinam, piperacillin, methicillin and cefazolin; 5 - 40 µg kg^{-1} for ticarcillin, cefalonium, cefacetrile, cefotaxime, cefuroxime, cefoperazone, biapenem and meropenem; 6.25 - 50 µg kg^{-1} for penicillin V; 50 - 400 µg kg^{-1} for cephalexin and ertapenem; 75 - 600 µg kg^{-1} for cloxacillin, dicloxacillin, nafcillin and oxacillin; 125 - 1,000 µg kg^{-1} for desfuroylceftiofur cysteine disulfide and desfuroylceftiofur dimer and 12.5 - 100 µg kg^{-1} for all the other analytes.

Introduction
The β-lactams are antibiotics used in both human and veterinary medicine. Penicillins, cephalosporins and carbapenems represent the most important β-lactam drug groups. Penicillins and cephalosporins are often administered to food-producing animals for the prevention or treatment of bacterial infections. Concerns have been raised over the usage of third and fourth generation cephalosporins, because of their importance in human medicine and potential contribution to antimicrobial resistance. The carbapenems are broad-spectrum antibiotics, but these drugs are not licensed for use in food-producing animals. Inappropriate use of antibiotics may also lead to residues in food and cause health hazards such as allergic reactions in sensitive individuals. Consequentially, it is essential that accurate chemical LC-MS/MS tests are available to confirm and measure the presence of non-compliant antibiotic residues in food.

Most published LC-MS/MS methods for β-lactams report the inclusion of a limited number of penicillins and cephalosporins. The most comprehensive method for 22 β-lactams, including penicillins, cephalosporins and carbapenems, was reported by Berendsen *et al.* (2013) for poultry muscle. Becker *et al.* (2004) also developed a sensitive and specific method for 15 β-lactams for *bovine* milk, muscle and kidney. However, in both cases, the sample preparation involves a laborious procedure and a reversed-phase solid phase extraction (SPE) clean-up.

The objective of this study was to develop a UHPLC-MS/MS method for the major β-lactams listed in Commission Regulation (EU) 37/2010 and for novel compounds that could potentially be used outside of the European Union. The method also includes the major metabolite of cephapirin, desacetyl cephapirin (DAC) and two of the ceftiofur-related metabolites, namely desfuroylceftiofur cysteine disulfide (DCCD) and desfuroylceftiofur dimer (DCD). A further objective of this study was to develop a fast and easy sample preparation procedure, with no need for extensive steps.

Materials and Methods

Chemicals and standards
Ampicillin trihydrate, cloxacillin sodium salt monohydrate, dicloxacillin sodium salt hydrate, mecillinam, methicillin sodium salt, nafcillin sodium salt monohydrate, oxacillin sodium salt monohydrate, penicillin V potassium salt, cefalonium hydrate, cephapirin sodium, biapenem, doripenem monohydrate, meropenem trihydrate and faropenem sodium hydrate were purchased from Sigma-Aldrich (Dublin, Ireland). Amoxicillin trihydrate, penicillin G potassium salt, piperacillin, ticarcillin monosodium, cefadroxil hydrate, cefazolin sodium salt, cefoperazone sodium salt, cefotaxime sodium salt, cefquinome sulphate, cefuroxime sodium salt, cephalexin monohydrate and imipenem were purchased from LGC Standards (Teddington, Middlesex, UK). DCD and DCCD were a gift from Zoetis (Kalamazoo, MI, USA). Cefacetrile, DAC sodium salt, ertapenem disodium

90% and the internal standards amoxicillin-d_4, ampicillin-d_5, benzyl penicillanate-d_7 potassium salt (penicillin G-d_7), nafcillin-d_5 sodium salt, penicillin V-d_5, cefadroxil-d_4 (major), cefazolin-$^{13}C_2{}^{15}N$ sodium salt, cephalexin-d_5 hydrate, DAC-d_6 sodium salt (major) and DCCD-d_3 were supplied by Toronto Research Chemicals (Toronto, ON, Canada). Ultra-pure water (18.2 MΩ) was generated in-house using a Millipore system (Cork, Ireland). Acetonitrile (MeCN) was HPLC grade and supplied by Romil Ltd (Cambridge, UK). Dimethyl sulfoxide (DMSO), formic acid (HCOOH) 98 - 100% and ammonium acetate were supplied by Sigma-Aldrich. Endcapped C_{18} sorbent was purchased from Agilent Technologies Ltd (Cork, Ireland).

Preparation of standard solutions

Stock solutions of the individual β-lactams and internal standards were prepared in H_2O/MeCN (75:25, v/v) or (50:50, v/v) or DMSO at a concentration of 0.5 mg mL^{-1} or 1 mg mL^{-1}, depending on the solubility of each compound. After preparation, solutions were stored in 2.5 mL aliquots in 15-mL polypropylene tubes at -80°C. Under these storage conditions, individual stock solutions were found to be stable for at least 2 months. Three intermediate standard solutions containing all the β-lactams at concentrations ranging from 4 µg mL^{-1} to 200 µg mL^{-1} were prepared in water. Working calibration solutions were prepared by diluting the intermediate stock solutions in water. A mixed internal standard solution was prepared at concentrations ranging from 0.2 µg mL^{-1} to 10 µg mL^{-1} in water. Intermediate and working solutions were prepared monthly and stored at -80°C.

Sample preparation

Bovine muscle samples not containing any detectable β-lactam residues were used as negative controls. Sample aliquots (2.00 g ± 0.01 g) were weighed into a 50-mL polypropylene centrifuge tube. Extracted matrix calibrants were fortified with 100 µL of the working standard solutions. A 100 µL volume of internal standard solution was added to all calibrants, controls and samples, which were then allowed to stand for 15 min. Water (1.9 mL for the extracted matrix calibrants and 2 mL for the controls and test samples) and MeCN (8 mL) were added and the polypropylene tubes containing the samples were subsequently homogenised over ice for 20 s. The homogenised samples were centrifuged (3,500 rpm, 15 min) and the supernatant was decanted into a 50-mL polypropylene tube containing 500 mg of C_{18} sorbent. The samples were vortexed (40 s) and centrifuged (3,500 rpm, 15 min). A 5 mL aliquot of the supernatant was placed into a 15-mL polypropylene tube and evaporated under nitrogen in a Turbovap at 40°C to a volume of < 1 mL. The volume was then made up to 1 mL with water and the extracts were vortexed for 10 s, prior to filtration through 0.2 µm PTFE syringe filters. The filtered extracts were collected directly into autosampler vials and 10 µL were injected into the UHPLC-MS/MS system.

Instruments and UHPLC-MS/MS conditions

Samples were analysed using a Waters Acquity UPLC system coupled to a Waters Quattro Premier triple quadrupole mass spectrometer (Milford MA, USA) equipped with ESI ionisation probe. Separation was performed on a CSH C_{18} analytical column (2.1 x 100 mm, particle size 1.7 µm) fitted with an in-line filter with a 0.2 µm pore size. The column temperature was maintained at 30 °C. A binary gradient separation comprising of 0.01% HCOOH in water with 0.2 mM ammonium acetate (mobile phase A) and 0.01% HCOOH in MeCN (mobile phase B) was employed at a flow rate of 0.4 mL min^{-1}. The gradient profile was as follows: (1) 0 - 1.5 min, 100% A; (2) 1.5 - 3.5 min, 80% A; (3) 3.5 - 8.5 min, 20% A; (4) 8.5 - 8.6 min, 0% A; (5) 8.6 - 10.5 min, 0% A; (6) 10.5 - 10.6 min, 100% A, which was held for 7.4 min (re-equilibration of the column), for a total run time of 18 min. The UHPLC autosampler was rinsed after each injection using strong (H_2O:MeCN, 20:80 v/v, 750 µL) and weak (H_2O:MeCN, 90:10 v/v, 1000 µL) washes. Sample temperature was maintained at 7°C in the autosampler.

A divert valve was used to reduce the introduction of co-extracted matrix components into the MS (solvent delay: [a] 0 - 1.97 min; [b] 7.56 - 18 min). The mass-spectrometer was operated in positive electrospray ionisation mode (ESI+) and the capillary voltage was set at 2.4 kV. Source and desolvation temperatures were 140°C and 450°C, respectively. MS tuning was performed by teed infusion of 1 µg mL^{-1} standard aqueous solutions of the β-lactams and internal standards using a mobile phase consisting of (A) 0.01% HCOOH in water and (B) 0.01% HCOOH in MeCN (50:50, v/v). The LC pump was operated at a flow rate of 0.3 mL min^{-1} and the syringe pump was operated at 20 µL min^{-1}. The cone voltage and collision energy were optimised for each analyte and two product ions were selected so that a minimum of four identification points were obtained for all the analytes as required by Commission Decision 2002/657/EC. A multiple-reaction monitoring (MRM) method was developed with 15 different time-sectored events. Inter-scan delay and inter-channel delay were set to 5 ms (Table 1).

Validation

The following parameters were evaluated during the validation of the method: selectivity, linearity, matrix effect, accuracy, within laboratory reproducibility (WLR), absolute recovery, decision limit (CCα) and detection capability (CCβ). For the legislated compounds, validation was carried out at 0.5, 1 and 1.5 times the maximum residue limits (MRLs) established by current regulation (37/2010) except for DCD and DCCD, which were validated at 250, 500 and 750 µg kg^{-1}. The non-MRL substances were validated around a target level that was identified based on the sensitivity of the method. The validation was performed on three different days by three different analysts. On each day, a different *bovine* muscle negative control was used and eight portions of the sample were fortified at each validation level.

Table 1. UHPLC-MS/MS conditions for β-lactam antibiotics.

Analyte	Measured ion	RT (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	Dwell time (s)	CV (V)	CE (eV)	MRM[b]
Imipenem	$[M+H]^+$	2.27	300.1	142.0[a]/126.1	0.194	22	28/17	1
Biapenem	$[M+H]^+$	2.86	351.2	110.2[a]/170.1	0.180	25	19/20	2
Amoxicillin	$[M+H]^+$	3.17	366.1	114.0[a]/349.1	0.035	18	20/9	3
Amoxicillin-d_4	$[M+H]^+$	3.17	370.6	354.0	0.020	16	9	3
Cefadroxil	$[M+H]^+$	3.22	364.1	208.1[a]/158.0	0.030	15	9/9	3
Cefadroxil-d_4	$[M+H]^+$	3.21	368.6	115.0	0.012	16	20	3
DAC	$[M+H]^+$	3.25	382.1	152.1[a]/226.1	0.030	29	24/23	3
DAC-d_6	$[M+H]^+$	3.23	387.9	115.1	0.015	31	45	3
Doripenem	$[M+H]^+$	3.32	421.1	342.2[a]/274.2	0.021	20	13/16	3
Meropenem	$[M+H]^+$	3.48	384.1	141.0[a]/114.1	0.103	23	15/23	4
Ampicillin	$[M+H]^+$	3.73	350.1	106.1[a]/192.1	0.033	22	21/16	5
Ampicillin-d_5	$[M+H]^+$	3.72	355.0	197.1	0.025	18	16	5
Cephalexin	$[M+H]^+$	3.73	348.1	158.1[a]/174.1	0.036	20	9/17	5
Cephalexin-d_5	$[M+H]^+$	3.72	353.0	179.1	0.025	17	14	5
Cephapirin	$[M+H]^+$	3.77	424.0	292.1[a]/152.1	0.040	24	15/24	5
Cefquinome	$[M+H]^+$	4.04	529.0	134.2[a]/167.1	0.074	21	13/23	6
Ertapenem	$[M+H]^+$	4.16	476.1	432.1[a]/233.2	0.016	20	9/14	6
Mecillinam	$[M+H]^+$	4.18	326.1	167.2[a]/139.2	0.022	34	15/29	6
DCCD	$[M+H]^+$	4.20	548.7	183.1[a]/241.2	0.032	36	29/20	6
DCCD-d_3	$[M+H]^+$	4.18	551.9	366.0	0.032	36	21	6
Cefalonium	$[M+H]^+$	4.23	458.9	152.0[a]/337.0	0.006	18	18/10	6
Cefacetrile	$[M+NH_4]^+$	4.83	357.0	280.1[a]/252.1	0.056	16	9/14	7
Cefotaxime	$[M+H]^+$	4.84	456.1	396.0[a]/167.1	0.080	23	10/19	7
Faropenem	$[M+Na]^+$	5.01	308.0	178.0[a]/222.1	0.047	26	18/12	8
Cefazolin	$[M+H]^+$	5.06	454.9	156.0[a]/295.1	0.047	17	15/15	8
Cefazolin-$^{13}C_2{}^{15}N$	$[M+H]^+$	5.06	457.8	298.1	0.047	15	16	8
Cefuroxime	$[M+NH_4]^+$	5.32	442.0	364.1[a]/336.1	0.129	16	14/14	9
Cefoperazone	$[M+H]^+$	5.51	646.0	143.2[a]/530.0	0.022	25	33/12	10
DCD	$[M+2H]^{2+}$	5.64	429.2	183.1[a]/397.0	0.026	23	21/13	10
Ticarcillin	$[M+H]^+$	5.73	385.1	160.1[a]/114.1	0.039	25	14/36	10
Methicillin	$[M+H]^+$	5.95	381.1	165.2[a]/222.2	0.038	27	23/18	10
Piperacillin	$[M+H]^+$	5.99	518.0	143.2[a]/160.1	0.020	28	17/10	10
Penicillin G	$[M+H]^+$	6.19	335.1	160.1[a]/176.1	0.135	21	12/12	11
Penicillin G-d_7	$[M+H]^+$	6.17	342.0	183.1	0.080	18	11	11
Penicillin V	$[M+H]^+$	6.51	351.1	160.1[a]/114.1	0.139	21	13/33	12
Penicillin V-d_5	$[M+H]^+$	6.49	355.9	160.0	0.050	20	15	12
Oxacillin	$[M+H]^+$	6.74	402.1	160.1[a]/243.2	0.150	20	16/14	13
Cloxacillin	$[M+H]^+$	7.00	436.0	160.1[a]/277.1	0.115	22	12/12	14
Nafcillin	$[M+H]^+$	7.13	415.1	199.2[a]/171.2	0.081	20	7/28	14
Nafcillin-d_5	$[M+H]^+$	7.11	420.0	261.1	0.050	19	17	14
Dicloxacillin	$[M+H]^+$	7.38	470.0	160.2[a]/114.2	0.151	22	14/34	15

[a] *= Quantitation ion; RT = Retention Time; CV = Cone Voltage; CE = Collision Energy*

[b] *= MRM Windows 1 = (1.99 – 2.98 min); 2 = (2.67 - 3.13 min); 3 = (2.98 – 3.46 min); 4= (3.41 – 3.58 min); 5 = (3.60 – 3.87 min); 6 = (3.91 – 4.41 min); 7 = (4.75 – 4.96 min); 8 = (4.93 – 5.20 min); 9 = (5.23 – 5.44 min); 10 = (5.38 – 6.15 min); 11 = (6.05 – 6.32 min); 12 = (6.36 – 6.65 min); 13 = (6.65 – 6.87 min); 14 = (6.85 – 7.32 min); 15 = (7.28 – 7.52 min)*

The available labelled internal standards were used in the quantification of their corresponding analytes. Cloxacillin, dicloxacillin and oxacillin were all corrected using nafcillin-d_5; cefacetrile, cefotaxime, cefuroxime and faropenem were corrected using cefazolin-$^{13}C_2{}^{15}N$, mecillinam and ertapenem using ampicillin-d_5 and cefalonium and cefquinome using cephalexin-d_5; doripenem and imipenem were corrected using DAC-d_6. No internal standards were used for the other analytes.

Results and Discussion

Method development

MS/MS conditions. Teed infusion experiments showed protonated molecules $[M+H]^+$ for most of the β-lactams. Monitored precursor ion for DCD was $[M+2H]^{2+}$; faropenem formed the sodium adduct $[M+Na]^+$, while cefacetrile and cefuroxime formed the ammonium adduct $[M+NH_4]^+$. Stability issues of the penicillin stock solutions were also discovered when tuning was performed. Penicillin standards were, indeed, initially dissolved in methanol (MeOH) or H_2O/MeOH and stored at -30°C. MeOH is a common solvent used in the analysis of β-lactams. Contradictory informations can be found in literature regarding the stability of penicillin standards in solvent (Becker *et al.*, 2004; Berendsen *et al.*, 2011; Fagerquist *et al.*, 2005; Mastovska and Lightfield, 2008; Okerman *et al.*, 2007; Tyczkowska *et al.*, 1992). In our research, MS tuning was initially performed immediately after standard preparation and subsequently repeated two weeks later. Interesting results were observed for cloxacillin, dicloxacillin and penicillin V. The second experiment showed that the optimal cone voltage for cloxacillin and dicloxacillin had changed from the original value (22 V) to a higher value (46 V) and from 21 V to 38 V for penicillin V. The MS spectrum of each compound also showed a mass corresponding to their methyl ester, while the MS/MS spectrum appeared completely different when compared to the one obtained two weeks earlier. This was likely due to a breakdown of the β-lactam ring and occurrence of methanolysis. The results are in agreement with what is reported by Pellicciotti *et al.* (2014). For this reason, MeOH was completely removed from the entire method. Alternative solvents and mixtures were selected and standard solutions were stored at -80°C.

UHPLC conditions. A range of different mobile phase additives (formic acid, acetic acid, trifluoroacetic acid, ammonium acetate and ammonium formate) were evaluated at different concentrations and their effect on sensitivity and chromatography was investigated. In addition, three different UPLC column chemistries were assessed, namely, BEH C_{18}, CSH C_{18} and HSS T3. Optimal results were achieved when using a binary gradient separation with mobile phase consisting of (A) 0.01% HCOOH and 0.2 mM ammonium acetate in water and (B) 0.01% HCOOH in MeCN, with separation on a CSH C_{18} column. The concentration of 0.01% HCOOH provided the maximum overall response, while higher concentrations of the same acid or use of different acids significantly decreased sensitivity. Response for all analytes was also decreased when using a concentration of salts higher than 0.5 mM. Therefore, 0.2 mM ammonium acetate was added to mobile phase A to promote the formation of the ammonium adduct for the cephalosporins cefacetrile and cefuroxime.

Sample preparation. The aim of this study was to develop a rapid and easy sample preparation procedure. The QuEChERS approach (Quick, Easy, Cheap, Effective, Rugged and Safe) was evaluated during preliminary studies, but it seemed to cause a significant loss of the most polar compounds. In contrast, a more suitable simple protocol was identified based on the methods developed by Fagerquist *et al.* (2005) and Mastovska and Lightfield (2008). We found that the homogenisation of muscle tissue samples over ice significantly improved the precision of the method. Similarly, Turbovap conditions were also critical, showing that an evaporation temperature of 40°C at maximum nitrogen flow allowed solvent removal in approximately 45 min. A number of sorbent materials were investigated for dSPE: C_{18}, C_8, PSA (primary/secondary amine), NH_2 and zirconium dioxide-based sorbents (Z-Sep, Z-Sep+, Z-Sep/C_{18}). PSA and NH_2 sorbents were immediately discarded, as they provided unacceptable recoveries for the majority of the analytes. Among all the other sorbents, the use of C_{18} only minimised matrix effects and showed best overall recoveries and precision.

Method validation

Selectivity, linearity and matrix effect. Analytes and internal standards were injected singly in order to monitor for interferences in the UHPLC-MS/MS traces. Cross-talk was minimised by optimising the chromatographic separation and identifying unique product ions for each compound. The selectivity of the method was also verified through testing 27 *bovine* muscles from different origin and no matrix interferences were observed. Linearity (R^2 > 0.988) was achieved for all the analytes over the calibration range of the method. Matrix effects were also evaluated: 27 blank samples were post-extraction spiked at the MRLs or target levels and the signal obtained from those samples was compared to the signal obtained from a standard solution at the same concentration. Both ion suppression and enhancement were observed. Amoxicillin and methicillin showed the greatest amount of ion suppression and enhancement, respectively (75% and 73%). However, accuracy was significantly improved for most of the analytes using the internal standards.

Accuracy, WLR, absolute recovery, CCα and CCβ. Satisfactory accuracy and coefficients of variation (CVs) were achieved for all the analytes. Absolute recoveries were calculated by comparing results from fortified samples to those of negative samples spiked post-extraction at 2 x lowest calibration level and 0.875 x highest calibration level on three different occasions. Overall absolute recoveries ranged from 61 to 89% for all the compounds, except for cefadroxil, DCCD, DCD, imipenem and ertapenem (see Table 2).

Table 2. Validation results for bovine muscle.

Analyte	MRL/TL (µg kg^{-1})	Accuracy% (WLR%)			CCα (µg kg^{-1})	CCβ (µg kg^{-1})	OAR% (RSD%, n = 3)	
		0.5 MRL/TL	MRL/TL	1.5 MRL/TL			2 LCL	0.875 HCL
Amoxicillin	50	102 (5.9)	101 (4.5)	103 (5.2)	53	57	69 (2.4)	70 (5.5)
Ampicillin	50	101 (2.4)	99 (2.4)	101 (3.3)	51	53	78 (7.9)	76 (4.8)
Cloxacillin	300	103 (2.2)	101 (2.2)	101 (1.7)	308	318	81 (5.6)	88 (3.6)
Dicloxacillin	300	104 (2.4)	103 (2.3)	104 (5.0)	320	344	83 (2.3)	87 (3.4)
Mecillinam	10	96 (2.5)	97 (3.3)	100 (4.0)	10.5	11.2	85 (1.1)	83 (2.5)
Methicillin	10	108 (10.2)	104 (9.5)	98 (6.7)	12.0	13.9	85 (5.0)	84 (4.4)
Nafcillin	300	101 (2.2)	101 (1.8)	100 (1.5)	309	318	84 (2.0)	88 (4.2)
Oxacillin	300	103 (2.7)	101 (3.2)	101 (3.2)	322	340	78 (12.6)	89 (5.8)
Penicillin G	50	106 (2.8)	105 (2.8)	105 (2.4)	53	56	79 (12.5)	85 (3.1)
Penicillin V	25	101 (2.4)	100 (2.3)	102 (2.8)	26.0	27.2	87 (1.5)	86 (3.4)
Piperacillin	10	104 (4.9)	104 (5.8)	105 (5.3)	11.0	12.1	89 (4.0)	85 (4.6)
Ticarcillin	20	99 (6.4)	99 (8.6)	100 (6.7)	23.5	26.7	61 (5.3)	62 (6.6)
Cefacetrile	20	101 (4.3)	100 (4.6)	99 (8.2)	21.4	24.1	80 (2.7)	79 (3.4)
Cefadroxil	50	100 (5.7)	98 (5.4)	101 (6.3)	52	57	58 (2.1)	61 (8.1)
Cefalexin	200	98 (1.9)	98 (1.9)	100 (3.5)	203	212	69 (4.8)	67 (3.9)
Cefalonium	20	100 (8.9)	100 (5.6)	100 (6.0)	21.7	24.0	79 (13.0)	81 (2.4)
Cefazolin	10	101 (5.0)	101 (4.5)	100 (5.2)	10.7	11.6	81 (4.6)	83 (4.2)
Cefoperazone	20	104 (5.5)	101 (7.0)	102 (6.3)	22.7	22.5	86 (3.7)	86 (6.7)
Cefotaxime	20	102 (6.4)	104 (5.2)	104 (9.2)	22.4	26.0	64 (5.0)	67 (7.0)
Cefquinome	50	97 (4.9)	97 (7.9)	101 (7.2)	57	65	87 (3.5)	86 (5.3)
Cefuroxime	20	104 (7.2)	102 (6.7)	101 (7.2)	22.0	25.0	87 (1.4)	84 (3.2)
Cephapirin	50	Quantitatively converted to DAC in spiked muscle (approx. 15 min)						
DAC	50	102 (4.9)	101 (4.4)	101 (3.7)	55	59	77 (5.0)	78 (2.7)
DCCD	500	104 (4.8)	101 (5.9)	105 (6.5)	577	646	40.3 (7.8)	45.1 (2.9)
DCD	500	108 (5.2)	102 (7.0)	103 (8.8)	570	665	53 (4.7)	56 (7.9)
Biapenem	20	106 (5.6)	103 (2.9)	103 (5.8)	20.4	22.1	71 (2.2)	71 (3.6)
Doripenem	50	106 (4.8)	103 (7.1)	100 (4.7)	59	65	62 (3.9)	65 (2.8)
Ertapenem	200	101 (4.7)	97 (4.3)	102 (8.0)	216	245	44.2 (6.4)	43.3 (6.8)
Imipenem	50	102 (6.5)	99 (7.2)	86 (9.5)	66	81	47.0 (8.3)	51 (4.9)
Meropenem	20	104 (3.6)	100 (6.0)	99 (3.6)	21.9	23.6	66 (0.46)	66 (8.4)
Faropenem	50	97 (17.3)	97 (17.0)	97 (11.7)	58	72	78 (0.10)	79 (3.3)

TL = target level; OAR = overall absolute recovery; LCL = lowest calibration level; HCL = highest calibration level

Stability in the autosampler. The matrix calibrant fortified at the MRLs or target levels was injected as check sample at evenly spaced intervals throughout the run to monitor the stability of the analytes in the autosampler. All the analytes could be considered stable in matrix for at least the duration of a validation run (approx. 21 h), as the absolute area obtained from the check sample stayed constant until the end of the run and the calculated relative standard deviation (RSD%) was not higher than 7.9% except for imipenem (RSD% = 10.2%). Although validation results were satisfactory, the peak area for imipenem seemed to decrease gradually throughout the run due to the instability of this compound.

Comparison with other existing methods

Relatively few LC-MS/MS methods have been published for the analysis of β-lactam residues in muscle (Becker *et al.*, 2004; Berendsen *et al.*, 2013; Chiaochan *et al.*, 2010; Chico *et al.*, 2008; Granelli *et al.*, 2009; Macarov *et al.*, 2012; Pérez-Burgos *et al.*, 2012; van Holthoon *et al.*, 2010). Most of these methods include a limited number of analytes or exclude cephapirin and ceftiofur metabolites. With the exception of Berendsen *et al.* (2013), no methods for carbapenems in food products have been reported at all. Although the Berendsen method represents the most valid approach for monitoring ceftiofur and the total amount of its metabolites, it involves a complex derivatisation process followed by SPE clean-up, difficult to apply in routine analysis. A time-consuming sample preparation including SPE clean-up is also involved in the method reported by Becker *et al.* (2004). The method described in this study adapted a fast and easy sample preparation protocol previously developed for *bovine* kidney to *bovine* muscle tissue and is able to detect and quantify up to 30 β-lactam antibiotics, including

carbapenems, DAC and two of the ceftiofur metabolites, in a reasonably short chromatographic run (18 min). Finally, data obtained from the WLR study showed that the method performs very well in terms of accuracy and precision.

Conclusions

The analysis of β-lactams is particularly difficult due to stability problems associated with standard solutions. Solvents used during standard preparation and storage conditions play key roles. Our research has shown that the use of MeOH causes a very rapid degradation of some of the penicillins and can potentially lead to the identification and selection of incorrect precursor/product ions. In this work, we addressed the stability issues through the use of alternative solvents, such as H_2O/MeCN and DMSO. A sample preparation protocol was developed so that the residues of 30 β-lactam antibiotics could be easily extracted from fortified *bovine* muscle tissue samples using simple solvent extraction and C_{18} dSPE clean-up prior to detection by UHPLC-MS/MS. The inclusion of isotopically labelled internal standards was found to improve the accuracy and precision of the analytical method. The method has advantages over current published methods because of the wide range of analytes included and the simplicity of the sample preparation. To the best of our knowledge, this is the first non-derivatized method for carbapenems in food products. Further work needs to be carried out to assess the method through the application to incurred samples and inter-laboratory studies.

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MULTI-RESIDUE DETERMINATION OF ANTIBIOTICS IN EUROPEAN SEA BASS LIVER SAMPLES (*DICENTRARCHUS LABRAX*) THROUGH UHPLC-MS/MS

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Abstract

The present work describes a method for the detection and quantification of antibiotics representing seven classes, in European sea bass (*Dicentrarchus labrax*) liver tissues. Sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol were simultaneously determined through ultra-high-performance liquid-chromatography with tandem mass-spectrometry (UHPLC-MS/MS). Several procedures for sample treatment and extraction were tested to achieve the most suitable conditions. Validation was achieved following Decision 2002/657/EC guidelines with precision, recovery, CCα and CCβ determined according to maximum residue limit (MRL) or the minimum required performance limit (MRPL), depending on compounds. With validation, the method was proven suitable for routine analyses in the detection and confirmation of antibiotics in liver of European sea bass, which is an economically important marine fish species, intensively reared in aquaculture.

Introduction

Pharmaceuticals are an important component in intensive animal rearing to prevent and treat disease outbreaks, among which antibiotics play a key role. Given the intensification of production to sustain the demands of a growing worldwide population, antibiotic usage has proportionally increased. To prevent health issues related with the potential presence of residues that can lead to allergic reactions and antibiotic resistance in both veterinary and human medicine and also to monitor illegal administration of forbidden drugs, the European Union has implemented mandatory control of veterinary drug residues in food items of animal origin (EC Regulation 96/23). Thus, the development of multi-class multi-residue methodologies for analyses of residues in animal tissues is paramount to ensure food safety.

Within the food industry, aquaculture has increased significantly due to the changes in global trends for animal products favouring fish consumption (FAO, 2014) and thus monitoring residues in fish is crucial. Presently, few multi-class methods are available for analysis of antibiotics in animal matrices including muscle, liver and kidney tissues. Determination of residues in liver samples is particularly useful due to its function in drug metabolism. However, when analysing fish liver with the existing methods the major setback is usually sample size. The methods available usually require over 2 g of liver sample (Shao *et al*., 2007; Kaufmann *et al*., 2008; Freitas *et al*., 2015), which in case of fish is not always possible since many times it is well under 300 mg. Thus, the present work aimed to develop a suitable methodology for routine analysis of fish liver of European sea bass (*Dicentrarchus labrax*) through ultra-high-pressure-liquid-chromatography tandem mass-spectrometry (UHPL-MS/MS).

Materials and Methods

Reagents and Solutions

With the exception of the reagents used in the mobile phase, which were of HPLC grade, all other chemicals were of analytical grade. Acetonitrile, methanol, *n*-hexane and formic acid were acquired from Merck (Darmstadt, Germany) while ethylenediamine tetra-acetic acid (EDTA) was purchased from Sigma–Aldrich (Madrid, Spain) as well as the standards for sulfonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol.

The following six internal standards were used: demethyltetracycline for the tetracyclines; penicillin V for the penicillins; lomefloxacin for the quinolones; roxithromycin for the macrolides; sulfameter for the sulfonamides and trimethoprim; and chloramphenicol-d5 for chloramphenicol, also acquired from Sigma–Aldrich.

Stock solutions (1 mg mL^{-1}) were prepared by diluting the appropriate amount of standard in methanol and stored at -20°C. By dilution of these, spiking solutions were prepared, which were used in the validation process and in routine analyses. They were stored at -20°C for a month.

Procedures

Two hundred milligrams of liver tissue from sea bass were weighed and placed in a 20-mL polypropylene centrifuge tube. Internal standards solution was added, vortexed for 30 s and allowed to stand in the dark. After this, acetonitrile and EDTA were added and the sample was sonicated. The sample was then centrifuged at 4,000 *g*, the supernatant transferred to a fresh tube and evaporated under gentle nitrogen flux until near dryness (1 mL). Ultrapure water was added and vortexed for 15 s. Clean-up by solid-phase extraction was applied with Oasis HLB cartridges, preconditioned with acetonitrile and water. Elution was done with acetonitrile. After evaporation and the lipid layer removed with *n*-hexane, the final extract was filtered and analysed by UHPLC-MS/MS (see Table 1 for MRM conditions).

The method was also applied to samples of sea bass treated with antibiotics via feed in an animal experiment.

Table 1. MRM acquisition conditions for each compound and internal standards.

Class	Name	ESI	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone Voltage (V)
Tetracyclines	Tetracycline	+	445.5	410.3/427.3	25
	Doxycycline	+	445.5	428.2/410.3	25
	Chlortetracycline	+	479.3	444.2/462.1	25
	Oxytetracycline	+	461.5	426.3/443.3	25
	Demethyltetracycline (IS)				
Macrolides	Tylosin	+	917.1	174.3/772.5	35
	Tilmicosin	+	869.3	174.2/156.1	35
	Spiramycin	+	843.5	174.0/540.3	35
	Erythromycin	+	734.5	158.2/576.5	25
	Roxithromycin (IS)				
Sulfonamides	Sulfanilamide	+	173.2	92.1/156.2	30
	Sulfapyridine	+	250.3	156.3/92.3	30
	Sulfadiazine	+	251.2	156.2/92.2	30
	Sulfathiazole	+	256.4	156.3/92.3	25
	Sulfamethoxazole	+	254.4	156.4/92.2	30
	Sulfisoxazole	+	268.3	156.2/113.2	25
	Sulfamethiazole	+	271	156.2/108.1	25
	Sulfisomidine	+	279.4	186.3/124.4	30
	Sulfamethazine	+	279.4	156.3/124.5	30
	Sulfamethoxypyridazine	+	281.2	156.2/92.2	30
	Sulfachloropyridazine	+	285.3	92.3/156.3	30
	Sulfaquinoxaline	+	301.3	92.2/156.3	30
	Sulfadoxine	+	311.4	156.4/92.3	30
	Sulfadimethoxine	+	311.4	156.4/92.3	30
	Sulfameter (IS)				
Quinolones	Nalidixic acid	+	233.2	215.1/187.1	40
	Flumequine	+	262.2	202.1/244.2	30
	Oxolinic acid	+	262.2	216.1/244.2	30
	Cinoxacin	+	263.2	217.1/245.2	30
	Norfloxacin	+	320.3	276.2/233.2	20
	Enoxacin	+	321.2	303.2/234.2	35
	Ciprofloxacin	+	332.2	288.2/245.2	35
	Danofloxacin	+	358.3	96.1/314.3	33
	Enrofloxacin	+	360.3	316.3/245.2	31
	Ofloxacin	+	362.1	261.3/318.2	34
	Marbofloxacin	+	363.3	72.1/320.2	30
	Lomefloxacin (IS)				
Penicillins	Penicillin G	+	335.1	176.0/160.0	30
	Ampicillin	+	350.4	106.3/160.4	25
	Amoxicillin	+	366.3	160.3/114.4	25
	Penicillin V (IS)				
Benzenoids	Trimethoprim	+	291.5	230.3/261.3	25
Chloramphenicol	Chloramphenicol	-	320.9	151.9/193.9	30

Validation

Validation followed the guidelines in EU Commission Decision 2002/657. Accordingly, recovery, repeatability, within-laboratory reproducibility, decision limit (CC_α) and detection capability (CC_β) were determined.

Results and Discussion

After testing several extraction procedures, the method described produced the best results. Also, HLB cartridges of 500 mg performed better given the small sample size. After successful validation, the method was then applied to sea bass samples in which the concentrations of five antibiotics in liver over time were studied: oxytetracycline (OTC), sulfadiazine (SDZ), trimethoprim (TRI), flumequine (FLU) and oxolinic acid (OXO). Results are shown in Table 2.

The method developed proved to be a valuable tool in routine analyses of liver samples obtained from juveniles of sea bass, which given the age, have very small livers.

Table 2. Antibiotics concentrations in liver samples of sea bass over a 21-day trial.

Treatment	Dose (mg kg^{-1} of diet)	t0	t1	t7	t8a	t8b	t9	t10	t12	t21
OTC	37.5		✔	✔	✔	✔	✔	✔		✔
	75		✔	✔	✔	✔	✔	✔	✔	✔
SDZ	110		✔	✔	✔	✔	✔		✔	
	220		✔	✔	✔	✔	✔	✔	✔	
TRI	22		✔	✔	✔	✔	✔	✔	✔	
	44		✔	✔	✔	✔	✔	✔	✔	✔
FLU	6		✔	✔	✔					
	12		✔	✔	✔	✔	✔			
OXO	6		✔	✔	✔		✔			
	12		✔	✔	✔	✔	✔			

Acknowledgements

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ANALYSIS OF CHLORAMPHENICOL IN COMPLEX FOOD MATRICES USING A SOLID-PHASE EXTRACTION SORBENT BASED ON MOLECULARLY IMPRINTED POLYMERS

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Abstract

Some broad-spectrum antibiotics such as chloramphenicol are prohibited for use in food-producing animals in the European Union, the USA and many other countries due to major human health concerns. So, no residue must be found in food. Practically, analytical methods used for detecting chloramphenicol must meet in the EU a minimum required performance limit (MRPLs) of 0.3 µg kg^{-1}. However, to reach this limit, even with the currently available highly advanced analytical equipment, sample pre-treatment remains a key-factor for the success of the analyses by reducing interferences, which can give ion suppression effects and detector fouling.

A new SPE method has been developed with the use of molecularly imprinted polymer (MIP) for a selective extraction of chloramphenicol from complex matrices such as honey prior to analysis by HPLC or ELISA methods. MIPs are affinity columns made of polymers very stable towards aqueous or organic solvents as well as temperature. These cost-effective products are widely used for clean-up and pre-concentration applications.

Introduction

Chloramphenicol (CAP, Figure 1) is a broad-spectrum antibiotic, which was widely used worldwide. Several health problems are related to its use. As a consequence, several countries (*e.g*. USA, EU and Canada) have prohibited its use for food-producing animals. As no permitted limit has been established, the EU has defined a minimum required performance limit (MRPL) of 0.3 µg kg^{-1} for a product of animal origin [1].

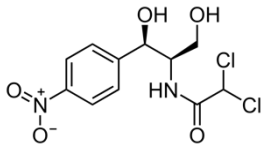

Figure 1. Chemical structure of chloramphenicol.

However, due to its broad-spectrum activity and its availability, CAP is still used in animal husbandry in several countries. Therefore, chloramphenicol analysis is still a current affair. In addition, food matrices are very complex and induce ion-suppression which distort analytical results when using mass spectrometry. It is essential to develop a highly selective and sensitive analytical assay to control and monitor CAP residues in difficult matrices such as food stuffs. For this low MRPL threshold, a clean-up step is crucial in order to improve sensitivity, reliability and the specificity of the method.

In this publication, a new method has been developed for the analyses of chloramphenicol in honey and shrimp. This method is based on the use of a molecularly imprinted polymer (MIP) which allows a selective solid-phase extraction process. In addition to specificity, MIPs are stable to organic and aqueous solvents, temperature and pH. These products are used as a powerful technique for sample clean-up and pre-concentration [2].

Materials and Methods

Materials

All reagents and chemicals were ACS grade quality or better. Water was deionized. Different kinds of honey (several mixture of flowers, acacias) were purchased at a supermarket. The SPE cartridge MIP Chloramphenicol was the product AFFINIMIP®SPE Chloramphenicol from AFFINISEP (Petit Couronne, France).

Preparation of samples prior to SPE

Ten g of honey were dissolved in 1 0mL water. This solution was mixed under magnetic stirring for 10 min and used as loading solution.

Solid-phase extraction (SPE) protocol

The SPE procedure (approximately 30 min) used a 1 mL MIP chloramphenicol cartridge as follow:

- The SPE cartridge was conditioned with 2 mL acetonitrile (ACN) followed by 2 mL of deionized water;
- 1 mL loading solution (or 10 mL for 0.3 µg kg^{-1}) was applied;
- The cartridge was washed with 1 mL water, 1 mL water containing 0.5% acetic acid/ACN (95/5, v/v), 2 mL 1% aqueous ammonia, and, finally, with 2 mL 1% aqueous ammonia/ACN (80/20, v/v);
- Vacuum was applied to the cartridge for 1 min;
- The cartridge was washed with 250 µL diethyl ether;
- CAP was eluted with 2 mL methanol under vacuum for 10 s;
- The eluate was dried and the residue was dissolved in the mobile phase.

Analysis

HPLC was performed on a Thermo Finnigan Surveyor Plus with a Thermo Accucore C18 column (50mm x 2.1mm; 2.5µm). The injection volume was 20 µL. Separation was carried out at a flow rate of 200 µL min^{-1} using a mobile phase of 10 mM aqueous ammonium acetate/ methanol (75/25, v/v). The detection system was a Thermo Finnigan MSQ Plus with an electrospray source (ESI) in negative mode. Quantification was done in selected ion monitoring (SIM) at m/z = 321.

For some experiments, UV chromatograms at 275nm have been recorded to analyse the efficiency of the clean-up by looking at the impurities.

Results and Discussion

To evaluate the clean-up of the MIP cartridge, honey spiked at 15.7 µg kg^{-1} CAP was diluted twice with water before HPLC UV analysis. The same honey was cleaned up with MIP chloramphenicol before analysis. In Figure 2, chromatograms show that a sample clean-up is necessary to observe a CAP peak and that the MIP removes most interferences from the honey.

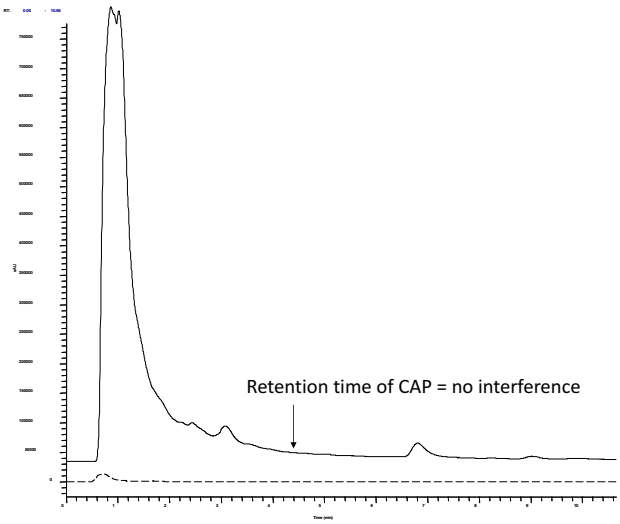

Figure 2. UV Chromatograms of Honey containing 15.7 µg g^{-1} CAP before clean-up (----) and after clean-up (- - -) with MIP Chloramphenicol.

Table 1. Recovery of Chloramphenicol spiked at different concentrations after MIP Chloramphenicol clean-up of 1g of Honey and relative standard deviation calculated from results generated under reproducibility conditions.

C° (µg kg^{-1})	Mean concentration measured (µg kg^{-1})	Recovery (%)	RSD_R (%)
15.7	16.9	108	6.5 (n=6)
18.2	16.6	91	11.4 (n=12)

Then, three different honeys, either spiked or not spiked, were analysed by LC-MS (Figure 3). SIM chromatograms show similar traces for the spiked honeys. The recovery and the relative standard deviation calculated from results generated under reproducible conditions were evaluated at 15.7 µg kg^{-1} and 18.2 µg kg^{-1}. Recoveries were higher than 90% (Table 1). In addition, repeatability was evaluated at 16.0 µg kg^{-1} (Table 2).

Honey was also spiked at the MRPL, 0.3µg kg^{-1} CAP (Figure 4). The sample was analysed with a single quadrupole mass-spectrometer for which 10 mL loading solution (instead of 1mL) were necessary to analyse the honey. This shows that the clean-up allows a concentration of high volume of loading solution and allow the analyses at very low concentration. Usually a LC-MS/MS is required for such determination.

Table 2. Recovery of CAP at 16 µg kg^{-1} after MIP clean-up of 1 g honey and relative standard deviation calculated from results generated under repeatability conditions (n=3).

C° (µg kg^{-1})	Mean concentration measured (µg kg^{-1})	Recoveries (%)	RSD_r (%)
16.0	15.4	96.1	3.3

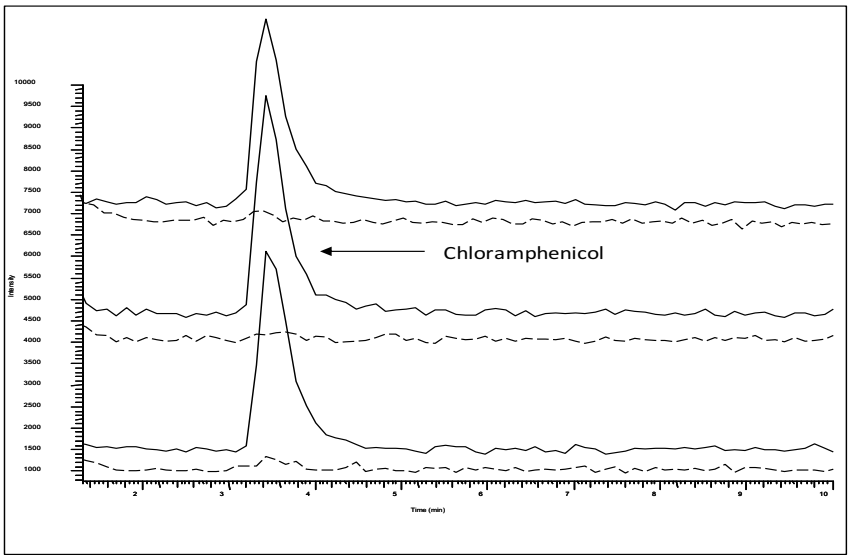

Figure 3. SIM Chromatograms obtained after MIP clean-up of 1 g honey spiked at 15.7 µg kg^{-1} CAP (-----) or not spiked (- - - -).

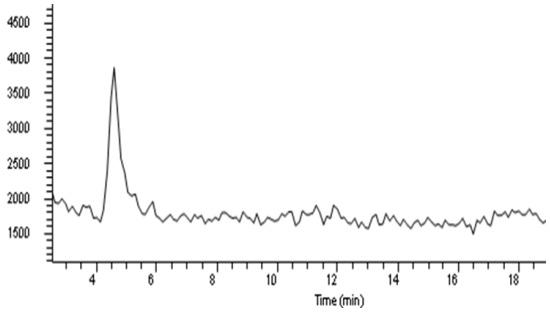

Figure 4. SIM Chromatogram obtained after clean-up with MIP Chloramphenicol of 10 g honey spiked with chloramphenicol at 0.3µg kg^{-1}.

Conclusion

Honey has been cleaned up with MIP Chloramphenicol. The use of UV detection proved its efficiency to remove interferences at the retention time of CAP. Indeed, the UV chromatogram showed a low background.

Reproducibility and repeatability were good at around 16 µg kg^{-1} CAP. From a very complex matrix such as honey, we obtained a high recovery yield (> 90%) with a low background, even with UV detection. The tests carried out on several kinds of honey demonstrated a good reproducibility, proving the efficiency of MIP Chloramphenicol for the clean-up. Reliable quantification of CAP from honey at low concentrations (0.3 µg kg^{-1}) using MIP Chloramphenicol and a single quadrupole mass detection was possible.

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ANALYSIS OF TETRACYCLINES IN MEAT USING SOLID-PHASE EXTRACTION BASED ON MOLECULARLY IMPRINTED POLYMERS

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Abstract

Tetracyclines, broad-spectrum antibiotics, are widely used in veterinary medicine in food-producing animals. However, these antibiotics residues can cause harmful effects in consumers. The level of these pharmacologically active substances is therefore regulated at low concentrations, such as 200 µg kg^{-1} for muscle tissue. Thanks to the technological improvement of analytical devices, very low concentrations of these residues can be detected. However, even with these highly performing equipment, sample pre-treatment remains a key-factor for the success of the analyses by reducing interferences and thus their subsequent consequences, like ion suppression and the detector fouling in mass spectrometry.

A new molecularly imprinted polymer (MIP) has been developed for selective extraction of oxytetracycline, tetracycline, chlortetracycline and theirs epimers from complex matrices such as meat prior to HPLC or ELISA analysis. Thanks to selectivity of MIP, excellent clean-up and recoveries were obtained.

Introduction

Tetracyclines (Figure 1) and their 4-epimers are broad-spectrum antibiotics and are widely used as veterinary medicines and feed additives. These residues can cause be toxic or give allergic reactions in hypersensitive individuals, and also transfer drug-resistant bacteria from food to humans. In response to these concerns and to prevent harmful effects of residual antibiotics on the human health, various international health organizations have established a maximum residual limit (MRL) for tetracyclins in food of animal origin. For instance, The Codex Alimentarius [1] has set a maximum residue levels (MRL) for tetracycline antibiotics at200 µg kg^{-1} in muscle, 600 µg kg^{-1} in liver and 1,200 µg kg^{-1} in kidney, whereas European regulation [2] has set a MRL at 100 µg kg^{-1} in muscle, 300 µg kg^{-1} in liver and 600 µg kg^{-1} in kidney.

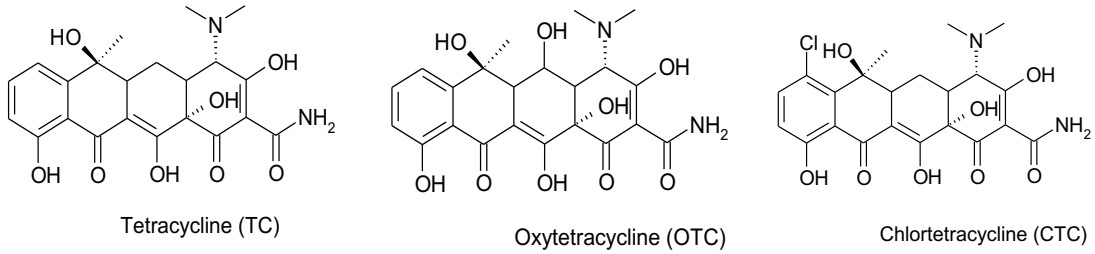

Figure 1. Chemical structure of tetracyclines

A molecularly imprinted polymer (MIP) based solid-phase extraction (SPE) method was developed for selective extraction of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and theirs epimers by SPE from muscle and pig kidney. MIPs are affinity columns made of polymers very stable to aqueous or organic solvents as well as temperature. These cost-effective products are widely used for clean-up and pre-concentration applications [3].

Materials and Methods

Materials

All reagents and chemicals were of ACS grade quality or better. Water was deionized. Muscle and pig kidney were purchased at a supermarket. The AFFINIMIP® SPE Tetracyclines was from AFFINISEP (Petit Couronne, France).

Pre-treatment of muscle and kidney prior to SPE use

The EDTA/McIlvaine buffer was prepared by mixing 50 mL 0.1 M citric acid with 31.25 mL 0.1 M $Na_2HPO_4 \cdot 7H_2O$ and the pH was adjusted to pH 4 with a NaOH solution. Then 3 g Na_2-EDTA were dissolved.

Preparation of loading solution based on AOAC 995.09 method

Ten g of meat (muscle or kidney) were blended for 30 s with 40 mL EDTA/Mc Ilvaine buffer and stirred for another 10 min with a magnetic stirrer. The mixture was centrifuged at 2,500 *g* for 10 min at a temperature below 15°C. The supernatant was collected. This operation was repeated with 40 mL buffer and again with 20 mL of buffer. Then, all supernatants were

combined and centrifuged at 2,500 *g* 20 min and filtered on Buchner. 750 µL 1 N NaOH solution were added to the filtrate and adjusted to pH 6.5 with a NaOH solution. This mixture was the loading solution.

Solid phase extraction (SPE) protocol

The SPE procedure (approximately 30 min) used a 1 mL cartridge MIP Tetracyclines as follows:

- The SPE cartridge was conditioned with 1mL acetonitrile (ACN), then with 1 mL water (2 drops s^{-1})
- 5 mL of meat loading solution was transferred onto the column at 0.5 drop s^{-1};
- The cartridge was washed with 1 mL 1% aqueous $NaHCO_3$ at 1 drop s^{-1}, directly followed by 2 mL water/ACN (60/40, v/v) at 1 drop s^{-1}
- Vacuum was applied for 1 min (only if elution was evaporated)
- Tetracyclines were eluted with 2 mL of 2% methanolic formic acid (1 drop s^{-1})
- The eluate was dried and the particulate material was dissolved in the mobile phase.

Analysis

HPLC was performed on a Thermo Finnigan Spectra System with a Thermo Hypersil Gold column (150 mm x 2.1 mm; 3 µm). Separation was accomplished using a gradient of the mobile phase (Table 1) at a flow rate of 0.2 mL min^{-1}. The detection system was a Thermo Finnigan Spectra System Model UV6000LP set to 355 nm. The injection volume was 100 µL.

Table 1. Gradient for the analysis of Tetracyclines.

Time (min)	10 mM oxalic acid in water (%)	10 mM oxalic acid in acetonitrile (%)	MeOH (%)
0	90	5	5
20	90	5	5
21	80	10	10
40	80	10	10
41	90	5	5

Results and Discussion

Results for the muscle

First, muscle was prepared according to the method AOAC 995.09 with EDTA/McIlvaine buffer as previously described to obtain the loading solution. The loading solution was spiked with 200 µg kg^{-1} TC, CTC and 4-epioxytetracycline (4-epiOTC) and with 400 µg L^{-1} 4-epichlortetracycline (4-epiCTC). Then 5 mL of this loading solution was used for the solid-phase extraction with the MIP Tetracyclines. Non-spiked muscle was analysed to evaluate the efficiency of the clean-up. Figure 2 shows several chromatograms; that of non-spiked muscle was clean.

Table 2. Recovery yield of Tetracyclines after MIP Tetracyclines clean-up of spiked muscle

Molecules	Concentration (µg kg^{-1})	Recovery (%; n=2)
Tetracycline (TC)	200	98
Chlortetracycline (CTC)	200	70
4-epichlortetracycline (4-epiCTC)	400	74
4-epioxytetracycline (4-epiOTC)	200	91

Muscle and water spiked at similar concentrations with tetracyclines show very similar chromatograms. As shown with the non-spiked muscle, the chromatogram of muscle confirms that it was clean and the analyte concentrations can be determined without difficulties. This indicates that the clean-up of muscle with the MIP tetracyclines was efficient.

The recovery yields for muscle is listed in Table 2 and is higher than 70% for all tested tetracyclines and even more than 90% for TC and 4-epiOTC. CTC and 4-epiCTC seemed the most sensitive molecules and we observed that recovery yield was affected by the evaporation of the solvent of the eluate. It explains the lower recovery yields for these molecule.

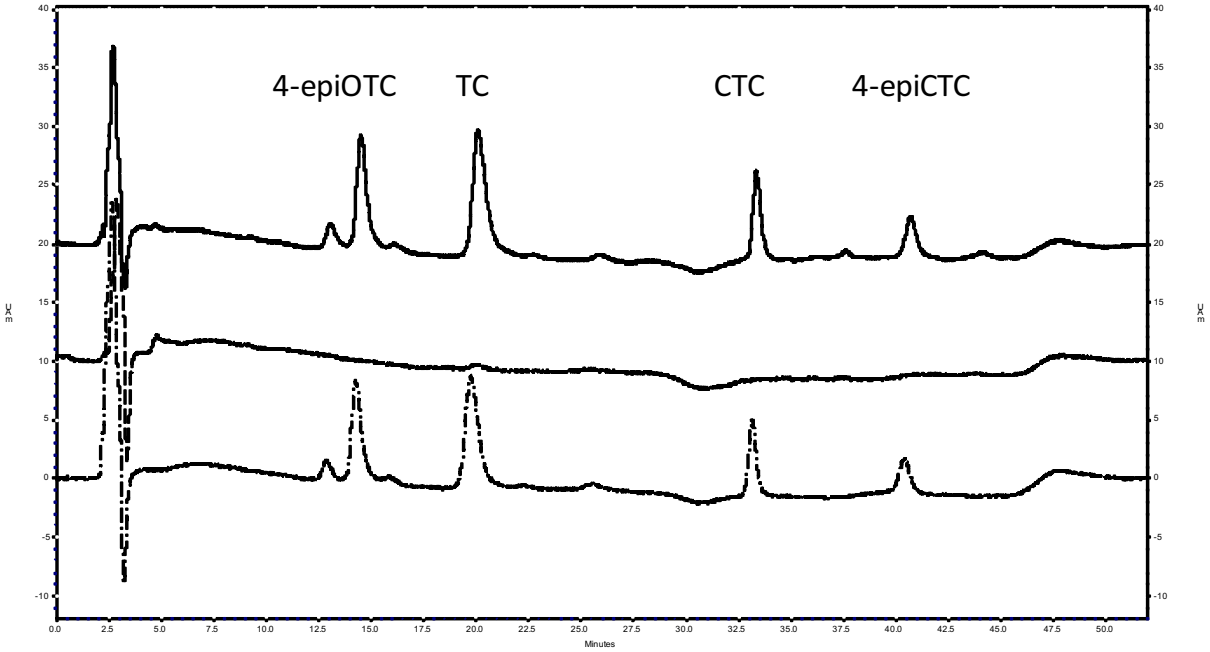

Figure 2. UV Chromatograms (355nm) obtained after clean-up with AFFINIMIP® SPE Tetracyclines of muscle spiked with TC, CTC and 4-epi-OTC at 200 µg kg^{-1} and 4-epiCTC at 400 µg L^{-1} (-----), muscle not spiked (- - - -) or of water spiked (--- - --- -)

Similar experiments were carried out with pig kidney tissue. For this matrix, the allowed concentration is higher than for muscle, and the sample was spiked at a concentration of 910 µg kg^{-1} for TC; 980 µg kg^{-1} for OTC and 860 µg kg^{-1} for CTC. As for muscle, we observed that the chromatogram of non-spiked tissue was clean (Figure 3). The chromatograms of spiked water and tissue were very similar except for the CTC peak where a shoulder is present for the tissue.

Table 3 shows recovery yields and the relative standard deviation calculated from results generated under reproducibility conditions for the three analytes. The recovery was higher than 79% for all analytes and the relative standard deviation was very good.

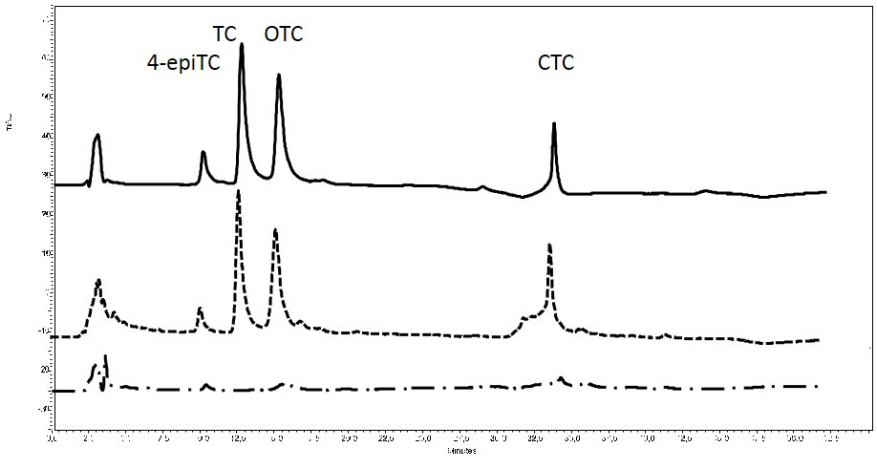

Figure 3. UV Chromatograms (355nm) obtained after clean-up with MIP Tetracyclines of pork kidney tissue (- - - -) , water (----) spiked with 910µg kg^{-1} TC, 980µg kg^{-1} OTC and 860 µg kg^{-1} CTC as well as pork kidney not spiked (--- - --- - ---).

Table 3. Recovery yields and the relative standard deviation calculated from results generated under reproducibility conditions of tetracyclines after MIP Tetracyclines clean-up of pork kidney tissue spiked at 910µg kg^{-1} for TC; 980µg kg^{-1} for OTC and 860µg kg^{-1} for CTC.

Molecules	Concentration µg kg^{-1}	R (%; n=5)	RSDr (%)
Tetracycline (TC)	910	85	5.4
Chlortetracycline (CTC)	860	79	2.8
Oxytetracycline (OTC)	980	80	4.2

Conclusion

Clean-ups of muscle and kidney tissues were carried out by solid-phase extraction using a MIP Tetracyclines. Tetracyclines and epi-tetracyclines were analysed. Recovery yields were higher than 70% and a good repeatability was observed for kidney tissues.

The efficiency of MIP Tetracyclines for meat (muscle and kidney tissues) was shown with UV chromatograms to be very similar to spiked water and with very clean chromatograms of non-spiked meat.

Special attention must be given to the evaporation step as a reduction of the recovery of CTC and 4-epiCTC was then observed. A slight volume of 10-30 µL must be kept in the vial to prevent this degradation. For use of LC-MS/MS analyses, it is even advisable to proceed via a dilute and shoot analyses of these compounds.

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ALBENDAZOLE IN AQUACULTURE: STUDIES FOR DRUG INCORPORATION IN FISH FEED COATING AND RESIDUE ANALYSIS IN FISH FILLET

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Abstract

Due the lack of regulated drugs for aquaculture, this study presents some important tools contributing to the evaluation of the potential usage of albendazole (ABZ) through the oral route using medicated fish feed. Aiming to avoid the risk of drug leaching from feed to aquatic environment, which could compromise the treatment efficacy and represent an environmental hazard, a procedure for ABZ incorporation in the feed by coating feed pellets with cellulose polymer containing the drug was evaluated. The medicated feed presented ABZ releasing from feed to water less than 2% when the medicated feed stayed in water for 15 min. Besides, analytical methods for ABZ quantitation in feed and for ABZ and its metabolites (albendazol sulfoxide, albendazole and albendazole aminosulfone) in fish fillet employing liquid chromatography associated with mass spectrometry in tandem was developed. These methods are necessary for depletion studies with ABZ aiming to estimate the withdrawal time considering food safety issues.

Introduction

Aquaculture comprises production systems for aquatic organisms (animal or plants) in the environments using freshwater or seawater. Nowadays, it is considered the animal food production system with the highest world growth rate (Defoirdt *et al*., 2011; Feucht and Zander, 2015). Fish and fishery products represent a valuable source of vitamins and essential micronutrients in a balanced and healthy diet (Reverter *et al*., 2014). According to the Food and Agriculture Organization of the United Nations, fish consumption worldwide has increased with a rate comparable to the world population growth (FAO, 2012).

All intensive animal production systems provide favourable environments for diseases spreading due the high animal population density. This is especially important for aquaculture, where the aquatic environment enhances pathogen proliferation (Quesada *et al*., 2013[a]). Abrupt physicochemical changes in the aquatic environment, such as a sharp drop in water temperature, can affect fish health due stress conditions. Such scenarios together with possible inadequate animal husbandry practices and poor environmental conditions favour the appearance and outbreak of parasitic, viral and fungal infectious diseases (Roberts and Bullock, 1980; Schalch *et al*., 2005; Quesada *et al*., 2013[a,b]).

Occurrence of diseases is unavoidable and leads to potential economic losses. The use of veterinary drugs in animal production systems for therapeutic purposes is almost inevitable because the spread of an eventual infectious disease in the production system would certainly jeopardize activity (Sismotto *et al*., 2014).

Intensive aquaculture has not yet justified the development of veterinary drug specifically for use in aquaculture. Rather, pharmaceutical companies offer products, which were developed for other areas of veterinary medicine, for use in aquaculture. The hazards associated with the presence of veterinary drug residues in edible tissues from aquaculture products include changes in colonization patterns of human-gut flora, acquisition of drug resistance in pathogens, allergies and toxic effects. Like other veterinary medicines, wherever there is an effective regulatory regime, authorization for the use of antimicrobials in aquaculture follows established processes. These include specifying the species, diagnosis, dose, duration, and withdrawal period to be observed when an antibiotic is used as a therapeutic agent. The establishment of appropriate withdrawal periods ensures that no harmful residues remain in edible tissues after use of a veterinary drug (Boison and Turnipseed, 2015).

Stringent safety and efficacy standards, including residue tests, are required before approval of any any agent. This rigorous approval process is very costly and time-consuming, whereas the sales potential for the aquaculture market in global terms is limited. This caused a lack of interest of pharmaceutical companies to developing antimicrobials and to register them (Rodgers and Furones, 2009). Therefore, the alternatives for aquaculture drugs are fairly limited. Considering the lack of drugs regulated for aquaculture, our research group evaluates the use of albendazole (ABZ) as a potential anti-parasitic drug to control helminthic infestation in pacu (*Piaractus mesopotamicus*), an important commercial fish in Brazil.

ABZ is a member of the benzimidazole anthelmintic family. Benzimidazole drugs are widely used for treating gastrointestinal parasite infections in animals (Delatour and Parish, 1986; Mckellar and Scott, 1990). ABZ is a broad-spectrum anthelmintic listed in the Code of Federal Regulations (CFR) and approved for use in cattle, sheep, and goats (CFR 21, 2010). ABZ has been used to treat fish parasites such as microsporidia (Schmahl and Benini, 1998) and tape worm infestation by *Eubothrium crassum* (Yu *et al*., 2011). Besides, residue depletion studies of ABZ and its metabolites have been carried out for some fish spe-

cies, like rainbow trout (*Onchorhynchus mykiss*), Atlantic salmon (*Salmo salar*), tilapia (*Oreochromis nilotica* x *O. mosambicus*), channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*), hybrid striped bass (*Morone chrysops* x *saxatilis*) (Shaikh *et al*., 2003, 2006, 2009) and yellow perch (*Perca flavescens*) (Yu et al, 2011) evidencing its potential as anti-parasitic drug for aquaculture.

The major metabolites of ABZ following oral treatment, are albendazole sulfoxide (ABZSO), an active metabolite, and the inactive metabolites albendazole sulfone ($ABZSO_2$) and albendazole aminosulfone (ABZ-2-NH_2SO_2) (Lubega and Prichard, 1991; Lanusse *et al*., 1992). The latter, ABZ-2-NH_2SO_2 is listed as the marker residue (MR) in cattle and sheep with a tolerance of 50 ng g^{-1} in the muscle tissue (Code of Federal Regulations, 21, 2010). The Committee For Medicinal Products For Veterinary Use of European Medicines Agency (EMEA/MRL/865/03-FINAL, 2004) recommends a maximum residue limit (MRL) of 100 ng g^{-1} for the sum of ABZ and its metabolites.

As discussed, depletion studies to establish appropriate withdrawal times ensuring that no harmful residues remain in edible tissues after drug administration to animals, is very important. The development of a suitable analytical method to quantify residues in animal food matrices is fundamental. Hence, the present work aims to describe the development and the reliability assessment of an analytical method for determination of ABZ in fish feed and of ABZ and its metabolites in fish fillet. Since the main route for ABZ administration to fish is oral, a procedure to incorporate the ABZ in fish feed involving coating technique intending to avoid eventual ABZ leaching to the aquatic environment during fish medication, was evaluated.

Material and Methods

Chemicals and reagents

LC grades of acetonitrile (ACN), methanol (MeOH) and formic acid were from JT Baker (Mexico). Reagents methanol (Grupo Química, Brazil), sodium acetate (NaAc) and sodium chloride (NaCl) (Vetec Química Fina, Brazil), anhydrous magnesium sulphate ($MgSO_4$) (Sigma-Aldrich, Brazil) and primary–secondary amine (PSA) (Agilent, USA) were used in the extraction procedure. Deionized water was obtained from a Milli-Q 3-UV System (Millipore, Germany). Regenerated cellulose syringe filter (Sartorius, Germany) with 33 mm diameter and 0.22 mm pore size were applied to filter the sample extracts before chromatographic analysis. The analytical standards albendazole (ABZ, 98%), albendazol sulfoxide (ABZSO, 99.9%), albendazole sulfone (ABZSO2, 99.6%), albendazole aminosulfone (ABZ-2-NH_2SO_2, 99.6%) and phenacetin (PNC, 98%) were purchased from Aldrich Chemistry – Sigma-Aldrich (China). Raw ABZ (> 99%) and ethyl alcohol (96° GL) were purchased from the retail market (Ribeirão Preto city, São Paulo, Brazil). They were used to prepare the feed coating. Ethylcellulose was from Henrifarma (Brazil).

Standard solutions

Individual stock standard solutions of ABZ, ABZSO and PNC (used as internal standard) were prepared by dissolving the standards in MeOH at a concentration of 1.0 mg mL^{-1}. Due their lower solubility in MeOH, $ABZSO_2$ and ABZ-2-NH_2SO_2 were prepared at 0.1 mg mL^{-1}. Stock solutions were stored in closed amber vessels kept at < -20°C for no longer than 3 months.

Working solutions were prepared daily as a mixture of ABZ and its metabolites through mixing stock solutions in 0.1% aqueous formic acid. The mixture was used immediately after preparation. For the development of the analytical method for feed, working solutions of ABZ were prepared at concentrations ranging from 2.0 to 6.0 μg mL^{-1}. In case of the analysis of fillets, ABZ and its metabolites were prepared in the range from 0.02 to 0.2 μg mL^{-1}.

Equipment

Standard solutions were prepared using analytical balance (Shimadzu, Japan). For reagent solution used during the extraction procedure, a semi-analytical balance (Marte Científica, Brazil) was employed.

For the extraction procedure, a food processor (Philco, Brazil), vortex AP56 model (Phoenix, Brazil), refrigerated centrifuge Himac CF5RX model (Hitashi, Japan), centrifuge concentrator CentrVap (Labconco Corporation, EUA), ultrasonic cleaner Maxiclean (Unique, Brazil) and a horizontal mixer PA247 model (Pachane, Brazil) were used.

A mechanic mixer RW 20 digital (IKA, Brazil), spouted bed FBD 1.0 model (Labmaq, Brazil), circulation oven and air renewal (Marconi, Brazil) were employed during the drug incorporation in the fish feed. To the test the leaching of ABZ from the feed to water, a dubnoff metabolic shaker incubator (Marconi, São Paulo, Brazil) was used.

Chromatographic analysis was performed using an LC system (Shimadzu, Japan) composed of a binary pump and automatic injection system coupled to a triple-quadrupole mass-spectrometer (Quatro-LC, Micromass, UK) inferfaced by an electrospray ionization (ESI) source. The instrument control and data processing were carried out by Masslynx 4.0 software.

Fish feed and ABZ incorporation

Feed fish (Si Crescimento) containing 28% protein was purchased from Purina (Brazil). Further details of its composition is available through: http://www.aquasem.com.br/racao_purina.html.

A total amount of 120 mg of ABZ base was added to a polymeric suspension of 3 g ethylcellulose and 100 mL ethyl alcohol. This suspension was sonicated for 15 min and the sprayed on 100 g fish feed pellets. After that, the feed pellets were sprayed with 25 mL of polymeric suspension free from ABZ. The coating procedure was facilitated by a spouted bed technique at a temperature of 50°C and at 45 psi.

The method described by Soares JR (2004) was used to evaluate the leaching of ABZ from the medicated feed to water. For this, 1 g medicated feed (around six pellets) were bagged into nylon bags with mesh diameter of 1 mm, tied and submerged in a beaker containing 900 mL water. This leaching test was performed in triplicate (n=3). Beakers were mildly agitated in a dubnoff metabolic shaker incubator. Temperature (26.0 ± 1.5 C) and pH (7.1 ± 0.6) were monitored and controlled during the process. After immersion of the bagged feed, aliquots of 10 mL of the water from each beaker (n=3) were collected at 5, 15, 30, 45 and 60 min. Immediately after each aliquot collection, 10 mL water was added to each backer in order to keep the total volume of 900 mL until next collection. Each aliquot was filtered and 5 µL was injected in the LC-MS/MS system.

Blank fish fillet

Blank samples of fish fillets (pacu) were obtained from the fish-farming sector of the Department of Animal Science of Escola Superior de Agricultura "Luiz de Queiroz" of University of São Paulo (Piracicaba/SP, Brazil). They were reared with no use of any drug. Fish fillets were grounded using a food processor, divided into aliquots of 5 g and stored in a freezer (-20°C).

Sample preparation

ABZ extraction from feed. Two mL 0.1% aqueous formic acid was added to 1.0 g feed. The suspension was mixed using a vortex for 30 s and sonicated for 5 min. Then, 8 mL MeOH was added and the solution was shaken on a horizontal shaker for 15 min. The solution was centrifuged at 4°C and 3,500 rpm for 25 min. An aliquot of 500 µL of the supernatant was collected and transferred into a 10-mL volumetric flask. The flask volume was completed with 0.1% aqueous formic acid. The extract was filtered and transferred to a vial. A volume of 5 µL of the extract was injected into the LC-MS/MS system.

ABZ and its metabolites extraction from fish fillet. Five mL ACN was added to 5.0 g fish fillet, mixed by vortex for 1 min and sonicated for 10 min. Immediately after the addition of 2 g anhydrous $MgSO_4$ and 0.5 g NaCl, the suspension was mixed using a vortex for 1 min. The sample was centrifuged at 4°C and 3,500 rpm for 10 min. An aliquot of 2.0 mL of the supernatant was collected and transferred into a falcon tube containing 450 mg $MgSO_4$ and 25 mg PSA. The mixture was homogenised by vortex-mixing for 30 s. The sample was again centrifuged at 4°C and 3,500 rpm for 10 min. The supernatant was collected and evaporated in a centrifuge concentrator for 60 min at 50°C. The residue was suspended in a mixture of 1 mL 0.1% aqueous formic acid and ACN (1:1, v/v) and filtered. Five µL of the filtrate was injected into the LC-MS/MS system.

Extraction procedures were optimized by assessing the extraction efficiencies of the analytes for each matrix. This assessment involved the analysis of blank samples fortified with the analytes (including the internal standard) at known concentrations. The concentrations of the analytes in the fortified blank sample were calculated using a calibration curve obtained with the analytes in solvent. The results were calculated and expressed as a percentage, that is, the obtained concentration multiplied by 100 and divided by the expected concentration (used in the fortification).

LC-MS/MS methods

ABZ quantitation in feed. Chromatographic separations for the quantitation of ABZ in fish feed employing PNC as internal standard were made using the X-Terra MS C18 analytical column (3.9 mm x 100 mm x 3.5 µm, Waters, USA) as stationary phase. The mobile phase was composed by a mixture of (A) 0.1% aqueous formic acid and (B) ACN (60:40 v/v) flowing isocratically at 0.3 mL min^{-1}. The column oven was set at 25°C. The ESI interface was operating in the positive mode. The temperatures of the source block and dessolvation gas were set at 100°C and 350°C, respectively. Nitrogen was used as both drying (about 380 L h^{-1}) and nebulizing (about 40 L h^{-1}) gas, while argon was used as collision gas. The cone voltage during the analyses was set at 20 V and the collision energy was set at 20 eV for both ABZ and PNC monitoring. Analyses were performed by monitoring selected reactions mode (SRM) of protonated molecules $[M+H]^+$ and their respective fragments. The monitored transitions ions (*m*/*z*) were 266>234 for ABZ and 180>110 for PNC.

ABZ and its metabolites quantitation in fish fillet. Chromatographic separation was made with the same stationary phase, mobile phases, flow rate and temperature as described above. Except, a linear gradient was used: 0 min, 90% A; 3.0-5.0 min, 45% A; 6.0-9.5 min, 10% A; 10.5-17 min, 90% A. All operating parameters for ESI interface and mass analyser were the same as described above. The monitored transitions ions (*m*/*z*) were: 266>234 for ABZ, 282>240 for ABZSO, 298>266 for $ABZSO_2$, 243>133 for ABZ-2-NH_2SO_2 and 180>110 for PNC.

Analytical method evaluation

In order to assess the reliability of the analytical method, the following main parameters recommended by the guide proposed by the Brazilian Ministry of Agriculture (CGAL/SDA/MAPA, 2011) for analytical method validation were evaluated:

Selectivity. The selectivity of the developed method was evaluated in two ways. Firstly, by comparing the chromatograms obtained for the blank samples and the fortified blank samples for the presence of any potential interference in the matrix that could compromise the detection of the analytes.

Secondly, matrix effect using calibration curve obtained from the analytes in working solutions to quantify fortified extract samples (blank samples extracted followed by fortification with analytes at the and of the process) at three different concentrations (50, 100 and 150 ng g^{-1} fish fillet). The matrix effect results were obtained from the quantification of the fortified extract at those levels, by employing the equation of the calibration curve of the analytes in solution, and expressed as a percentage of the expected value subtracted from 100%. The matrix effect was evaluated only for fish fillet samples.

Linearity. The calibration curves were prepared by fortifying blank samples (feed or fillet) at five concentration levels, ranging from 0.4 to 1.2 mg g^{-1} for ABZ in feed and ranging from 0 to 200 ng g^{-1} for ABZ, ABZSO, $ABZSO_2$ and ABZ-2-$NH2SO_2$ in fish fillet. For both methods, PNC was used as internal standard. The PNC concentration was 0.8 mg g^{-1} and 100 ng g^{-1} in the feed and fish fillet method, respectively. Linearity of the standard curve for each analyte was expressed by the linear regression coefficient (r).

Precision. The intra-day and inter-day evaluation of the precision was performed with fortified blank feed or fish fillet samples. The intra-day precision was evaluated at three concentration levels of ABZ in feed (0.4, 0.8 and 1.2 mg g^{-1}) and three levels of ABZ, ABZSO, $ABZSO_2$ and ABZ-$_2$-$NH2SO_2$ in fish fillet (10, 100 and 200 ng g^{-1}) on the same day, by the same analyst using the same equipment. The procedure was executed in triplicate (n=3) for each level of concentration and expressed as coefficient of variation (CV). The inter-day precision was evaluated for the same three concentrations, however, with the replicates being prepared at three days (n=3). The analyses were performed by the same analyst on the same equipment. The results were expressed as coefficient of variation (CV%).

Trueness. The same assay designed to evaluate the inter-day precision was used to assess trueness. However, the results were calculated as a percentage of the obtained concentration of the fortified analyte, expressed by ranges.

The decision limit (CCα) and detection capacity (CCβ) of each analyte were calculated using their respective absolute standard deviation, which were obtained from the inter-day precision test. The CCα (α = 5%) was expressed as the MRL (assuming 100 ng g^{-1} for each analyte) plus 1.64 times the standard deviation of the intra-day precision. The CCβ (β = 5%) was expressed as the concentration corresponding to the CCα value plus 1.64 times the inter-day precision standard deviation.

Results and discussion

Analytical method for feed

Initially, 1 g feed and 10 mL extraction solution were used. The first optimization step for the ABZ and PNC extraction process was the selection of an extraction solution. Different organic solvents (MeOH, ACN, ethyl acetate), pure or mixed with 1% aqueous formic acid in different proportions were tested. Some authors reported cleaner extracts with ACN as solvent, while the use of MeOH results in a better extraction capacity. In the present study, we obtained the dirtiest extracts with the use of ACN as extraction solution, whereas the use of MeOH confirmed to have the best extraction capacity. Actually, the best extraction results (around 90% for ABZ and near of 105% to PNC) were obtained using MeOH mixed with 1% aqueous formic acid at 8:2 (v/v).

Robert *et al*. (2015) developed a screening method for the presence of antibiotics and anthelmintics in feed samples. ACN and MeOH were tested as extraction solvents, with and without addition of formic acid. Corroborating to our results, the authors concluded that the use of MeOH helped to precipitate proteins and to eliminate interferences.

To monitor the presence of sulfonamides in animal feed, Borràs *et al*. (2011) developed two analytical methods. Mixtures with ACN and water at different proportions and mixtures of ethyl acetate with water and MeOH were tested as solvent extractors. The researchers reported dirtiest extracts when ACN was used as extraction solvent.

In addition to testing different compositions of the extraction solution and its influence on extraction efficiency, we evaluated also the use of sonication. The use of ultrasound for 5 min favoured the interaction of ABZ with extraction solution and thereby increased the extraction efficiency.

The selectivity of the method was evaluated to ensure that the analyte response peak was derived solely from it without any interference of the matrix. Therefore, a comparison of the chromatograms obtained for the blank feed sample and blank sample fortified with ABZ showed high selectivity of the method. Considering the use of mass-spectrometry as analytical instrument in this method, matrix effect was evaluated. However, no significant effect was observed.

The calibration curve for ABZ in feed showed adequate linearity with a linear regression coefficient higher than 0.99. The coefficients of variation for intra-day and inter-day precision were lower than 0.81 and 1.03%, respectively. The trueness of the method ranged from 99 to 101%. The results obtained for all assessed parameters indicate that the method for ABZ quantification in feed is suitable.

Incorporation of ABZ in fish feed and leaching evaluation

A possible alternative to avoid drug leaching from feed to the aquatic environment is the use of feed coated with polymers. In the present study, ABZ was added to a commercial fish feed by coating the feed with a polymer suspension containing ABZ. The suspension was sprayed onto the surface of the feed pellet resulting in a homogeneous film of polymer and drug. After that, a smaller volume of polymer suspension free from ABZ was sprayed onto the surface of the previous feed pellets in order decrease the potential ABZ leaching to water.

The polymer used in the coating process must have a low water solubility, be available at low cost, capacity to blind potential the flavour of the drug and, particularly, not be toxic. Thus, the ethylcellulose was the polymer of choice.

After the drug was incorporated into the feed, it was analysed in order to evaluate the concentration obtained. The analyses were in triplicate and indicated a loss about 22% of ABZ in the incorporation process. The coefficient of variation obtained from the triplicate analyses revealed a good uniformity of the drug incorporation into the feed (CV < 2.6%, n=3).

The release of the drug from the feed to the aquatic environment can impair the quality of water and fish rearing. Thus, the leaching process is an important aspect to be addressed when the drugs are administered through the feed. The results for the percentage of drug released from feed to water during the leaching are summarised in Table 1.

Table 1. Leaching test for ABZ from coated medicated feed into the water.

Time points (min)	Drug released from feed to water (%)	Coefficient of Variation (%, n=3)
5	2	19
15	2	3
30	2	10
45	3	7
60	3	8

Feed is considered as adequate when it is available for up to ten minutes for consumption by the fish (Cantelmo, 2002). This implies that the stability of the feed pellet is essential to reduce possible loss of nutrients and, especially, the loss of the drug. The feed coated by a polymer enables such stability and reduces drug leaching. Leaching is an important phenomenon to be considered in residue depletion studies, as the drug concentration in medicated feed will undergo significant changes during its stay in water until consumed by fish.

The leaching test showed that the drug incorporation into the feed through a coating is a promising strategy. Up to 15 min, only 2% of the drug present in the feed was leached into the water. Even if the feed remained in the water for 60 min, drug loss did not exceed 3%. These data indicate that a coating feed with a suitable polymer containing the drug of interest, preserves the integrity of the feed and a constant concentration of the drug. It provides more time for the sick fish to be fed.

Analytical method for fish fillet

Although all benzimidazole representatives have a common basic chemical structure with a benzene ring and an imidazole group, they show differences in chemical properties, especially concerning pK_a and polarity. These differences are more prominent between the parent drug and their metabolites. Besides, the complexity of fish fillet and low concentrations at which drug residues can be found, sample preparation is essential to obtain reliable results. Recent studies have used the QuEChERS (Quick Easy Cheap Effective Rugged and Safe) method and its modifications for extraction of different veterinary drugs in different matrices of animal origin. Due to its application versatility, speed, ease of implementation, low cost, efficiency and robustness, this method was chosen in this study to determine ABZ and its metabolites in pacu fillet.

The initial tests to extract ABZ and it metabolites from pacu fillet were performed with 5-g sample portions. As the extraction solution, 10 and 5 mL of pure ACN, ACN containing 1.0% formic acid and ACN containing 0.1% formic acid were evaluated. These tests showed that the use of formic acid in the extraction solution gave lower extraction efficiency and accuracy. Prestes *et al*. (2009) suggested that the use of ACN as an extraction solvent is interesting since it provides extraction of a wide range of compounds with different polarities. In addition, a lesser amount of lipophilic interferences from the matrix, such as fats and pigments, are extracted. Thus, we chose to continue the extraction tests with ACN.

Besides the composition of the extraction solution, we evaluated the influence of salt in the partition process. It aims to promote the effect of "salting out." The study showed that both NaCl and the NaAC can be used in the extraction process, without significant differences in extraction efficiency. It was decided to use NaCl.

We tested two forms of agitation as well. Initially, assays employed an ultrasound bath for 10 min to assist the extraction process. The ultrasound bath was replaced by vortexing. Sample-containing tubes were subjected to vigorous shaking for 1 min. The simultaneous mixing using a vortex and by sonication resulted in increased efficiency extraction.

After method development and optimization, the analytical evaluation in order to check its reliability was done taken as reference the main parameters recommended by the guide proposed by the Brazilian Ministry of Agriculture (CGAL/SDA/MAPA, 2011) for analytical method validation. Table 2 presents the evaluated parameters.

Table 2. Parameters of evaluation to assess the reliability of the analytical method developed to quantify ABZ and metabolites in fish fillet.

Parameter	ABZ	ABZSO	$ABZSO_2$	ABZ-2-NH_2SO_2
Linear range (ng g-^{1})	0-200	0-200	0-200	0-200
Linearity (r)	>0.99	>0.99	>0.99	>0.99
Matrix Effect (%)				
50 (ng g^{-1}, n=3)	-6	-4	-1	13
100 (ng g^{-1}, n=3)	-12	-11	-10	10
150 (ng g^{-1}, n=3)	-9	-14	-7	3
Intra-day Precision (CV, %)				
10 (ng g^{-1}, n=3)	9	20	6	12
100 (ng g^{-1}, n=3)	9	12	5	11
200 (ng g^{-1}, n=3)	4	15	5	10
Inter-days Precision (CV, %)				
10 (ng g^{-1}, n=3)	5.2	20	10	20
100 (ng g^{-1}, n=3)	8.3	15	15	15
200 (ng g^{-1}, n=3)	4.5	15	11	4
Trueness (Recovery tests, %)				
10 (ng g^{-1}, n=3)	113	88	101	116
100 (ng g^{-1}, n=3)	91	84	87	84
200 (ng g^{-1}, n=3)	100	95	97	95
CCα (ng g^{-1})	100.5	100.3	100.3	100.1
CCβ (ng g^{-1})	101.0	100.6	100.6	100.2

The selectivity of the method was investigated by comparing the chromatograms obtained for a blank sample and a blank sample fortified with ABZ in case of the feed method, and with ABZ and its metabolites in the case of fish samples. This comparison did not reveal any interference in the matrix that could compromise the detection of the analytes. Furthermore, the matrix effect was less than 14% for all analytes. As the developed method employed calibration curve with fortified sample, the matrix effect did not present a problem. Considering the guide proposed by the Brazilian Ministry of Agriculture (CGAL/SDA/MAPA, 2011) for analytical method validation, the evaluated parameters indicate that the developed method is suitable for the quantification of ABZ and its metabolites in fish fillet.

Conclusions

The present work described the development and reliability assessment of an analytical method for the determination of ABZ in fish feed and of ABZ and its metabolites in fish (pacu) fillet. The method evaluation indicated good suitability, which is fundamental, especially for fish fillet, in which the analytes must be quantified at low concentration levels. In addition, a method for fish feed medication employing a coating of feed pellets with cellulose polymer containing the drug was proposed. It resulted in less leaching of ABZ from medicated feed into the water. The presented analytical methods and the feed medication procedure is intended to contribute to evaluation of the potential usage of ABZ as anti-parasitic drug for fish medication through the oral route.

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SULFADIMETHOXINE AND ORMETOPRIM IN AQUACULTURE: STUDY FOR RESIDUE ANALYSIS IN FISH FILLET

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Abstract
Due the lack of regulated drugs for aquaculture, in particular in Brazil, this study presents some important tools contributing to the evaluation of the potential usage of the sulfadimethoxine (SDM) and ormetoprim (OMP) as antimicrobial drugs for oral medication of fish using medicated feed. Medicated feed released up to 7% SDM and 27% OMP when in water for 5 min. In addition, analytical methods for the determination of SDM and OMP in feed and in fish fillet employing liquid chromatography tandem mass-spectrometry were developed. They are necessary for depletion studies of SDM and OMP aiming to estimate the withdrawal time considering food safety issues. The parameters reflecting the reliability of the analytical methods are good.

Introduction
Antimicrobial substances are a powerful resource applied worldwide to ensure animal health and by that high-yield animal production systems. However, their use inevitably leads to the presence of residues of those substances in animal products, leading to the question whether there is a potential risk to public health when the residue levels are above established maximum residue limits (MRLs) (Paschoal *et al*., 2009).

Aquaculture plays an important role in global food production. Due to changes in human dietary habits, fish consumption has increased, with more people changing to a healthier diet with an appropriate nutritional profile (Crepaldi *et al*., 2006; Quesada *et al*., 2013). According to Food and Agriculture Organization of United Nations (FAO, 2012), the world consumption of fish has increased in a rate comparable to the growth of the world population. Nevertheless, Intensive aquaculture has not yet justified the development of veterinary drug specifically for use in aquaculture. Rather, pharmaceutical companies offer products, which were developed for other areas of veterinary medicine, for use in aquaculture.

The hazards associated with the presence of veterinary drug residues in edible tissues from aquaculture products include changes in colonization patterns of human-gut flora, acquisition of drug resistance in pathogens, allergies and toxic effects. Like other veterinary medicines, wherever there is an effective regulatory regime, authorization for the use of antimicrobials in aquaculture follows established processes. These include specifying the species, diagnosis, dose, duration, and withdrawal period to be observed when an antibiotic is used as a therapeutic agent. The establishment of appropriate withdrawal periods ensures that no harmful residues remain in edible tissues after use of a veterinary drug (Boison and Turnipseed, 2015).

Stringent safety and efficacy standards, including residue tests, are required before approval of any any agent. This rigorous approval process is very costly and time-consuming, whereas the sales potential for the aquaculture market in global terms is limited. This caused a lack of interest of pharmaceutical companies to developing antimicrobials and to register them (Rodgers and Furones, 2009). Therefore, the alternatives for aquaculture drugs are fairly limited.

Occurrence of diseases is unavoidable and leads to potential economic losses. The use of veterinary drugs in animal production systems for therapeutic purposes is almost inevitable because the spread of an eventual infectious disease in the production system would certainly jeopardize activity (Sismotto *et al*., 2014). Careful use of these substances in animals intended for human consumption is advisable. Uncontrolled and inappropriate use carries potential risks related to microbial resistance, which can affect not only the production system itself but also human health and the environment. Regarding the impact on aquaculture production, once the antimicrobial resistance is established, the antimicrobial efficacy will be reduced. Regarding the impact on human health, the intake of veterinary pharmaceutical residues above the limit that is considered safe is an important public health issue because it can cause possible adverse effects, such as allergies and problems with the human intestinal flora. With respect to the environmental contamination caused by the use of veterinary drugs in aquaculture, emphasis is placed on the extensive length of time that drugs stay in the environment, thereby altering the chemical processes of the sediments (Lunestad 1992; FAO/OIE/WHO 2006; FAO 2010).

Considering the lack of drugs regulated for aquaculture, in particular in Brazil, our research group evaluates the use of a combination of sulfadimethoxine (SDM) and ormetoprim (OMP) (5:1, w/w) in pacu (*Piaractus mesopotamicus*), which is an important commercial fish in Brazil.

Bacterial diseases stand out as an important limiting factor for productivity in aquaculture as they cause stunted growth and a high mortality rate (Ranzani-Paiva *et al*., 1997). They are one of the major threats to intensive aquaculture. The bacteria of the genus *Aeromonas* are among the pathogens that cause most economic losses, accounting for most of the diseases of growing animals (Austin and Austin, 1987; Carraschi *et al*., 2011).

The commercial product Romet (Romet-30®) is FDA-approved, but is still not regulated for use in Brazil. It is an antimicrobial drug with a broad spectrum of action. It contains two active substances, SDM and OMP (5:1, w/w). The combination is widely used in the aquaculture industry and is effective for the treatment of bacterial diseases as those caused by *Aeromonas salmonicida* in salmonids and *Edwardsiella ictaluri* in catfish (Kosoff *et al.*, 2007). The recommended daily dose for this drug is 50 mg per kg fish for 5 consecutive days.

Pharmacokinetic parameters and depletion studies for antimicrobials used in fish regulated for aquaculture in the USA and EU are important guides for the Brazilian aquaculture regulatory agencies. Nevertheless, as fish are poikilothermic animals, their metabolic activity is determined largely by the water temperature. Depletion studies are very important to support the regulation for antimicrobial usage in Brazil. They should be carried out with antimicrobials administered to fish reared under local environmental conditions.

Pacu is a species with economic importance for Brazil. It is susceptible to haemorrhagic septicemia caused by *Aeromonas hydrophila*. The availability of a drug with the action offered by SDM and OMP, is of interest as it can contribute to the health of Brazilian aquaculture. However, residue depletion studies of these drugs in pacu, reared under Brazilian climatic conditions, intending for estimation of the withdrawal time are lacking. In order to undertake such studies, the availability of an analytical method to determine these drugs residues in fish fillet is necessary.

The present work aimed to develop a reliable an analytical method for the determination of SMD and OMP in fish fillet. Considering the principal route of drug administration for aquaculture species is oral by feed, it was necessary to develop an analytical method for SDM and OMP in fish feed too. Reliability assessment for this method was carried out as well.

Material and Methods

Chemicals and reagents

LC grade acetonitrile (ACN), methanol (MeOH) and formic acid were from JT Baker (Mexico). Hexane was from Macron (USA). Water was produced with deionized water by a Milli-Q 3-UV System (Millipore, Germany). Regenerated cellulose syringe filter (Sartorius, Germany) with 33 mm diameter and 0.22 mm pore size were applied to filter the sample extracts before chromatographic analysis. Analytical standard ormetoprim (99%) was from Dr. Ehrenstorfer (Germany), whereas sulfadimethoxine (≥99.9%) and sulfametoxazol (SMZ, ≥99.9%) were purchased from Sigma-Aldrich (USA).

Commercially available Trissulfin® SID 34.5% containing SDM and OMP (5:1, w/w) prescribed for pets (dogs and cats) was used to medicate the feed.

Standard solutions

Individual stock standard solutions of SDM, OMP and SMZ (used as internal standard) were prepared at 1.0 mg mL^{-1} by dissolving the standards in MeOH. Stock solutions were stored in closed amber vessels at < -20°C for less than 3 months.

Working solutions were daily prepared as a mixture of SDM and OMP by mixing the stock solutions in 0.1% aqueous formic acid. They were used immediately after preparation. For feed analytical method development and validation, SDM and OMP concentrations for working solution ranged between 2-6 μg mL^{-1} and 0.5-1.5 μg mL^{-1}, respectively. For the fillet analytical method, SDM and OMP concentrations ranged from 0.01-1 μg mL^{-1} and 0.05-0.15 μg mL^{-1}, respectively

Equipment

Standard solutions were prepared using analytical balance (Shimadzu, Japan). A food processor (Philco, Brazil) was used to homogenize samples. Sample preparation was further accomplished with a vortex AP56 model (Phoenix, Brazil), refrigerated centrifuge Himac CF5RX model (Hitashi, Japan), centrifuge concentrator CentrVap (Labconco Corporation, EUA), ultrasonic cleaner Maxiclean (Unique, Brazil) and a horizontal mixer PA247 model (Pachane, Brazil). A mechanic mixer RW 20 digital (IKA, Brazil), spouted bed FBD 1.0 model (Labmaq, Brazil), circulation oven and air renewal (Marconi, Brazil) were employed in the procedure to incorporate the drugs in the fish feed. To the test the leaching of SDM and OMP from the feed into the water, a dubnoff metabolic shaker incubator (Marconi, São Paulo, Brazil) was used.

Chromatography analysis was performed using a LC system (Shimadzu, Japan) composed by binary pumping and automatic injection and systems coupled to a triple-quadrupole mass-spectrometer (Quatro-LC, Micromass, UK) inferfaced by an electrospray ionization (ESI) source. The instrument control and data processing were carried out by Masslynx 4.0 software.

Fish feed and drugs incorporation

Fish feed with 28% of protein (Si Crescimento) was purchased from Purina (Brazil) Further details of its composition are available through http://www.aquasem.com.br/racao_purina.html.

The inclusion of Trissulfin® in fish feed was realised by spraying the feed pellets with the drug mixed in vegetal oil. An amount of 6.12 mg of Trissulfin® were added to 50 g feed and 5 mL (10%, w/v) of vegetal oil. Initially the drug is mixed in the vegetable oil for 5 min and then added to the feed under agitation for 5 min until the oil was absorped by the feed. After that, the drug-containing feed rested at room temperature for 24 h for complete oil plus drugs absorption.

Drugs leaching from the prepared feed to water was monitored taking the method described by Soares (2004) as reference. For each test, 1 g feed (about six pellets) were bagged, using nylon bags with mesh diameter of 1 mm, tied and submerged in a beaker containing 900 mL water. This leaching test was performed in triplicate (n=3). Beakers were mildly agitated in a dubnoff metabolic shaker incubator. Temperature (26.0 ± 1.5 C) and pH (7.1 ± 0.6) were monitored and controlled during the process. After immersion of the bagged feed, aliquots of 10 mL of the water from each backer (n=3) were collected at 5, 15 and 30 min. After each aliquot collection, 10 mL water was added to each beaker to restore the total volume to 900 mL. Each aliquot was filtered and a volume of 5 µL was injected into the LC-MS/MS system for analysis of SDM and OMP.

Blank fish fillet

Blank samples of fish fillets (pacu) were obtained from the fish-farming sector of the Department of Animal Science of Escola Superior de Agricultura "Luiz de Queiroz" of University of São Paulo (Piracicaba/SP, Brazil). They were reared with no use of any drug. Fish fillets were grounded using a food processor, divided into aliquots of 5 g and stored in a freezer (-20°C).

Sample preparation

Drugs extraction from feed. Ten mL MeOH was added to 1.0 g feed, stirred by vortex for 30 s and sonicated for 10 min. The solution was then centrifuged at 4 °C and 3,500 rpm for 25 min. An aliquot of 1,000 µL supernatant was transferred into a 25-mL volumetric flask. The flask volume was completed with 0.1% aqueous formic acid. The extract was filtered and 5 µL extract was injected into the LC-MS/MS system.

Drugs extraction from fish fillet. Ten mL ACN was added to 2.0 g fish fillet and mixed for 30 s using a vortex, sonicated for 10 min and centrifuged at 4,000 rpm for 10 min. Then, 5 mL supernatant and 2.0 mL hexane were transferred to a clean tube, mildly agitated for 15 s and centrifuged at 4,000 rpm for 5 min. Then, the lower phase was dried by centrifuge concentrator for 60 min at 60°C. The residue was suspended in 1 mL of a mixture of 0.1% aqueous formic acid and ACN (1:1, v/v) and filtered. A volume of 5 µL of the filtrate was injected into the LC-MS/MS system.

Extraction procedures were optimized by assessing the extraction efficiencies of the analytes for each matrix. This assessment involved the analysis of blank samples fortified with the analytes (including the internal standard) at known concentrations. The concentrations of the analytes in the fortified blank sample were calculated using a calibration curve obtained with the analytes in solvent. The results were calculated and expressed as a percentage, that is, the obtained concentration multiplied by 100 and divided by the expected concentration (used in the fortification).

LC-MS/MS methods

For the analysis of feed or fish fillet, chromatography was made possible with an X-Terra MS C18 analytical column (3.9 mm x 100 mm x 3.5 µm, Waters, USA) housed in a column oven set at 25 °C. The mobile phase was composed of a mixture of (A) 0.1% aqueous formic acid and (B) acetonitrile containing 0.1% of formic acid. Analytes were separated using a gradient elution as follows: 0 min, 98% (A); 10-15 min, 0% (A); 16-19 min, 98% (A). The flow rate was 0.25 mL min^{-1}. The ESI interface operated in the positive mode. The temperatures of source block and dessolvation gas were set at 100°C and 350°C, respectively. Nitrogen was used as both drying and nebulizing gas, while argon was used as collision gas. The cone voltage and collision energy were 30 V and 20 eV, respectively, for SDM, while these were 27 V and 25 eV for OMP, and 20 V and 15 eV for SMZ. Analyses were performed using monitoring selected reaction mode (SRM) of protonated molecules $[M+H]^+$ and their respective fragments. The transitions monitored (*m*/*z*) were 311>156 for SDM, 275>259 for OMP and 254>156 for SMZ.

Analytical method evaluation

In order to assess the reliability of the analytical method, the following main parameters recommended by the guide proposed by the Brazilian Ministry of Agriculture (CGAL/SDA/MAPA, 2011) for analytical method validation were evaluated:

Selectivity. The selectivity of the developed method was evaluated in two ways. Firstly, by comparing the chromatograms obtained for the blank samples and the fortified blank samples for the presence of any potential interference in the matrix that could compromise the detection of the analytes.

Secondly, by observing matrix effects using calibration curve obtained from the analytes work solutions to quantify fortified extract samples (blank samples extracted followed by fortification with analytes at the end of the process) at three concentrations (0.75, 1.00 and 1.25 mg g^{-1} for fish feed samples and 75, 100 and 125 ng g^{-1} for fish fillet samples). The matrix effect

results were obtained from the quantification of the fortified extract in those levels, by employing the equation of the calibration curve of the analytes in solution, and expressed as a percentage of the expected value subtracted from 100%.

Linearity. The calibration curves were prepared by fortifying blank samples (feed or fillet) at five concentration levels, ranging from 0.5 to 1.5 mg g^{-1} SDM and from 0 to 150 ng g^{-1} SDM for feed and fish fillet, respectively. OMP curves ranged from 0.125 to 0.375 mg g^{-1} and 0 to 150 ng g^{-1} for feed and fish fillet, respectively. For both matrices, SMZ was used as internal standard. For feed method, the SMZ concentration was 1.0 mg g^{-1} and for fish fillet it was 100 ng g^{-1}. Linearity of the standard curve for each analyte was expressed by the linear regression coefficient (r).

Precision. The intra-day and inter-day evaluation of the precision was performed with fortified blank feed or fish fillet samples. The intra-day precision was evaluated at three concentration levels of SDM (0.5, 1.0 and 1.5 mg g^{-1}) and OMP (0.125, 0.250 and 0.375 mg g^{-1}) in feed and three levels of SDM and OMP (75, 100 and 125 ng g^{-1}) in fish fillet on the same day, by the same analyst with the same equipment. The method was executed in triplicate (n=3) for each level of concentration to acquire the coefficients of variation (CV%). The inter-day precision was assessed for the same three concentration levels, however, with the replicates being prepared at three different days (n=3). The analyses were performed by the same analyst, using the same equipment and the results were expressed as coefficients of variation (CV%).

Trueness. The same assay designed to evaluate the inter-day precision was used to assess trueness. However, the results were calculated as a percentage of the obtained concentration of the fortified analyte, expressed by ranges.

Results and discussion

Analytical method for feed

The extraction procedure used for the extraction of sulfonamides and OMP from fish feed was adapted from the method described by Borràs *et al*. (2011), which detected sulfonamides in feeds for several animal species except fish. This method used a mixture of ethyl acetate and water (99:1, v/v) as extraction solution. However, the method was not satisfactory for the present study as it gave poor recoveries of approximately 30% for both analytes.

To monitor the presence of sulfonamides in animal feed, Borràs *et al*. (2011) developed two analytical methods. Mixtures with ACN and water at different proportions and mixtures of ethyl acetate with water and MeOH were tested as extraction solvents. The researchers reported dirtiest extracts in trials where the ACN was used as extraction solvent.

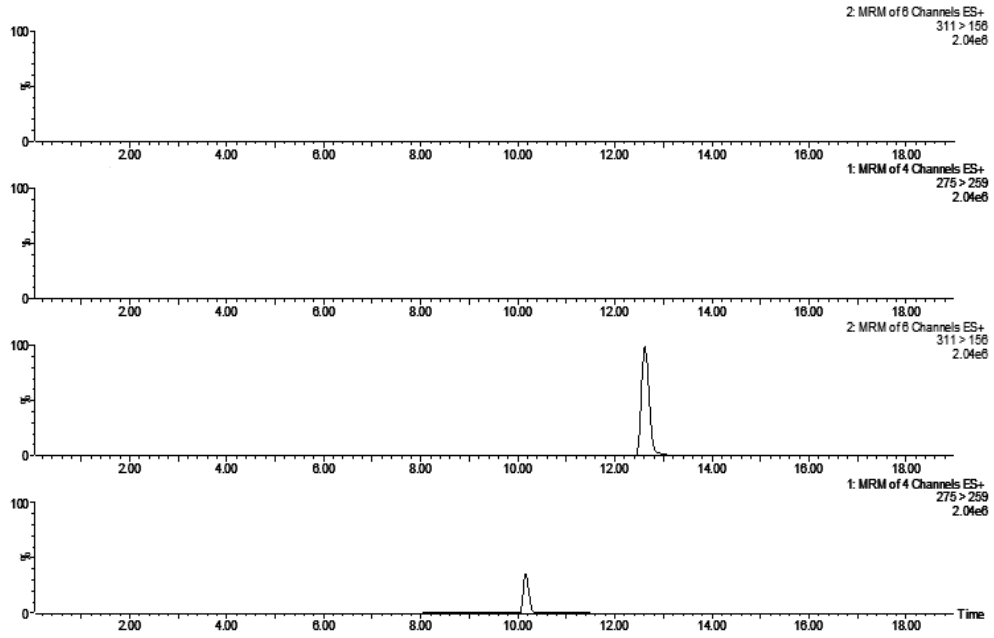

Figure 1. Selectivity of the method for fish feed sample. (A) Chromatogram obtained from analyse of fillet blank sample after extraction procedure. (B) Chromatogram obtained from analyse of feed blank sample fortified with SDM, OMP and SMZ (100 ng g^{-1}).

In the present study, replacing the initial tested extraction solution composition (ethyl acetate and water, 99:1, v/v) with MeOH improved the extraction efficiency up to approximately 100% for SDM and OMP. The selectivity of the method was

inspected to ensure that the analyte response peak was derived solely from that without any interference of the matrix (Figure 1). Therefore, a comparison of the chromatograms obtained from the blank sample feed and the blank sample fortified with SDM and OMP showed high selectivity of the method with no interference present.

Evaluated validation parameters are summarized in the Table 1. The obtained results indicate that the methods employed in the present study for SDM and OMP quantification in fish feed was suitable.

Table 1. Parameters of evaluation to assess the reliability of the analytical method developed to quantify SDM and OMP in fish feed.

Parameter	SDM	OMP
Linear range (mg g^{-1})	0.5-1.5	0.125-0.375
Linearity (r)	>0.99	>0.99
Matrix Effect (%)		
0.75 (SDM) and 0.185 (OMP) (mg g^{-1}, n=3)	2	1.3
1.00 (SDM) and 0.250 (OMP) (mg g^{-1}, n=3)	6	5.1
1.25 (SDM) and 0.315 (OMP) (mg g^{-1}, n=3)	-2	2.5
Intra-day Precision (CV, %)		
0.5 (SDM) and 0.125 (OMP) (mg g^{-1}, n=3)	5	2
1.0 (SDM) and 0.250 (OMP) (mg g^{-1}, n=3)	3	6
1.5 (SDM) and 0.375 (OMP) (mg g^{-1}, n=3)	2	2
Inter-days Precision (CV, %)		
0.5 (SDM) and 0.125 (OMP) (mg g^{-1}, n=3)	4	12
1.0 (SDM) and 0.250 (OMP) (mg g^{-1}, n=3)	9	3
1.5 (SDM) and 0.375 (OMP) (mg g^{-1}, n=3)	10	7
Trueness (Recovery tests, %)		
0.5 (SDM) and 0.125 (OMP) (mg g^{-1}, n=3)	99	97
1.0 (SDM) and 0.250 (OMP) (mg g^{-1}, n=3)	100	100
1.5 (SDM) and 0.375 (OMP) (mg g^{-1}, n=3)	100	99

Incorporation of SDM and OMP in fish feed and leaching evaluation

After inclusion of the drug, the feed was analysed in order to evaluate the concentration obtained and identify possible drug losses during the process of incorporation. The analyses were carried out in triplicate (n=3). The results indicate a loss of about 1% SDM and 13% OMP in the incorporation process.

The release of the drug from the feed into the aquatic environment can impair the quality of water and fish rearing. Thus, the leaching process is an important aspect to be addressed when the drugs are administered for medicated fish feed. The results for the leaching of the drug from the fortified feed into water are summarised in Table 2.

Fish feed is considered adequate when it is available for up to ten minutes for consumption by the fish (Cantelmo, 2002). This implies that the stability of the feed pellet is essential to reduce possible loss of nutrients and, especially, the loss of the drug.

Table 2. Leaching test for SDM and OMP from feed to water.

Time points (min)	SDM released from feed to water (%)	OMP released from feed to water (%)
5	7	27
15	11	38
30	14	50

The leaching test showed that the drug included in the feed by spraying feed pellets with oil containing SDM and OMP gave different results for the release of the two substances. The leaching process was more progressive for OMP, indicating that fish should consume medicated feed quickly in order to avoid the risk of drug under-dosing in the treatment.

Analytical method for fish fillet

Initial tests for SDM and OMP determination in fish fillet were adapted from the work of Potter *et al.* (2007), where they analysed simultaneously 17 sulfonamides, ormetoprim and trimethoprim in salmon fillet. It was used an extraction solution composed by a mixture of water and ACN (1:1, v/v). The extraction with the extraction solution was followed by a clean-up step with hexane and, then, chloroform. This method did not present good results for pacu samples. It was obtained efficiencies extraction lower than 30% for both analytes. Seeking to improvements in the extraction efficiencies, the composition of the extraction solution was evaluated by testing different organic solvents like MeOH or ACN (100%) or mixture of MeOH:water

(1:1, v/v). It was observed that the best results for extraction efficiency (higher than 75% for both analytes) were obtained using ACN (100%). The addition of chloroform was removed from the extraction process.

After method development and optimization, the analytical evaluation in order to check its reliability was done taken as reference the main parameters recommended by the guide proposed by the Brazilian Ministry of Agriculture (CGAL/SDA/MAPA, 2011) for analytical method validation. Table 3 presents the evaluated parameters.

Table 3. Parameters of evaluation to assess the reliability of the analytical method developed to quantify SDM and OMP in fish fillet.

Parameter	SDM	OMP
Linear range (ng g-^{1})	0-150	0-150
Linearity (r)	>0.99	>0.99
Matrix Effect (%)		
75 (ng g^{-1}, n=3)	2	9
100 (ng g^{-1}, n=3)	1	1
125 (ng g^{-1}, n=3)	2	5
Intra-day Precision (CV, %)		
75 (ng g^{-1}, n=3)	5	4
100 (ng g^{-1}, n=3)	3	5
125 (ng g^{-1}, n=3)	4	3
Inter-days Precision (CV, %)		
75 (ng g^{-1}, n=3)	9	18
100 (ng g^{-1}, n=3)	8	15
125 (ng g^{-1}, n=3)	7	14
Trueness (Recovery tests, %)		
75 (ng g^{-1}, n=3)	96	97
100 (ng g^{-1}, n=3)	99	99
125 (ng g^{-1}, n=3)	99	99
CCα (ng g^{-1})	100.1	100.2
CCβ (ng g^{-1})	100.2	100.4

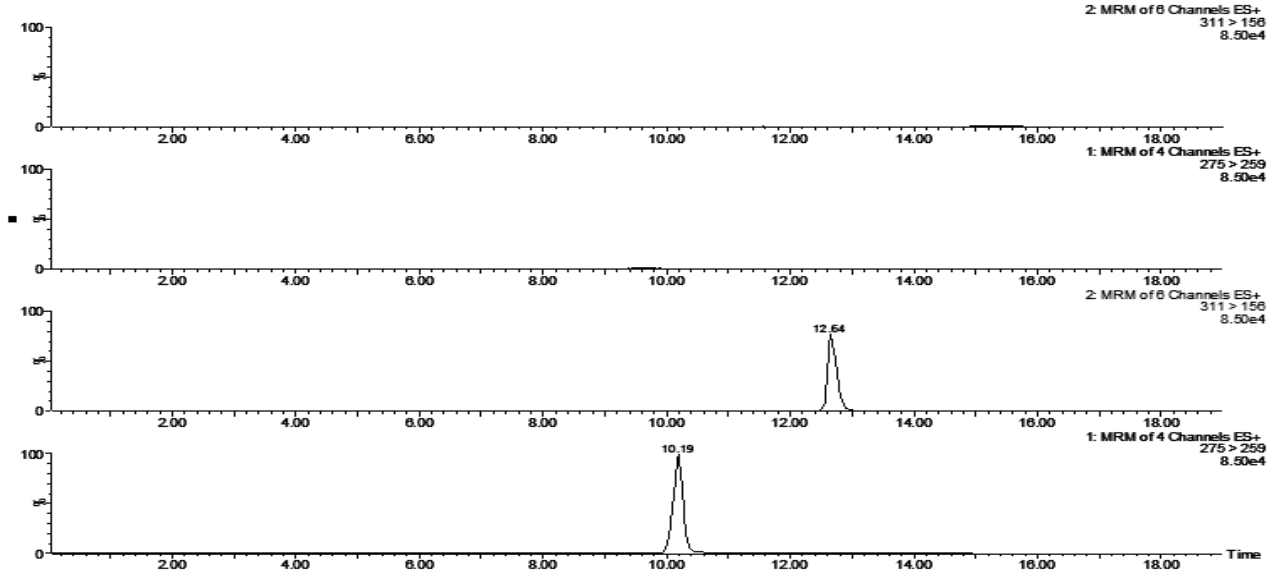

Figure 2. Selectivity of the method for fish fillet sample. (A) Chromatogram obtained from analyse of fillet blank sample after extraction procedure. (B) Chromatogram obtained from analyse of feed blank sample fortified with SDM, OMP and SMZ (100 ng g^{-1}).

The selectivity of the method obtained by comparing the chromatograms obtained for the blank sample and the fortified blank sample with the SDM and OMP for feed method and fish fillet din not presented any potential interfering of the matrix that could compromise the analytes detection (Figure 2). Furthermore, the matrix effect was evaluated for all analytes with results lower than 9%. As the developed method employs calibration curve with fortified sample, the matrix effect did not

represent a problem. Considering the guide proposed by the Brazilian Ministry of Agriculture (CGAL/SDA/MAPA, 2011) for analytical method validation, the evaluated methods are suitable for SDM and OMP monitoring in fish fillet.

Conclusions

The present work described the development and validation of an analytical method for the determination of SDM and OMP in fish feed and in pacu fish fillet. The method evaluation indicates good suitability, which is fundamental especially for fish fillet in which analytes must be quantified at low concentrations. The method developed for medicated fish feed fortified using vegetal oil containing SDM and OMP sprayed on feed pellets is an alternative medication approach, but care must be taken that the time for consumption by the fish is short because the leaching of the drug from the feed into the water is significant. The presented analytical methods and the feed medication procedure is intended to contribute to evaluation of the potential usage of SDM and OMP as antimicrobial drug for fish medication through the oral route.

Acknowledgements

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THE SIMULTANEOUS ANALYTICAL METHOD FOR DETERMINATION OF FLUNIXIN AND TOLFENAMIC ACID IN ANIMAL TISSUE WITH LC-MS/MS

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Abstract

Flunixin and tolfenamic acid are non-steroid anti-inflammatory drugs. This study presents a QuEChERs extraction and an LC-MS/MS analysis method for these compounds in animal tissues. Homogenized sample was incubated with β-glucuronidase at 37°C and then extracted with acetonitrile containing 1% formic acid. The second extraction was by salting-out with a powder mixture of magnesium sulphate, sodium citrate and disodium citrate sesquihydrateacetate. After centrifugation, the supernatant was diluted and analysed. Quantitative analysis was performed by matrix-matched calibration curve. CORTECS C18 column (2.7 mm, 2.1 × 100 mm) with gradient elution using 0.05% formic acid solution and acetonitrile can reach good separation of the analytes. In a validation study, recoveries were found between 68.2% and 95.9% for flunixin and tolfenamic acid spiked at 0.002, 0.005 and 0.01 μg g^{-1} in all investigated matrices. The RSD values were lower than 10%. Limits of quantification (LOQ) was 0.002 μg g^{-1} for both drugs.

Introduction

Anti-inflammatory drugs are commonly used in husbandry for suppressing inflammation, preventing allergies or alleviating pain. These drugs mainly include bradykinin, antihistamine, non-steroid anti-inflammatory drugs (NSAIDs) and corticosteroids. NSAIDs and corticosteroids are two most commonly used anti-inflammatory drugs (Kahn and Line, 2005; Malone, 2009). In Taiwan, competent authority has stipulated maximum residue limits (MRL) of two NSAIDs, flunixin and tolfenamic acid, in food of animal origin, whereas no method is available to detect these drugs.

Over the recent years, tandem mass-spectrometry with multiple reaction monitoring (MRM) acquisition is a preferred choice for analysing trace residue in food stuff. Compared to GC/MS/MS, LC/MS/MS may be more convenient due to its straightforward feature. Most NSAIDs are polar compounds having an acidic group with pK_a values between 3 to 5. Electrospray ionization coupled to liquid chromatography tandem mass spectrometry can therefore be a suitable detection technique.

For sample pre-treatment, several studies utilized acetonitrile as extraction solvent. The extract is then cleaned-up with *n*-hexane or solid-phase extraction (Malone *et al*. 2009; Olejnik *et al*. 2013; Van Hoof *et al*. 2004). Because many NSAIDs administered to animals are conjugated in the liver to the glucuronide form and then be excreted via urine or bile (Boelsterli and Ramirez-Alcantara, 2011), optimal hydrolysis should be taken into consideration (Malone et al. 2009) prior to sample analysis. β-Glucuronidase was used in several studies for cleaving drugs with conjugated acyl-glucuronidic acid in animal tissue to free the targeted molecule prior to sample extraction (Asea *et al*. 2001; Olejnik *et al*. 2013).

Till now, many methods have been published to analyse NSAIDs or corticosteroids in food matrices, including single analyte and multi-residues analyses. Common pre-treatment methods include liquid-liquid extraction followed by purification using a solid-phase cartridge or *n*-hexane saturated with acetonitrile. However, these methods can be replaced by a more rapid and simple method to avoid the tediousness of the procedure and consumption of large quantities of organic solvent.

The first report of the QuEChERs (quick, easy, cheap, effective, rugged and safe) method was the analysis of pesticides in vegetables and plants (Anastassiades *et al*., 2003). Recent advances in LC-MS have driven interest in QuEChERs as a means of simplifying the pre-treatment process used in the analysis of veterinary drugs in foods of animal origin.

This paper presents a LC/MS/MS method for funixin and tolfenamic acid based on QuEChERs extraction, which dramatically reduces the time required for sample pre-treatment compared with conventional analytic methods and can be suitable for large-scale analysis in laboratory on market surveillance.

Materials and Methods

Reagents and chemicals

Flunixin, tolfenamic acid, sodium acetate, β-glucuronidase (type H-2 from *Helix pomatia*, containing glucuronidase ≧85,000 and sulfatase ≦7,500) were purchased from Sigma (St. Louis, MO, USA). Gradient grade Lichrosolv acetonitrile was obtained from Merck (Darmstadt, Germany). Formic acid and glacial acetic acid were also from Merck. Ultra-pure water was obtained using a Milli-Q Ultrapure system (Millipore, Bedford, MA, USA). QuEChERs Extraction Kit, EN (4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 1 g of sodium citrate and 0.5 g of disodium citrate sesquihydrate) was obtained from Agilent Technologies (Santa Clara, CA, USA).

0.2 M Sodium acetate buffer was prepared in water and pH was adjusted to 5.2 ± 0.1 with glacial acetic acid. Standard stock solution (0.1 mg mL^{-1}) was prepared in methanol and stored at -20°C. Fresh working solution (1 µg mL^{-1}) was prepared from the stock solution in methanol each month. A dilution of 0.1 µg mL^{-1} in 0.1% methanolic formic acid was used to optimize the mass spectrometer.

Apparatus

High-speed shaker was a 2010 Geno/Grinder, SPEX SamplePrep (Metuchen, NJ, USA). The homogenizer was a Polytron PT-MR 3100 (Kinematic AG, Littau, CH). LC system comprised an Eksigent ultraLC system (AB SCIEX, Redwood City, CA) equipped with a quaternary pump, an autosampler, a degasser, and a column oven. A CORTECS C18 column (2.1 × 100 mm, 2.7 µm; Waters, Milford, MA, USA) was used to separate the analytes and was held at 40°C. Water containing 0.05% formic acid and acetonitrile was used as mobile phase at a flow rate of 0.40 mL min^{-1}. The injection volume was 20 µL.

Mass spectrometry was performed using a QTRAP 5500 (AB SCIEX, Framingham, MS, USA) hybrid triple quadrupole mass spectrometer equipped with a Turbo V ion source and TIS (TurboIon Spray) probe operating in electrospray ionization (ESI) mode. A source temperature of 500°C was maintained throughout testing. Nitrogen was used as a nebulizer (50 psi), auxiliary (50 psi), curtain (20 psi), and collision gas (high). Ionspray voltage was set to 5,500V. MS-MS parameters were optimized by infusing standard solution (0.1 µg mL^{-1}) at a flow rate of 10 µL mL^{-1}. Experimental MS parameters are listed in Table 1.

Table 1. Analytical conditions of the mass spectrometer

Parameter	Condition
Scan type	MRM
Polarity	Positive/Negative
Scan mode	N/A
Ion source	Turbo ion
Resolution Q1	Unit
Resolution Q3	Unit
Setting time (msec)	50.00
MR pause (msec)	5.00
MCA	No
Step size (Da)	0.00
Collision, curtain and ion source gas	N_2
CUR (psi)	20
GS1 (psi)	50
GS2 (psi)	50
IS (V)	5,500/-4,500
TEM (℃)	500
CAD (high/medium/low)	High
CEM (V)	2,000

Sample preparation

Pork, *bovine*, pig kidney, pig liver, pig lipid and milk were purchased from local markets in Taipei, Taiwan. Milk was stored at 4°C, and other samples were ground, homogenized, and stored at -20°C until analysis. Precisely 2 g of the homogenized sample was loaded into 50-mL centrifuge tubes. Ten mL 0.2 M sodium acetate (pH 5.2) was added to each test tubes, which were shaken. After standing 10 min, 100 µL β-glucuronidase solution was added, mixed with vortexer, and incubated for 1 h in a waterbath at 37°C to simulate incurred sample analysis.

After digestion, 10 mL acetonitrile containing 1% formic acid was added to each tube and then QuEChERs powder (4 g anhydrous magnesium sulphate, 1 g sodium chloride, 1 g sodium citrate and 0.5 g disodium citrate sesquihydrate) was poured into the tubes. The tubes were immediately capped and vigorously shaken by hand to avoid agglomeration before agitation using a high-speed shaker at 1,000 rpm for 1 min. The suspension was then centrifuged at ca 5,000 rpm at 10°C for 1 min. A volume of 500 µL supernatant was transferred into an Eppendorf vial. Water was added to 1,000 µL and the solution was passed through a 0.22 µm polytetrafluoroethene (PTFE) filter prior to LC/MS/MS analysis.

Results

Matrix-matched calibration

Tables 2 and 3 summarises slopes, correlation coefficient (r), matrix effect of standard curve and matrix-matched calibration curve at concentrations from 0.2 to 10 ng mL^{-1} for both drugs in all matrices. Matrix effects were found for both substances in various matrices. Matrix-matched calibration curves were therefore used to quantify the amount of flunixin and tolfenamic acid to compensate the matrix effect.

Table 2. Results of linear regression analysis of (matrix-matched) standard curves (0.2 ~ 10 ng mL^{-1}) and matrix effects for flunixin.

Matrix	Standard curve	r	Matrix-matched calibration curve	r	Matrix effect (%)
Pork	$Y = 2.72\times10^{6}X + 6.84\times10^{4}$	0.9997	$Y = 2.69\times10^{6}X - 2.42\times10^{4}$	0.9993	-1.1
Bovine muscle	$Y = 2.57\times10^{6}X + 6.09\times10^{4}$	0.9969	$Y = 4.60\times10^{5}X - 226$	0.9995	78.9
Milk	$Y = 2.92\times10^{6}X - 2.32\times10^{4}$	0.9939	$Y = 3.09\times10^{6}X + 8.74\times10^{4}$	0.9963	5.8
Pig liver	$Y = 2.53\times10^{6}X + 2.92\times10^{4}$	0.9992	$Y = 1.44\times10^{6}X + 2.77\times10^{4}$	0.9982	-43.1
Pig kidney	$Y = 2.68\times10^{6}X - 8.01\times10^{4}$	0.9997	$Y = 2.18\times10^{6}X + 3.17\times10^{4}$	0.9995	-18.7
Pig fat	$Y = 2.08\times10^{6}X + 9.13\times10^{4}$	0.9991	$Y = 2.20\times10^{6}X + 5.96\times10^{4}$	0.9996	5.8

Table 3. Results of linear regression analysis of (matrix-matched) standard curves (0.2 ~ 10 ng mL^{-1}) and matrix effects for tolfenamic acid.

Matrix	Standard curve	r	Matrix-matched calibration curve	r	Matrix effect (%)
Pork	$Y = 1.04\times10^{5}X + 598$	0.9999	$Y = 6.28\times10^{4}X - 1.21\times10^{3}$	0.9984	-39.6%
Bovine muscle	$Y = 8.87\times10^{4}X - 1.69\times10^{3}$	0.9994	$Y = 4.44\times10^{4}X - 68.5$	0.9992	-49.9%
Milk	$Y = 2.45\times10^{5}X + 2.08\times10^{3}$	0.9988	$Y = 2.33\times10^{5}X + 8.68\times10^{3}$	0.9995	-4.9%
Pig liver	$Y = 1.01\times10^{5}X + 589$	0.9999	$Y = 1.94\times10^{4}X + 102$	0.9987	-80.8%
Pig kidney	$Y = 4.91\times10^{5}X + 7.29\times10^{3}$	0.9996	$Y = 1.53\times10^{5}X + 3.20\times10^{3}$	0.9996	-68.8%
Pig fat	$Y = 8.45\times10^{4}X - 284$	0.9999	$Y = 7.86\times10^{4}X + 1.01\times10^{3}$	0.9999	-7.0%

Table 4. Recoveries, coefficients of variation and limits of quantification of flunixin in beef, milk, pork, pig liver, pig kidney and pig fat.

Matrix	Intra-day precision						Inter-day precision
	0.002 µg g^{-1}		0.005 µg g^{-1}		0.01 µg g^{-1}		0.005 µg g^{-1}
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	CV (%)
Pork	95.9	2.1	88.9	3.1	87.1	2.4	3.1
Bovine muscle	95.3	8.1	84.5	9.4	94.5	4.1	4.8
Milk	87.2	3.1	91.3	1.6	94.3	1.4	2.4
Pig liver	86.2	1.7	91.9	2.9	94.0	2.5	3.4
Pig kidney	84.3	2.6	85.0	1.4	86.0	1.8	1.2
Pig fat	76.2	5.1	78.5	0.9	81.5	1.5	3.1

Table 5. Recoveries, coefficients of variation and limits of quantification of tolfenamic acid in beef, milk, pork, pig liver, kidney and fat.

Matrix	Intra-day precision						Inter-day precision
	0.002 µg g^{-1}		0.005 µg g^{-1}		0.01 µg g^{-1}		0.005 µg g^{-1}
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	CV (%)
Pork	92.7	4.1	83.8	8.8	87.5	9.1	7.7
Bovine muscle	88.2	5.9	95.1	3.1	90.3	1.0	9.0
Milk	78.0	3.7	85.0	2.4	87.0	2.1	2.6
Pig liver	89.7	5.0	86.3	5.3	81.0	3.7	5.0
Pig kidney	83.0	3.6	82.7	4.2	82.1	3.3	6.0
Pig fat	68.2	4.8	69.8	4.7	71.7	2.5	4.2

Validation

Tables 4 and 5 summarise the results of the validation study. Validation was on the basis of Taiwanese guideline (Taiwan food and drug administration, 2012). Intra-day recoveries of five replicates at three concentrations (2, 5, and 10 ng g^{-1}) ranged from 68.2 to 95.9% in all matrices. Inter-day precision of ten replicates at 5 ng g^{-1} was 1.2 to 4.8% for flunixin and 2.6

to 9.0% for tolfenamic acid. Both intra-day and inter-day precisions were below 10%. In the proposed method, the LOQ of flunixin and tolefenic acid was 2 ng g^{-1}, which gave a signal-to-noise ratio (S/N) for the quantitation ions more than 10 and confirmatory ions more than 3 (Figures 1 and 2).

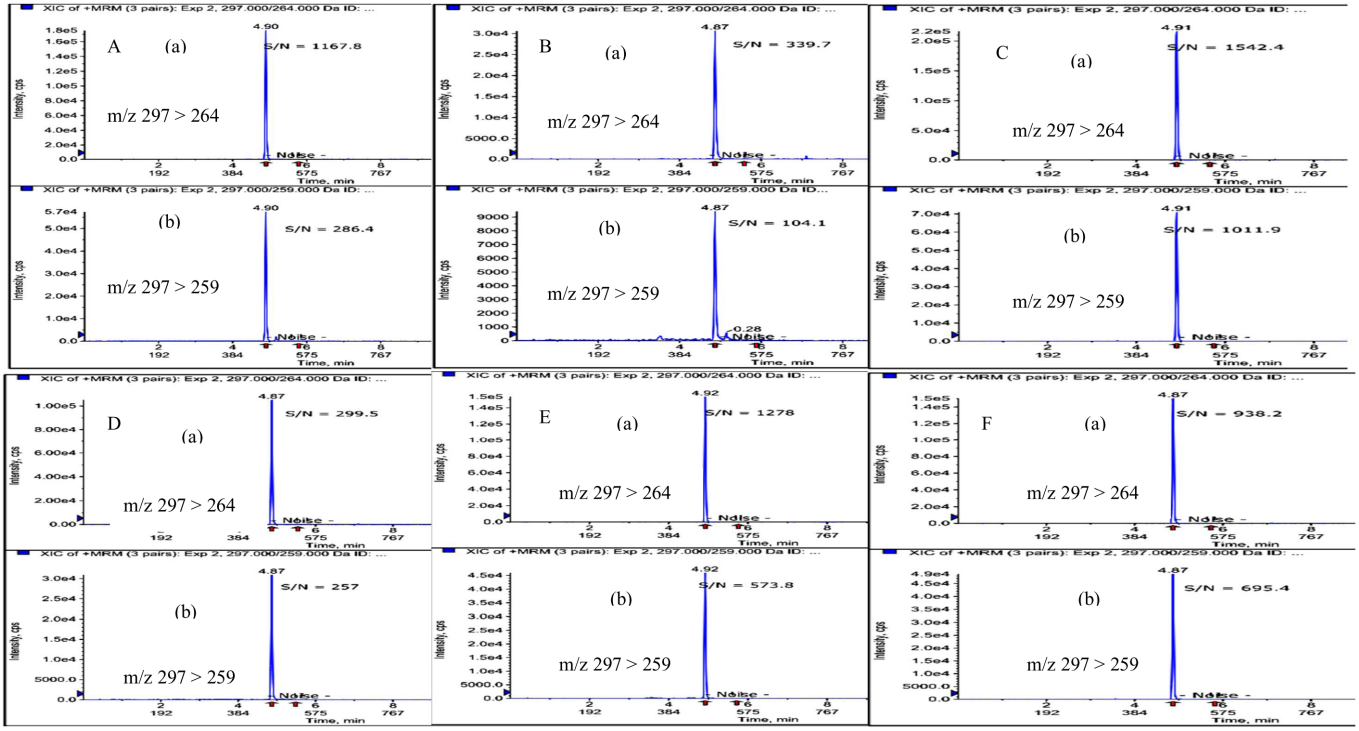

Figure 1. MRM chromatograms and signal-to-noise ratios of the (a) quantification transition and (b) qualitative transition of flunixin at the limit of quantitation of (A) pork, (B) bovine muscle, (C) milk, (D) pig liver, (E) pig kidney and (F) pig fat.

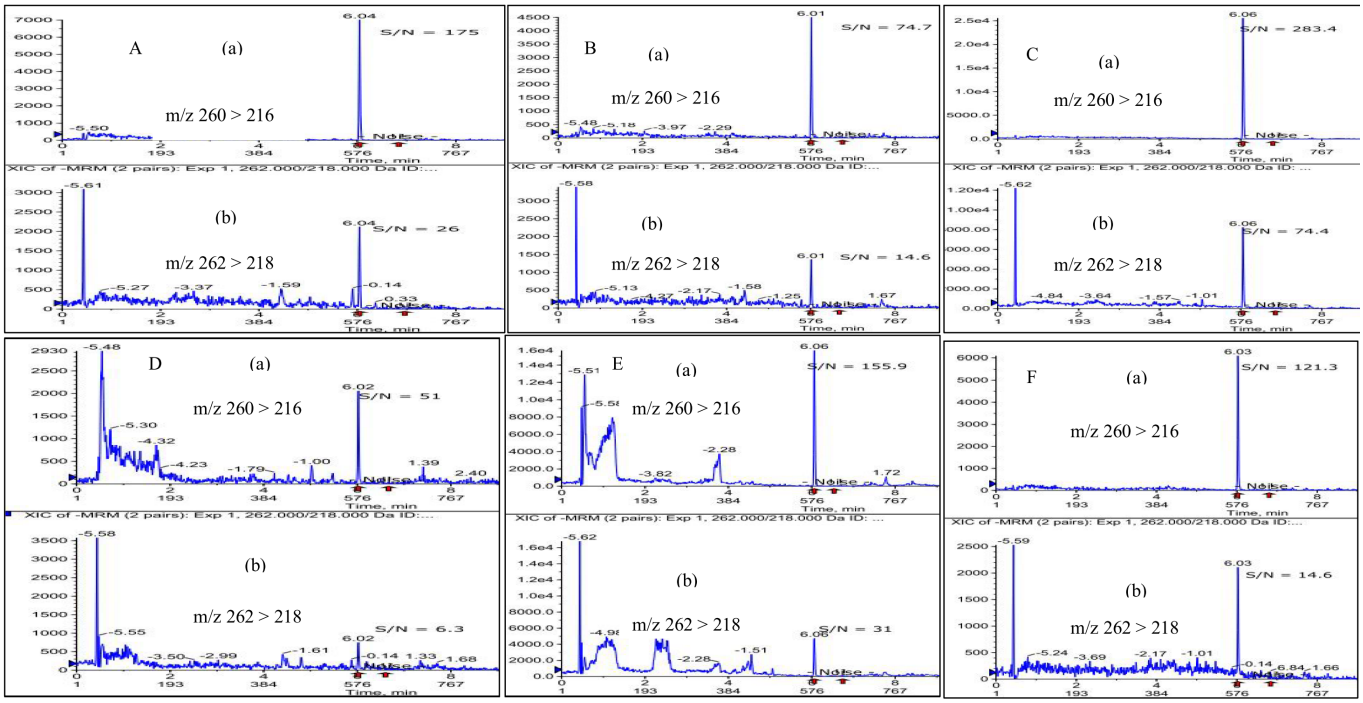

Figure 2. MRM chromatograms and signal-to-noise ratios of the (a) quantification transition and (b) qualitative transition of tolfenamic acid at the limits of quantitation of (A) pork, (B) bovine muscle, (C) milk, (D) pig liver, (E) pig kidney and (F) pig fat.

Discussion

Optimization of Mass Spectrometry

The tuning of the mass spectrometer was performed by infusing standard solution (0.1 µg mL^{-1}) in methanol containing 0.1% formic acid at a flow rate of 10 µL min^{-1} with a built-in syringe pump in ESI mode. The precursor ion of flunixin ($[M+H]^+$) is *m/z* 297, whereas the precursor ions of tolfenamic acid are *m/z* 260 and *m/z* 262, which is the deprotonated molecule $[M-H]^-$. Collision-induced dissociation (CID) of the precursor ions with collision gas yielded fragment ions for both drugs. Two ion pairs for each compound were selected for quantification and confirmation. Optimum mass spectrometry settings are summarized in Table 6.

Table 6. LC/MS/MS settings and retention times for flunixin and tolfenamic acid.

Compound	Ion pair: Precursor ion (*m/z*) > product ion (*m/z*)	Declustering potential (DP)	Entrance potential (EP)	Collision energy (CE)	Collision cell exit potential (CXP)	Retention time (min)
Flunixin	297 > 264 [a]	60	10	32	20	4.84
	297 > 259 [b]	60	10	48	20	
Tolfenamic acid	260 > 216 [a]	-60	-10	-23	-20	6.02
	262 > 218 [b]	-60	-10	-23	-20	

[a] *Quantitation ions;* [b] *Confirmation ions*

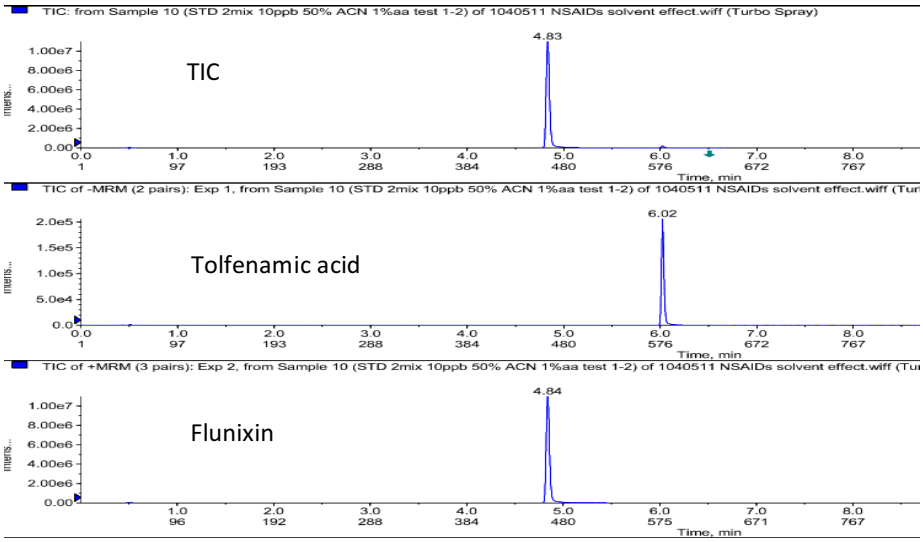

Figure 3. The total ion and multiple reaction monitoring chromatograms of tolfenamic acid and flunixin separated with the CORTECs C18 column at the concentration of 10 ng mL^{-1}.

Liquid chromatography

Flunixin and tolfenamic acid have different acid-dissociation constants, the former is 5.8 and the latter is 4.3. For this reason, peaks shift can be seen if no modifier or buffer salt is present in the mobile phase. In this experiment, mobile phase containing 0.05% formic acid and acetonitrile can get stable retention times as shown in Figure 3.

Sample pretreatment

Most non-steroidal anti-inflammatory drugs (NSAIDs) contain a carboxylic acid group, which is usually conjugated to an acyl glucuronide in the second phase metabolism (Boelsterli and Ramirez-Alcantara, 2011). In this experiment, enzymatic digestion by β-glucuronidase was adopted from other reports (Asea *et al.* 2001; Olejnik *et al.* 2013). However, a variety of β-glucuronidases, spiking volumes and digestion times are documented. Because neither an explicit standard method in the literature nor certified reference material can be found, an in-house method of enzymatic procedure was used in the study.

After digestion, a quick and easy extraction method was another point to be considered. In the experiment, QuEChERs salt containing 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 1 g of sodium citrate and 0.5 g of disodium citrate sesquihydrate was used. In a preliminary study, we selected pig muscle, pig liver and pig lipid to evaluate extraction efficiency of three replicates at spiking level of 5 ng g^{-1}. The recovery results of both drugs for the three matrices ranged from 70 to 99% and the coefficients of variation were under 12% (Table 7).

Table 7. Recoveries and coefficients of variation of flunixin and tolfenamic acid fortified at 5 ng g^{-1} into pork, pig liver and pig lipid samples.

Matrix	Recovery% (CV%)	
	Flunixin	Tolfenamic acid
Pork	80.3 (1.1)	72.5 (3.9)
Pig liver	99.2 (6.4)	97.3 (10.6)
Pig lipid	82.8 (1.7)	74.6 (2.0)

Conclusions

In this study, we developed an efficient and effective LC-ESI-MS-MS method for the detection of flunixin and tolfenamic acid in animal tissues. QuEChERs procedure provides a rapid and easy operation, and also achieves a high quality of results. For separation, mobile phase containing 0.05% formic acid can prevent the shift of retention time. A matrix-matched calibration curve was adopted to provide accurate quantification and to compensate for signal suppression. This is the first study to report on the application of QuEChERs extraction method for analysing flunixin and tolfenamic acid in animal tissues. This method is suitable for screening and accurate quantification in large-scale market surveillance.

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DEVELOPMENT OF A NEW HIGHLY SENSITIVE ELISA FOR THE RAPID SCREENING OF TETRACYCLINE IN HONEY

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Abstract
Tetracycline is a broad-spectrum antibiotic used in apiculture for treatment of bacterial diseases such as foulbrood. The use of antimicrobials in beekeeping is strictly regulated or banned. The main purposes for monitoring bee products are for consumer health protection, international commercial competition, and better product quality. This study reports the development of a new highly sensitive, rapid one step, competitive Enzyme-Linked Immunosorbent Assay (ELISA) for the screening of tetracycline residues in honey. A minimal sample preparation is required and the assay time is 1 h. A standard series, spiking material and ready-to-use conjugate were included. The limit of detection was 2 ppb (tetracycline equivalence). Recovery ranged from 75 - 119% and intra-assay precision, expressed as CV (%) was ≤ 6. The results indicate applicability of this ELISA to the rapid, sensitive and convenient screening of tetracycline residues in honey following a minimal sample preparation. This facilitates the monitoring of honey samples to ensure regulatory compliance.

Introduction
Tetracyclines are a group of broad-spectrum antibiotics, commonly used to treat disease in livestock. Due to potential contamination of the food supply, tetracycline antibiotic levels must be routinely monitored to ensure regulatory compliance.

Tetracyclines have been widely used since their discovery in the late 1940's as therapeutic agents in human and veterinary medicine but also as growth promoters in animal husbandry although controversial because of concerns that this practice is contributing to the emergence of resistance and the risk to human health (Chopra *et al*., 2001, Economou and Gousia, 2015). In recent years tetracyclines have been used in apiculture for the treatment of bacterial brood infections and although not intended for use during the production of marketable honey, residues could still be present. Regulatory authorities have specified maximum residual limits (MRLs), or tolerances, for tetracyclines, which are considered to represent safe levels for human consumption.

As antibiotics are not permitted in honey (Al-Waili *et al*., 2012) but are used in the control of bee diseases, then rapid screening methods to detect their presence are essential. There are 630,000 beekeepers in the EU and subsequently 16,000,000 hives in the EU producing 204,000 tonnes of honey per year (EU Commission, 2016). Sensitivity is crucial in the detection of tetracycline residues as these drugs are either strictly regulated or banned in honey. No MRL for honey has been set (European Commission), but 20 µg kg^{-1} was recommended by Community Reference Laboratories (CRL) for all tetracyclines in honey (CRL Guidance Paper, 2007).

The aim of this study was to develop a new highly sensitive ELISA for the rapid screening of tetracycline residues in honey samples, requiring a minimal sample preparation with detection limits $\underline{<}$ 2 ppb. If performing the test using duplicate wells, this kit has the ability to analyse 40 samples within 1 h. The analytical performance of this ELISA is reported.

Material and Methods

Competitive ELISA
The determination of tetracycline in honey was carried out using a newly developed ELISA kit (TCS10117, Randox Food Diagnostics, Crumlin, UK). This ELISA is based on a competitive reaction where antigen present in the standard and sample competes with horseradish peroxidase labelled conjugate for a limited number of capture antibody binding sites (antibody solution). The antibody then binds to a second antibody immobilised and stabilised on 96 well microtitre (breakapart) plate. Antibody solution and conjugate were supplied ready-to-use. Lyophilised standard series and spiking material were supplied in the kit.

In brief, the experimental procedures were as follows: 50 µL sample/standard, 50 µL antibody solution and 50 µL conjugate were added per microtitre plate well. After incubation for 30 min at +15°C to +25°C the microtitre plate was washed to remove excess reagents. After washing, 125 µL enzyme substrate was immediately added across the plate and incubation for 20 ± 2 min followed. The reaction was then stopped by the addition of stop solution. The optical density was measured at 450 nm. Colour intensity is inversely proportional to the concentration of the analyte present in the sample. The assay time is 1 h.

Honey Sample Preparation

Honey (1 g) was dissolved in 9 mL of diluted diluent/wash buffer. The preparation was diluted 1:1 with ready-to-use sample diluent, *e.g*. 200 µL sample preparation and 200 µL sample diluent. The sample was mixed prior to application to the microtitre plate. Sample volume per well was 50 µL. The dilution factor applied to each honey sample was 20.

Analytical parameters

Calibration curve. The calibration curve was generated by using a 4-parameter curve fit. The calibration range of the assay was 0 ppb - 2.04 ppb (tetracycline equivalence).

Sensitivity. The half maximal inhibitory concentration (IC_{50}) was calculated by taking 50% of the absorbance of the zero standard and reading this value from the x-axis (ppb) off the corresponding calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Limit of detection (LOD). LOD for tetracycline was calculated as the mean concentration (ppb) of 24 negative honey samples plus 3 standard deviations. This represents the lowest concentration of analyte in a sample matrix that can be accurately detected by the assay.

Specificity. IC_{50} for tetracycline and cross-reactants tested was calculated. Assay specificity, expressed as cross-reactivity (CR) % was determined as follows:

$$CR\ (\%) = [IC_{50}\ (\text{tetracycline}) / IC_{50}\ (\text{cross-reactant})] \times 100$$

Intra-assay Precision. Intra-assay precision was evaluated for twelve replicates of 0, 0.06, 0.15, 0.35, 0.85 and 2.04 ppb concentration levels and two control samples within a single run. Results were expressed as CV (%).

Recovery. Negative honey samples (n = 2) were spiked at three different concentrations within the calibration range. The recovery (%) was calculated as follows;

$$\text{Recovery}\ (\%) = (\text{assigned sample concentration/spiked concentration}) \times 100\%$$

Stability. Shelf life was determined by accelerated stress testing. Each kit component was stressed at 37°C for 14 days and compared with the same material stored at +2°C to + 8°C.

Reconstituted real time stability of the standard/spiking material was assessed at various time points and compared to freshly reconstituted material. Reconstituted standard/spiking material was set up at +2°C to + 8°C and also at -20°C.

Results

Sensitivity

The IC_{50} value attained with this ELISA was 0.15 ppb (tetracycline equivalence) and the LOD was 2 ppb (tetracycline equivalence). This is relevant for an improved detection of tetracycline residues in honey as these drugs are either strictly regulated or banned.

Table 1. Tetracycline ELISA: specificity data

Analyte	% Cross-reactivity
Tetracycline	100
4-epitetracycline	87
Rolitetracycline	67
4-epioxytetracycline	52
Oxytetracycline	52
Chlortetracycline	51
Demeclocycline	41
Doxycycline	23
4-epichlortetracycline	20
Methacycline	11
Minocycline	<1
Norfloxacin	<1
Ceftiofur	<1
Florfenicol	<1
Streptomycin	<1
Tylosin	<1

Specificity

The newly developed sensitive ELISA presents a broad specificity profile for tetracyclines with cross-reactivity values ranging from 11% (methacycline) to 100% (tetracycline), providing an increased detection capability (Table 1).

Intra-assay Precision

The intra-assay precision for different concentration levels and control samples, expressed as CV (%) was ≤6 (Table 2).

Table 2. Tetracycline ELISA: intra-assay precision.

Standard Level	CV (%)
1	1.6
2	1.8
3	3.3
4	2.2
5	5.5
6	6.0
Control 1	4.0
Control 2	5.8

Recovery

The mean recovery (%) for three different concentration levels ranged from 75% to 119% (Table 3).

Table 3. Tetracycline ELISA: mean recovery.

Sample	Recovery (%)		
	Level 1	Level 2	Level 3
1	86	89	75
2	110	119	111

Stability

A shelf life of 2 years was determined for each of the kit components after accelerated stress testing. An example for the antibody solution is shown (Figure 1).

Reconstituted stability of the standard/spiking material was determined as 24 h stability when stored at +2°C to + 8°C and 1-week stability when stored at -20°C.

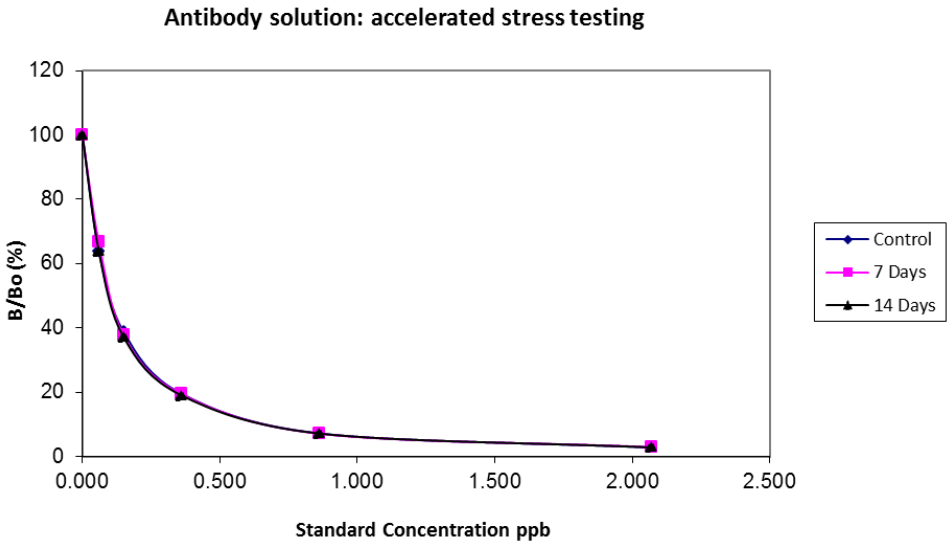

Figure 1. Tetracycline ELISA: shelf life stability. Example is the antibody solution after accelerated stress.

Discussion

The results show that this innovative one step ELISA presents exceptional sensitivity for tetracycline in honey (LOD = 2ppb) without compromising the assay precision (overall intra-assay precision: CV ≤ 6%) or the recovery (75% - 119%). This ELISA provides a comprehensive specificity profile for the tetracyclines and their epimers enhancing detection capability. This is highly advantageous for the screening of honey samples as antibiotics are not permitted in honey but are used in the control of bee diseases and therefore residues may still be present. Minimal sample preparation is required and if performing the

test using duplicate wells, 40 samples can be analysed within 1 h. Moreover, this ELISA kit has a user friendly format, standard series, spiking material and ready to use conjugate are included. The shelf life stability of all the components is two years. This represents an analytical improvement in the rapid screening of tetracycline in honey samples, only positive results after screening require further confirmatory analysis. This facilitates the monitoring of honey samples to ensure regulatory compliance and therefore consumer protection.

Conclusion

This sensitive one step ELISA provides an excellent screening tool for the detection of low levels of tetracycline residues in honey matrices, which is relevant as the presence of antibiotic residues is not permitted in honey.

The developed ELISA represents a stable, valuable and convenient (standard series, spiking material and ready-to-use conjugate are included) analytical tool for the rapid screening (within 1 h) of honey samples following minimal sample preparation. This is relevant for consumer protection and to monitor regulatory compliance.

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MULTI-ANALYTE SCREENING OF TWELVE COCCIDIOSTAT RESIDUES IN ANIMAL FEED USING BIOCHIP ARRAY TECHNOLOGY

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Abstract

Coccidiostats are antiprotozoal agents that act upon Coccidia parasites in livestock. These drugs are often administered through the use of medicated animal feeds with often more than one drug being used at once. Prophylactic use of medicated coccidiostat feeds increases the risk of drug residue carry over into meat for human consumption. Maximum content limits have been set in place for a number of coccidiostat residues.

With the aim to increase the screening capacity, this study reports the application of biochip array technology to the simultaneous determination of coccidiostat residues from a single animal feed sample with minimal sample preparation. Simultaneous chemiluminescent immunoassays, defining discrete test sites on the biochip surface, were applied to the analyser Evidence Investigator analyser. Initial evaluation showed multiplex semi-quantitative detection of the following coccidiostats: lasalocid, nicarbazin, imidocarb, toltrazuril, maduramicin, salinomycin/narasin, clopidol, monensin, robenidine, decoquinate, halofuginone and diclazuril. Sensitivities ranged from 0.50 mg kg^{-1} – 400 mg kg^{-1} (sensitive screening), following dilution sensitivities ranged from 3.80 mg kg^{-1} – 3,000 mg kg^{-1} (elevated detection). The analysis of a quality control sample from FAPAS excellent accuracy of the biochip array.

This initial study indicates the great capability of biochip array technology to the multiplex determination of coccidiostat residues in feed.

Introduction

Industrially bred poultry and rabbits are particularly prone to coccidiosis, a contagious protozoan infection, which causes diarrhoea and dysentery. Major infections can cause significant mortality with economic implications. In Europe, some coccidiostats are authorised as feed additives in accordance with Regulation 2003/1831/EC on additives for use in animal nutrition. They are authorised as prophylactic products to prevent coccidiosis.

In the production of feed, carry-over of coccidiostats may occur from a batch where the coccidiostats are used as authorised feed additives to a batch of non-targeted feed. To avoid coccidiostat carry-over Regulation 2005/183/EC sets specific requirements for feed hygiene. Maximum levels of coccidiostats in feed have been set within the European Union (Commission Directive 2009/8/EC, Commission Regulation 574/2011) to protect animals and animal products from the unintentional treatment induced by carry-over.

It is therefore important to detect the presence of coccidiostats in feed and analytical methods for their screening and/or confirmation in this matrix have been reported: HPLC-UV spectrofluorimetry (De Jong *et al*., 2004; Thalmann *et al*., 2004), LC-MS/MS (Turnipseed *et al*., 2001; Mortier *et al*., 2005, Vincent *et al*., 2008), HPLC-MS/MS (Delahaut *et al*., 2010), flow cytometric immunoassay (Bienenmann-Ploum *et al*., 2012). Biochip array technology allows multi-analytical determination from a single sample and facilitates the rapid multiplex screening of batches of samples as fifty four biochips can be handled at the same time through the use of the semi-automated biochip analyser Evidence Investigator. The core of the technology is the biochip (9mm x 9mm) and represents the platform in which the capture molecules are immobilized and stabilised defining arrays of discrete test regions (DTR) on a pre-activated surface (Fitzgerald *et al*., 2005, Molloy *et al*., 2005). The biochip is also the vessel where simultaneous chemiluminescent immunoreactions take place. The chemiluminescent signal from each DTR is simultaneously detected and recorded using a Charge-Coupled Device (CCD) camera. Image processing, quantification and validation are carried out by instrument specific software. Applications of this technology to the determination of residues have been reported (O'Mahoney *et al*., 2011, Porter *et al*., 2012, Popa *et al*., 2012a, Popa *et al*., 2012b, Oruc *et al*., 2013, Gaudin *et al*., 2014). This study reports the initial evaluation of the application of biochip array technology to the multi-analyte screening of coccidiostat residues in feed.

Materials and Methods

Biochip based immunoassays

Simultaneous chemiluminescent immunoassays were employed an applied to the analyser Evidence Investigator (EV3602, Randox Food Diagnostics, UK). The immunoassays included were: clopidol, decoquinate, diclazuril, halofuginone, imidocarb, lasalocid, maduramicin, monensin, nicarbazin, robenidine, salinomycin/narasin, toltrazuril. The experimental procedure is summarised as follows: 150 µL of assay buffer was added per sample/ standard or control biochip followed by addition of 50 µL of sample, standard or control. Following incubation in the custom thermoshaker provided for 30 min at 25°C and

370 rpm, 100 µL of working strength conjugate was then added into each biochip and the assay was incubated for a further 60 min at 25°C and 370 rpm. After washing, chemiluminescent substrate was added to each biochip and the light reaction generated was captured and quantified by the using digital imaging technology. The Evidence investigator incorporates dedicated software, which automatically processes, reports and archives the data generated.

Feed Sample Preparation
A simple liquid/liquid extraction is required followed by centrifugation before application to the biochip. The final extract dilution can be chosen to fit the user's specific needs.

Analytical parameters

Limit of detection. The limit of detection (LOD) for each analyte was calculated based on the mean concentration plus 3 x standard deviations from the data for 20 negative feed samples. This represents the lowest concentration of analyte that can be distinguished due to matrix effects.

Specificity / Cross-reactivity (CR). The half maximal inhibitory concentration (IC_{50}) for each analyte and cross-reactant tested was calculated by taking 50% of the signal of the zero calibrator and reading this value from the x-axis (ppb) off the corresponding calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Specificity, expressed as cross-reactivity (%) (CR %) was calculated as follows:

CR (%) = [(IC_{50} (analyte) / IC_{50} (cross-reactant)] x 100

Intra-assay Precision. Intra-assay precision was determined by testing 20 replicates of sample fortified at three different concentration levels. Results were expressed as CV (%).

Sample analysis. One quality control FAPAS sample was tested and the results were compared with FAPAS assigned values.

Results

Limit of detection
For the sensitive detection the LODs ranged from 0.5 mg kg^{-1} (nicarbazin) to 400 mg kg^{-1} (clopidol). When elevated detection was applied the LODs ranged from 3.8 mg kg^{-1} (nicarbazin) to 3,000 mg kg^{-1} (clopidol) (Table 1).

Table 1. Sensitivity profile of the biochip based immunoassays.

Assay	Sensitive detection	Elevated detection
	LOD (mg/kg)	**LOD** (mg/kg)
Clopidol	400.0	3000.0
Decoquinate	125.0	937.5
Diclazuril	17.5	131.3
Halofuginone	2.5	18.8
Imidocarb	1.0	7.6
Lasalocid	25.0	188.0
Maduramicin	3.8	28.1
Monensin	3.8	28.1
Nicarbazin	0.5	3.8
Robenidine	20.0	150.0
Salinomycin/narasin	6.3	46.9
Toltrazuril	6.3	46.9

Specificity/Cross-reactivity
The specificity profile showed detection of a broad range of coccidiostats (Table 2).

Intra-assay Precision
Intra-assay precision (n=20), expressed as CV (%), was <15% for all the assays across different concentration levels.

Sample analysis
The comparison of the quality control sample results from FAPAS and the corresponding biochip array results for lasalocid, monensin and salinomycin are shown (Table 3).

Table 2. Specificity profile of the biochip array

Assay	Compound	CR (%)
Clopidol	Clopidol	100
	Nequinate	135
Decoquinate	Decoquinate	100
Diclazuril	Diclazuril	100
	Clazuril	12
Halofuginone	Halofuginone	100
Imidocarb	Imidocarb dipropionate	100
Lasalocid	Lasalocid A	100
Maduramicin	Maduramicin ammonium	100
Monensin	Monensin	100
	Monensin A	89
Nicarbazin	Dinitrocarbanilide	100
	Nicarbazin	98
Robenidine	Robenidine	100
Salinomycin/narasin	Salinomycin	100
	Narasin	130
Toltrazuril	Toltrazuril sulphone	100
	Toltrazuril sulphoxide	145
	Toltrazuril	7

Table 3. Quality control sample analysis

Coccidiostat	FAPAS assigned range (mg kg^{-1})	Biochip Array results (mg kg^{-1})	FAPAS results (mg kg^{-1})
Lasalocid	48.0-112.0	60.9	80.0
Monensin	59.6-139.2	122.6	99.4
Salinomycin	45.8-106.9	83.6	76.3

Discussion

The results of this initial evaluation indicate that the biochip array detects a broad range of coccidiostat residues in feed. The methodology requires simple sample preparation and can be applied to the semi-quantitative detection of coccidiostats at different concentration levels. Favourable comparison was observed with FAPAS assigned values for a quality control sample. The application of the immunoassays to the semi-automated biochip analyser Evidence Investigator facilitates the rapid multiplex screening of batches of samples as fifty-four biochips can be handled at the same time. Biochip array technology increases the screening capacity and facilitates the surveillance of feed samples.

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DEVELOPMENT OF A B-AGONIST FAST ELISA FOR THE SCREENING OF MILK WITH MINIMAL SAMPLE PREPARATION

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Abstract
Beta-agonists can be used for a number of purposes including therapeutic treatment and for promoting growth. In the European Union, the use of these compounds for growth-promotion in farm animals intended for human consumption is banned, as there is a potential health risk to consumers. These compounds are listed for monitoring in live animals and animal products. The availability of rapid screening methods to detect β-agonists facilitates the testing process. This study reports the development of a β-agonist fast Enzyme-Linked Immunosorbent Assay (ELISA) for the rapid screening of milk with minimal sample preparation. In this competitive ELISA the components are ready-to-use and the incubation time is short. Milk samples only require centrifugation prior to addition to the microtitre plate. Forty milk samples were run in duplicate within one hour. Analytical performance is not compromised by the short assay time. The assay was standardised to clenbuterol, with the intra-assay precision typically less than 9%. The limit of detection was ≤0.1ppb. The mean recovery for the samples tested, ranged from 102% to 121%. The ELISA represents an excellent analytical tool for the rapid determination of β-agonists when large numbers of test samples have to be analysed to monitor legislative compliance.

Introduction
Beta-agonists are sympathomimetic amines and are used in veterinary medicine as bronchodilatory and tocolytic agents (Botsoglou and Fletouris, 2001). Their ability to increase the muscle-to-fat body ratio has led to their illegal use as a growth-promoters in livestock production. For use in lean meat production, dosages of 5 – 15 times greater than the therapeutic dosage would be required, together with a more prolonged period of in feed administration, which is often quite near to slaughter, to obviate the elimination problem. Such a use would result in significant residue levels of these compounds in edible tissues of treated animals, which might, in turn exert adverse effects on the cardiovascular and central nervous system of consumers (Martinez Navarro, 1990). Outbreaks of clenbuterol toxicity have been reported (Barbosa *et al*., 2005; Kuiper *et al.,* 1998, Pulce *et al*., 1991, Salleras *et al*., 1995). To protect public health, in the European Union (EU), it is forbidden to place β-agonists on the market for use in farm animals intended for human consumption (EU Council Directive 96/22/1, 1996) and this prohibition is extended to other countries by regulatory agencies. To prevent illegal tissue residues, the primary parameter used by veterinarians is the withdrawal time or the time require for the depletion of the drug from the animal before the animal's meat can be marketed for human consumption. In dairy practice, this is the milk discard time (Riviere and Sundolf, 2009).

Screening for the presence of β-agonists is therefore necessary to monitor regulatory compliance for consumer protection. After screening only samples with positive results need further analysis with a confirmatory method. The importance of screening milk samples is substantiated by the fact that more than 6 billion people worldwide consume milk and milk products. FAO data shows that livestock production is growing rapidly, which is interpreted to be the result of the increasing demand for animal products. Since 1960, milk production has nearly doubled and this is attributed partly to the rise in population, as well as to the increase in affluence in many countries. A joint IFPRI/FAO/ILRI study suggested that global production and consumption of milk will continue to rise, from 568 million metric tons in the year 2000 to 700 million metric tons in 2020 (Speedy, 2003).

The European Commission has specified an MRL of 0.05 ppb clenbuterol in milk (Pulce *et al*, 1991). This MRL has also been accepted by the USA, Japan and Australia. Brazil and Canada have not accepted this MRL for clenbuterol in milk. (Joint FAO/WHO Food Standards Programme, 2001; www.omicusa.com; www.dairyaustralia.co.au, 2012).

This study reports the evaluation of the analytical performance of a new rapid, user-friendly ELISA for the detection of β-agonists in milk.

Materials and Methods

Competitive ELISA
Quantitative determination of the target analyte was carried out using a Beta-agonist Fast Milk ELISA kit (BF3507, Randox Food Diagnostics, Crumlin, UK). The kit components in this competitive ELISA are ready-to-use, with a 96-well breakapart pre-coated microtitre plate, six liquid standards and a working strength conjugate. The assay was performed in accordance with

the manufacturer's instructions, with 100 µL of standard or sample pipetted per well, followed by addition of 50 µL of working strength conjugate per well. The microtitre plate was tapped gently for approximately 30 s to mix the reagents and then was incubated for 30 min at 25°C. After the initial incubation to allow a competitive reaction to take place, the plate was washed to remove excess reagents. The wells were then filled with enzyme substrate and a colorimetric reaction was allowed to develop over 10 min. The intensity of the colour that may consequently develop is inversely proportional to the concentration of the target analyte present in the sample. Stop solution was added after the substrate incubation was complete, to stop the reaction. The resulting colour intensity on the microtitre plate was then read spectrophotometrically using a microtitre plate reader at a wavelength of 450 nm, with a reference wavelength of 630 nm. A 4-parameter curve fit method was applied for generation of the standard curve.

Milk Sample Preparation
Minimal sample preparation is required before testing of milk samples can take place and as a result, data can be obtained rapidly, without the need for complicated extraction processes. Chilled milk samples are prepared for analysis by carrying out a single centrifugation step before the sample is applied directly to the microtitre plate. Typically, forty samples can be analysed in less than 1 h when run in duplicate. The sample volume required for each well is 100 µL.

Analytical parameters

Calibration curve. The calibration curve was generated using a 4-parameter curve fit.

Limit of detection. The limit of detection (LOD) for clenbuterol was calculated as the mean concentration plus 3 standard deviations from the data for 24 negative milk samples. This represents the lowest concentration of analyte that can be distinguished due to matrix effects.

Recovery. Negative milk samples (n=3) were fortified with three concentrations of clenbuterol. The three levels of fortification spanned the calibration range of the assay. The percentage recovery of each fortified sample was calculated as follows:
Recovery (%) = (assay concentration (ppb) / fortification level (ppb)) x 100%.

Specificity / Cross-reactivity. Specificity, expressed as cross-reactivity (CR) % was calculated as follows:
CR (%) = [(IC_{50} (clenbuterol) / IC_{50} (cross-reactant)] x 100%.

The half maximal inhibitory concentration (IC_{50}) for each analyte and cross-reactant tested, was calculated by taking 50% of the absorbance of the zero standard and reading this value from the x-axis (ppb) of the corresponding standard curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Intra-assay Precision. Intra-assay precision was determined by running six concentration levels across four different microtitre plates. Results were expressed as CV (%).

Results
After application of a 4-parameter curve fit method, the standard curve for clenbuterol showed a correlation coefficient, r > 0.99. The typical calibration range of the assay was of 0-2.5 ppb (Figure 1).

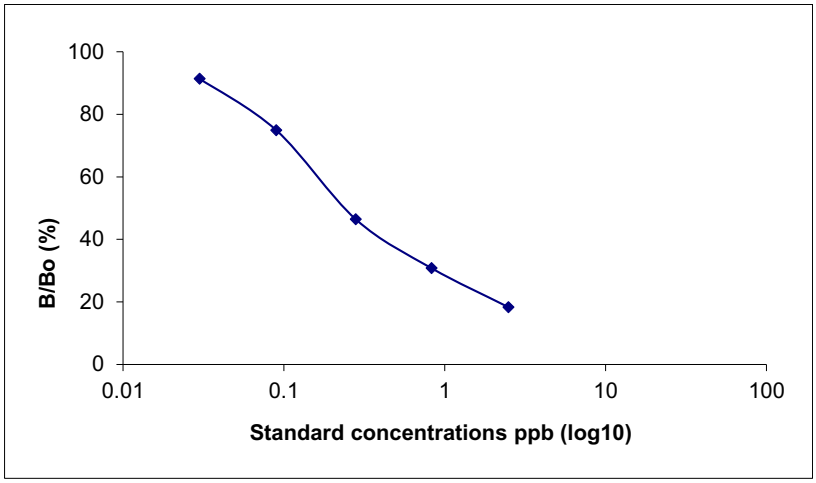

Figure 1. Beta-agonist fast ELISA: typical standard curve for clenbuterol.

Limit of Detection
The LOD for clenbuterol in milk was determined as ≤ 0.1ppb.

Recovery.
The mean recovery (%) from three negative milk samples fortified with three different concentration levels of clenbuterol were in the range 102% to 121% (Table 1).

Table 1. Beta-agonist Fast ELISA and its mean recovery.

Sample number	Mean % Recovery
1	102
2	110
3	121

Specificity
The assay presented a cross-reactivity of 100% for clenbuterol, with other β-agonist compounds also detected (Table 2).

Table 2. Beta-agonist fast ELISA: Specificity data.

Analyte	% Cross-Reactivity
Clenbuterol	100
Cimbuterol	125
Salbutamol	115
Tulobuterol	97
Bromochlorbuterol	86
Brombuterol	75
Carbuterol	56
Mabuterol	50
Clenpenterol	33
Terbutaline	33
Mapenterol	30
Pirbuterol	28
Penbutolol	6
Cimaterol	5
Clenproperol	5
Clorprenaline	2
Hydroxymethyl clenbuterol	1
Fenoterol	<1
Zilpaterol	<1
Deisozilpaterol	<1
Ractopamine	<0.0125

Intra-assay Precision
The intra-assay precision for six concentration levels, expressed as CV (%) was <9%-(Table 3).

Table 3. Beta-agonist fast ELISA: intra-assay precision.

Plate	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
	CV(%)					
1	4.3	6.3	4.1	4.1	3.4	5.4
2	5.3	5.2	3.6	4.6	4.7	8.8
3	6.5	5.8	6.9	4.7	3.4	4.8
4	6.3	4.8	3.8	3.4	3.2	6.1

Discussion
The results indicate optimal analytical performance of this user-friendly Beta-agonist Fast Milk ELISA, for the rapid screening of β-agonists in milk samples. The ready-to-use reagents, along with the simple one-step sample preparation method and short incubation times, minimises the time required to generate results. Less than 1 h for 40 samples run in duplicate. This does not compromise the assay precision as the CV (%) values were typically < 9% for different concentration levels. The mean recovery was in the range, 102% –121%. The combination of ready-to-use components, short incubation times and low limit of detection (≤0.1 ppb) make this ELISA a cost-effective, robust, precise, analytical tool for the screening of β-agonist drugs in milk when a large number of test samples have to be analysed to monitor legislative compliance.

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FUMAGILLIN AND DICYCLOHEXYLAMINE IN APICULTURE

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Abstract
Fumagilin-B® is currently the only registered chemical treatment to combat microsporidian fungal infections by *Nosema apis* and *N. ceranae*. The commercial formulation of fumagillin, Fumagilin-B®, contains the biologically active fumagillin as a salt, with dicyclohexylamine (DCH) being the counter-ion. DCH exhibits genotoxic and cytotoxic properties, and may be of importance to human health when present as a residue in honey and other hive products. An analytical method was developed to identify and quantitate DCH and fumagillin (along with relevant degradation products of fumagillin) in honey. DCH appears to be significantly more stable than fumagillin in honey, making DCH a much good marker residue that can be used to monitor fumagillin usage in apiculture. DCH also represents a significant residue in honey compared to fumagillin, since DCH is reportedly almost five times as toxic as fumagillin.

Introduction
Fumagillin (Figure 1) is a fungal metabolite first isolated from *Aspergillus fumigatus* (Hanson and Eble 1949). Fumagillin was successfully used to control the microsporidian fungal disease caused by *N. apis* (Katznelson and Jamieson 1952, Bailey 1953). A related, emerging, but epidemiologically different disease caused by *N. ceranae* is implicated in the large-scale loss of bee colonies worldwide (Cox-Foster *et al.* 2007; Higes *et al.* 2008, Higes *et al.* 2009, Higes *et al.* 2010; VanEngelsdorp *et al.* 2009; Fries 2010; Botías *et al.* 2012; Martínez *et al.* 2012). Infections by *N. ceranae* and *N. apis* are collectively referred to as nosema disease. Fumagillin being the only currently available effective chemical treatment against this parasite (Williams *et al.* 2008; Williams *et al.* 2011; Higes *et al.* 2011).

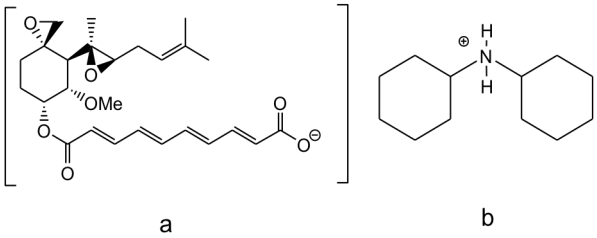

Figure 1. Chemical structure representation of fumagillin (a) as dicyclohexylamine salt, DCH (b) in the commercial formulation Fumagilin-B®.

The commercial formulation of fumagillin, Fumagilin-B® (Medivet Pharmaceuticals Ltd., High River, Alberta, Canada, DIN 02231180), consists of a salt of fumagillin, the counter-ion of this salt being DCH in a 1:1 stoichiometric ratio with fumagillin (Figure 1). The importance of recognizing DCH as a possible contaminant having potential human health and food safety implications for consumers of honey was recently recognized (van den Heever *et al.* 2014), with DCH reportedly being at least five times more toxic than fumagillin when tested on rats. Recently, a maximum residue limit (MRL) of 25 ng g^{-1} was established for fumagillin in Canada. To the best knowledge of the authors no MRL exists for DCH in honey or other hive products. It should be noted that DCH could also originate from environmental sources such as refineries and other related industries, since it is used as a corrosion inhibitor and as vulcanization additive in vehicle tires to name but a few. The significance of these possible environmental sources of DCH, as it relates to apiculture, needs to be investigated.

Current analytical methods describe the analysis of fumagillin and its biologically active UV degradation products, but none recognize the importance of DCH as an analyte (Kochansky and Nasr 2004; Kanda *et al.* 2011; Dmitrovic and Durden 2013), whereas thermal decomposition of fumagillin yields a biologically inactive compound. The lack of an analytical method to accurately quantitate DCH (Figure 1) in honey destined for human consumption, led to the development of this LC-MS/MS method. Roxithromycin was effectively used as an internal standard for fumagillin, but not for DCH, owing to analyte specific matrix effects observed for DCH only. This problem was overcome by the synthesis of the deuterium labelled analogue of DCH (Figure 2).

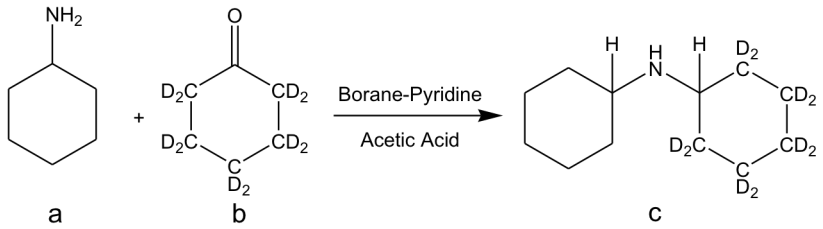

Figure 2. Chemical structure representation of the reaction of cyclohexylamine (a) with D_{10}-cyclohexanone (b) to afford the D_{10}-DCH (c) which is used as an internal standard for DCH analysis

Materials and Methods

The synthesis of the internal standard, D_{10}-DCH, was carried out in accordance with published methodology (Pelter *et al.* 1984), and the final product was isolated as the hydrochloric acid salt in 60% yield (Figure 2). The two internal standard working solutions (D_{10}-DCH and Roxithromycin) were prepared separately at concentrations of 5 µg mL^{-1} in acetonitrile for roxithromycin, and in methanol for D_{10}-DCH in methanol. A combined internal standard working solution consisting of roxithromycin (20 µg mL^{-1}) and D_{10}-DCH (300 µg mL^{-1}) was then prepared in acetonitrile. This internal standard mixture (50 µL) was then added to all sample and matrix matched calibration standards.

The fumagillin standard working solution (free from DCH) was prepared at 5 µg mL^{-1} in acetonitrile. Similarly, a DCH standard working solution at 5 µg mL^{-1} was prepared in methanol using the nitrite salt of DCH (since pure DCH is in liquid form and is volatile and difficult to handle safely and accurately in the laboratory as an analytical standard). Matrix-matched calibration standards were spiked at 10, 20, 50, 100, 200 (QC) and 500 ng g^{-1} respectively in honey. Care was taken not to expose the samples or standards to fluorescent or other light sources, which would cause or accelerate decomposition of fumagillin. Honey (5 g) was therefore weighed into amber coloured 50-mL centrifuge tubes, followed by addition of the internal standards (and standards for calibration curves). Distilled water (10 mL) was added, and the tubes vortexed for approximately 20 s, followed by shaking on a mechanical shaker for one h. The tubes were briefly centrifuged at 2,964 rcf for 5 min in order to improve the flow through the SPE cartridge by isolating any suspended solid particles in the diluted honey. Strata X (Part 8B-S100-FCH; 33 µm; 200 mg; 6 mL) polymeric reversed phase extraction cartridges (Phenomenex, Torrence, CA) were conditioned by sequential elution with methanol (5 mL) and water (5 mL) at a flow rate of 1 mL min^{-1}. The contents of the amber centrifuge tubes were then run through the SPE cartridges at a flow rate of 1 mL min^{-1}, with the waste being discarded. The SPE tubes were then washed once with 10 mL of a water/methanol solution (7:3 v/v) at a flow rate of 1 mL min^{-1}. The filtrate was discarded, and the SPE tubes were dried by pulling air through them for 5 min at a higher vacuum than that used for elution. Clean collection tubes were then placed in the vacuum manifold and the analytes of interest were eluted off the SPE cartridges by using 2 mL of acetonitrile containing 5% of formic acid (using vacuum to remove all possible eluent from the SPE tubes). The eluent was transferred into an amber 1.5 mL sample vial for analysis, without any further processing, using an injection volume of 10 µL.

A 1.0 M ammonium formate solution was prepared in distilled water. Mobile phase A was prepared by adding 2.0 mL of the 1.0 M ammonium formate solution and 100 µL of formic acid into a 1,000-mL volumetric flask, and diluting to volume with distilled water. Mobile phase B was prepared by adding 2.0 mL of the 1.0 M ammonium formate solution, 100 µL of formic acid and 100 mL of methanol into a 1,000-mL volumetric flask, followed by dilution to volume with acetonitrile. The mobile phase flow was kept constant at 0.250 mL min^{-1} with the column temperature at 30°C. The initial conditions were 90% mobile phase A, linearly changing to 25% A over 5 min. This condition was maintained for a further 15 min, followed by a linear return to 90% A over 5 min. The final condition was held for 5 min, for a total runtime of 30 min. Injection volumes of 5.0 µL were used for all standards and samples. Sample temperature was maintained at 20°C.

The liquid chromatography system used was a Waters 2695 separations module in tandem with a Waters Quattro Ultima Pt mass spectrometer. The column used for this analysis was a Waters Xterra MS-C18 with column internal dimensions of 4.6 x 100 mm with 3.5 µm packing particle size (Waters; Milford; MA), protected by a matching 4 x 2.0 mm guard column (Phenomenex; Torrance; CA, USA). The MS source temperature was set as 120°C, and the desolvation gas temperature was 350°C at a flow of 725 L h^{-1}. Cone gas flow was set at 55 L h^{-1} and a capillary voltage of 3.5 kV was used. Collision gas used was argon at a pressure of 2.7 10^{-3} mbar. All parameters and focussing potentials were adjusted for optimum detection of the protonated molecular ions $[M+H]^+$ and collision fragments for all compounds and internal standards of interest. Multiple reaction monitoring (MRM) chromatograms were recorded in positive electrospray mode (ES+) for all compounds. A full account of this method, including the validation data and MS/MS transitions used, can be found elsewhere (Van den Heever *et al.* 2015). After completion of method validation, sixteen domestically produced honey samples (for which the origin and history were not known) were randomly selected from a pool of samples available in our laboratory and analysed for fumagillin and DCH, and screened for UV-decomposed fumagillin.

Results and Discussion

Method development sample recoveries (±RSD%) were calculated for the 18 validation replicate samples at 10 ng g^{-1} (105.7±10.8), 100 ng g^{-1} (100.8±9.3) and 500 ng g^{-1} (104.3±8.7) for fumagillin, and at 10 ng g^{-1} (104.0±5.9), 100 ng g^{-1} (98.3±6.9) and 500 ng g^{-1} (104.0±9.7) for DCH. The linearity fit (R^2) was observed to be greater than 0.995 for both DCH and fumagillin over the linear range of 10-500 ng g^{-1} for all instances. Limits of detection (LOD) were calculated as 1.2 ng g^{-1} for fumagillin and as 0.24 ng g^{-1} for DCH, using the signal-to-noise criteria of 3:1. A quantitation limit (LOQ) of 10 ng g^{-1} was chosen, based on the lowest matrix-matched calibration standard, which is well above the calculated LOQ of 4.03 ng g^{-1} for fumagillin and 0.79 ng g^{-1} for DCH.

No UV-decomposed fumagillin could be detected during screening of the 16 samples. Fumagillin was detected and quantitated in 2 samples at levels above the LOQ at concentrations of 11.9 and 11.6 ng g^{-1}, respectively. No traces of fumagillin were detected in 5 samples, however it was detected at concentrations below 10 ng g^{-1} in 9 samples. DCH was detected in all of the 16 samples, with only 1 sample having a concentration lower than 10 ng g^{-1}. Observed DCH concentrations above the LOQ of 10 ng g^{-1} ranged from 20.0 to 234.6 ng g^{-1}(median value of 49.4 ng g^{-1}). A summary of these results is given in Table 1.

Table 1. Results of 16 random domestic honey samples analysed for fumagillin and DCH. Limits of quantitation (LOQ) were set as 10 ng g^{-1} and higher for both fumagillin and DCH, while limits of detection (LOD) was calculated as 1.2ng g^{-1} for fumagillin, and as 0.24 ng g^{-1} for DCH. The presence or absence resulting from the screening for the UV decomposed fumagillin is indicated as detected (D) or not detected (ND), while "Trace" denotes levels greater than the LOD but below the LOQ of fumagillin and DCH.

Sample Number	Fumagillin (ng g^{-1})	DCH (ng g^{-1})	UV-decomposed fumagillin (ng g^{-1})
1	11.6	234.6	ND
2	<LOD	20.0	ND
3	Trace	116.4	ND
4	Trace	72.8	ND
5	11.9	124.4	ND
5	Trace	76.8	ND
7	Trace	116.0	ND
8	<LOD	Trace	ND
9	Trace	59.6	ND
10	<LOD	21.7	ND
11	<LOD	39.1	ND
12	<LOD	28.8	ND
13	Trace	64.5	ND
14	Trace	28.9	ND
15	Trace	27.5	ND
16	Trace	25.9	ND

It should be noted that Fumagilin-B® may not the only source of DCH residues in honey and other agricultural products. Other potential sources of DCH could be related to environmental contamination associated with industrial activity (vehicle antifreeze, corrosion inhibitor, rubber additive, etc.), and needs to be investigated in order to establish whether this is of any real concern for human or bee health (Woldegiorgis *et al.* 2007; Brorström-Lundén *et al.* 2011).

Conclusions

The high frequency of detection of DCH in the domestically produced samples at concentrations above the LOQ of 10 ng g^{-1} suggests that DCH is significantly more stable than fumagillin (or its UV-decomposition products) in honey. The fivefold higher toxicity of DCH which is being applied in equimolar quantities to fumagillin when using Fumagilin-B®, makes this a potential important contaminant of honey and other hive products. DCH is also clearly a much better marker residue than fumagillin itself, when evaluating Fumagilin-B® usage in apiculture. If environmental contamination with DCH is indeed significant, it could have an impact on apicultural and other agricultural activities located in close proximity to busier major roadways and industrial centres, including oil refineries. The effect of DCH on honey bees at various stages of development also needs to be studied, because the somewhat lipophilic nature of DCH, having an estimated log K_{ow} value of between 3.5 to 4.37 and solubility of 800 mg L^{-1} in water at 25°C (Brorström-Lundén *et al.* 2011) indicate that there is a high probability that DCH could accumulate in wax. The close proximity of developing bee larvae to the comb wax may then make them more susceptible to the genotoxic effects of DCH.

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IMMUNOCHEMICAL DETECTION OF TOTAL PENICILLINS BY USING BIOHYBRID MAGNETIC PARTICLES

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Abstract

An enzyme-linked Immunosorbent assay (ELISA) with the ability to detect some of the most widely used penicillins, such as amoxicillin and ampicillin, was developed and validated for milk samples. The assay can achieve limits of detection much lower than the MRLs (maximum residue levels) established by the EU. The procedure allows the analysis of samples with a simple and fast treatment of the matrix with biohybrid magnetic particles hydrolysing the β-lactam ring. A new immunizing hapten was described and polyclonal antibodies have been produced showing high specificity for open ring penicillins and no cross-reactivity with the other antibiotic families. The assay has been designed to detect simultaneously both penicillin species in milk, the antibiotic itself and their hydrolysed form. Concerning the open-ringed forms their detection in food samples will be especially important in the next few years because of the illegal use of penicillinase enzymes by farmers to mask the real concentrations of β-lactams in milk.

Introduction

Since Alexander Fleming discovered penicillin in 1928, antibiotics have been widely used to treat humans against bacterial infections. Nowadays they are prescribed to treat mastitis, otitis, pharyngitis, lung infections or skin and organ abscesses. The most widely used are β-lactams due to their broad-spectrum and limited side effects. They include penicillins, cephalosporins and carbapenems and represent 65% of the world antibiotic market (1). Antibiotics are also used in veterinary medicine. Veterinarians and farmers need to control livestock diseases, but sometimes they use more antibiotic than strictly required (*i.e.* prophylaxis).

Most of the applied drugs are metabolized and excreted (2), but in larger amounts they may be accumulated in tissues and finally appear in meat (3), eggs (4) or milk (5,6). The presence of residues has been related to many undesirable consequences, with bacterial resistance being one of the main problems. Antibiotics in our dairy food favour the natural selection towards bacteria which are resistant to those drugs. Concerning β-lactams, organisms are capable of mutation and have developed different mechanisms to resist their action. Examples of this are production of β-lactamases, modification of penicillin-binding protein sites or blocking the β-lactam entry into the bacteria (7).

Other problems caused by antibiotics are the ADRs (adverse drug reactions) which include diarrhea, rash, urticaria and hypersensitivity, symptoms that can lead to an anaphylactic shock in some cases. The risk of an allergic reaction to penicillins (type I hypersensitivity reaction) has been extensively described and reported (8,9). In addition, there is a significant economic risk associated with penicillin residues, as these may inhibit the milk fermentation process for the production of yogurt and cheese. Antibiotics affect the processing efficiency (10,11) leading to many losses for this industry. According to European Medicines Agency, the maximum concentration in milk that inhibits starter cultures and delays acid production is not less than 6 μg kg^{-1} for penicillin (12). Furthermore, antibiotics are also surface water contaminants (13,14) stimulating bacterial resistance in the environment (15). Adding to all of these problems, some dairy producers dope the milk illegally with the so-called "antimicrobial destroyer". This means the addition of β-lactamases to the milk in order to cleavage the penicillin ring to avoid the current legislation (16-18). But unfortunately, these degradation products, called penicilloic acids, are not entirely free from causing severe ADRs (19, 20).

All of the undesirable consequences related to residue accumulation are pushing forward the need for high quality and ecological food. For years, great efforts have been made concerning food safety in order to develop sophisticated and robust methods to detect residues in different matrices. Global organizations have classified pharmacologically active substances depending on their safety (21). As a result, several regulations have been established in order to control the exposure to these compounds (22). For non-illegal, prohibited or highly toxic substances, the EU set MRLs (maximum residue limits) for veterinary medicinal products in foodstuff of animal origin. Concerning penicillins, they have a MRL of 50 μg kg^{-1} for meat and 4 μg L^{-1} for milk (Commission regulation 37/2010/EU for amoxicillin, ampicillin and penicillin G). These levels are lower than in many countries including USA, Canada and Japan. Even though the penicilloic acid metabolite is considered to be significant

with regards to hypersensitivity reactions, the MRLs for these antibiotics refer to the parent compounds only, with no control of the metabolite levels.

Regarding β-lactam antibiotics, there are several methods for their determination in milk and feed samples (23), which include chromatographic techniques (24) and bioassays (7). The only ones accepted by the EU are chromatography techniques (25). Although they provide specific and quantitative analysis, they are not suitable for rapid screening of a large amount of samples, even though Directive 96/23/EC establishes that at least 0.4% of the foodstuff produced must be analysed. Consequently, new quantitative and high-throughput screening methods need to be developed, including microbial assays, rapid tests, immunochemical assays and biosensors (7). Concerning the immunoassays that have been reported lately, none of them are able to detect amoxicillin below the established MRL. Some immunoassays can detect other penicillins below that value (26, 27) but they are not broad-specific assays, showing low cross-reactivities with the most widely used penicillins. On the other hand, concerning the β-lactamase treatment of contaminated milks, there are some methods for its detection (28) including immunoassays and spectrometric techniques. They require technical manipulation and are time-consuming. Moreover, those assays detect the concentration of enzyme but not the quantity of penicilloic acid produced.

Materials and Methods

Buffers

Unless otherwise indicated, phosphate buffer saline (PBS) is 0.01 M phosphate buffer in a 0.8% saline solution, pH 7.5. Coating buffer is a 0.05 M carbonate-bicarbonate buffer, pH 9.6. PBST is PBS with 0.05% Tween 20, pH 7.5. PB2T is 0.01 M phosphate buffer with 0.1% Tween 20, pH 7.5. Citrate buffer is 0.04 M sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3',5,5'-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer. Borate buffer is 0.2 M boric acid/sodium borate, pH 8.7. Storage buffer is 0.1 M Tris HCl with 0.1% BSA, pH 7.0. Carbonate buffer is 0.01 M carbonate-bicarbonate buffer, pH 10. Deuterated carbonate buffer was prepared by lyophilizing the carbonate buffer and dissolving the salts with an equal volume of deuterated water (D_2O).

Preparation of the immunizing hapten AX-N (Figure 1)

(a) Synthesis of 4,4'-dithiodibutyroate succinimide (3). 4,4'-dithiodibutyric acid (500 mg, 2 mmol) was dissolved in 10 mL dioxane anh. Solutions of NHS (500 mg, 4.4 mmol) in 6 mL dioxane and DCC (900 mg, 4.4 mmol) in 4 mL dioxane were added dropwise. The reaction was gently stirred at RT for 4 h. A white solid was observed. The mixture was filtered and concentrated. The final product was crystallized in acetone:Et_2O 1:1 (0.626 g, 73% yield).

(b) Synthesis of penicillinic dimer AX-N (4). In a round-bottomed flask was added a suspension of amoxicillin (548 mg, 1.5 mmol) in 10 mL ACN anh. under argon atmosphere. Next, Et_3N (0.421 mL, 3 mmol) was added and the total solubility of the antibiotic was observed. The succinimide ester 3 (185.8 mg, 0.43 mmol) was dissolved in 4 mL ACN and the resulting solution was added to penicillin. The reaction was stirred for 2 h at RT protected from the light. The mixture was evaporated and a saturated sodium bicarbonate solution was added to dissolve the crude. The aqueous solution was washed with AcOEt, acidified to pH 3 with HCl, and the final compound was extracted with AcOEt. The solvent was dried with $MgSO_4$, filtered and evaporated to obtain the final compound with 53% yield.

Preparation of the immunogen AX-NBr-HCH (Figure 1)

(a) Reduction of the disulfide bond. Di(*n*-butyl)phenylphosphine polystyrene (DBPP) resin (25 mg, 12.5 µmol of reducing agent) was added to a solution of the dimer (10 µmol) in 160 µL DMF anh. under argon. The reaction mixture was gently stirred at RT for 2 h. Next, MilliQ water (40 µL) was added to the suspension, which was stirred for 1 h at 50°C until the complete reduction of the disulfide bond was observed by HPLC. The suspension was filtered with a 0.45 µm porous size filter. The retained resin was washed with MilliQ water (2·120 µL) and the collected fractions were combined and used immediately for conjugation with the modified protein (step c).

(b) Activation of the protein. Simultaneously with the first step, a solution of 20 µmol of the cross-linker N-SBr (1) in 100 µL DMF anh. was added dropwise to a solution of 20 mg of protein (horseshoe crab hemocyanin, HCH) in 1.5 mL PBS. The reaction mixture was stirred at RT for 2 h and then purified by passing the solution through a Sephadex G-25 desalting column and eluting with PBS buffer. The fractions containing the modified protein were collected and combined.

(c) Hapten coupling to the protein. The reduced hapten obtained in the first step was added to the derivatised protein prepared in step b. The final amount of DMF did not exceed 10% to avoid protein denaturing. The reaction mixture was stirred for 2 h at RT and the conjugate was purified by passing the solution through a Sephadex G-25 desalting column eluted with water. Fractions were combined and lyophilized.

Preparation of the competitor PG-A-AD (Figure 1)
Penicillin G (PG) (10 µmol) was dissolved in 140 µL DMF anh. Then, a solution of DCC (50 µmol) in 30 µL DMF and a solution of NHS (25 µmol) in 30 µL DMF were added sequentially. The mixture was stirred for 1 h until a white precipitate appeared. The suspension was centrifuged at 10,000 rpm for 10 min. The supernatant was added dropwise to a solution of 10 mg of aminodextran (AD) in 1.8 mL PBS. The reaction mixture was gently stirred for 3 h at RT. The protein conjugate was purified by dialysis against 0.5 mM PBS (4 × 5 L) and Milli-Q water (1 × 5 L), and lyophilized.

Polyclonal antisera
The antisera (As) obtained by immunizing female white New Zealand rabbits with AX-NBr-HCH were named As217, As218 and As219. Evolution of the antibody titre was assessed on a non-competitive indirect ELISA, by measuring the binding of serial dilutions of the different antisera to microtiter plates coated with a fixed concentration of antigen (1 mg mL^{-1}). After six immunizations, the animals were exsanguinated, and the blood was collected in vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation at 4°C for 10 min at 10,000 rpm, and stored at -80°C in the presence of 0.02% NaN_3. Unless otherwise indicated, working aliquots were stored at 4°C.

Conjugation of biohybrid magnetic particles
Penicillinase (β-lactamase from *Bacillus cereus*) was covalently coupled to magnetic particles (MP) following a similar protocol supplied by the manufacturer. The MP (15 mg, 150 µL) were washed twice with borate buffer using magnetic separation. Then, the particles were suspended in a solution of 7 mg of the enzyme in borate buffer (200 µL) (Figure 2). Next, a solution of 3 M $(NH_4)_2SO_4$ in borate buffer (100 µL) was added, and the mixture was stirred for 24 h at RT light protected. The supernatant was removed with the help of the magnet and analyzed by the Bradford protein test to estimate the efficiency of the coupling. The penicillinase stock solution (40 mg mL^{-1}) and the supernatant were interpolated to a calibration curve of BSA. Next, a solution of 0.5 M ethanolamine in borate buffer (200 µL) was added followed by a solution of 3M $(NH_4)_2SO_4$ in the same buffer (100 µL). The mixture was stirred again, and left for 24 h at RT light protected. Finally, the MPs were washed twice with 1 mL of the storage buffer, resuspended in the same buffer (1 mL) and stored at 4°C. The final concentration of MP-penicillinase was 15 mg mL^{-1}, showing a bioconjugation yield over 70%.

Enzymatic synthesis of penicilloic acids
The enzymatic hydrolysis was made by mixing 20 µL (7.5 mg mL^{-1}) of biohybrid MPs with a solution of 0.01 mmol of the penicillin in 1 mL carbonate buffer, during 10 min at RT by mechanical agitation (Figure 2). The solution was magnetically separated obtaining the corresponding penicilloic acid quantitatively. Characterization details of all the open-ringed penicillins were made with deutered carbonate buffer and are provided in the supporting information.

Competitive ELISA PG-A-AD/As218
Microtiter plates were coated with the antigen PG-A-AD (0.5 µg mL^{-1} in coating buffer, 100µL/well), overnight at 4°C and covered with adhesive plate sealers. The next day, milk samples (with or without enzymatic treatment) or standard curve (amoxicilloic acid from 2,000 to 0.0128 nM in milk) were added to a dilution plate (80 µL/well) followed by the antiserum As218 (1/4,000 diluted in PB2T, 80 µL/well), and pre-incubated for 1 h at RT with gentle shaking. The coated plates were washed four times with PBST (300 µL/well), and the content of the dilution plate was transferred to the coated plate (100 µL/well), which was incubated for 1 h at RT under shaking. The plates were washed as before, and a solution of anti-IgG-HRP (1/6,000 in PBST) was added to the wells (100 µL/well) and incubated for 30 min at RT. The plates were washed again, and the substrate solution was added (100 µL/well). Color development was stopped after 30 min at RT with 4 N H_2SO_4 (50 µL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four-parameter equation according to the following formula: $y = (A - B/[1 - (x/C)D] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

Milk experiments
(a) Sample Treatment. Biohybrid magnetic particles (20 µl, 7.5 mg mL^{-1}) were added to whole milk samples (1 mL). The mixtures were shaken 10 min at RT and then magnetically separated (1 min).

(b) Robustness. Minor changes of the sample treatment were tested by introducing variations in the quantity of penicillinase (0.1 to 5 µg) and in the time required (2 to 60 min) for the enzymatic hydrolysis of the penicillins. The best results were selected for following assays.

(c) Matrix Effect Studies. Non-specific interferences produced by the milk were assessed by preparing standard curves at different milk dilutions (1, 1/5, 1/10 and 0) with a synthetic buffer mimicking the physicochemical parameters of the milk (40 mM phosphate buffer, pH 6.6 and 4.9 mS cm^{-1}).

(d) Specificity studies. Solutions of different penicillins (AX, AP, PG, PV), and other antibiotics including cephalosporins, tetracyclines, fluoroquinolones and sulphonamides, 10 mM in whole milk (1 mL) were treated with penicillinase immobilized onto magnetic particles (10 µL). The standard curves were performed following the protocol described before. The cross-reactivity (CR) values were calculated according to the equation: IC_{50} [nM] (Penicilloic acid)/ IC_{50} [nM] (cross-reactant) × 100.

(e) Recovery studies. The recovery of the analyte concentration after the sample treatment was assessed by spiking blank milk samples in triplicates at six different concentrations (from 1 to 60 µg L^{-1}). The sample concentrations were calculated interpolating the results to amoxicillin standard curves prepared in blank whole milk. The results were fitted to a linear regression curve between the spiked concentrations and the measured ones.

(f) Reproducibility studies. The assays were carried out thrice on three days, with three replicates for each one. The main features of the final assay were described as the mean of all the replicates.

(g) Decision limit (CCα) and detection capability (CCβ). Both parameters are defined by Commission Decision 2002/657. CCα and CCβ values were calculated in terms of absorbance and the values obtained were interpolated in the appropriate calibration curve. CCα was calculated as $CC\alpha_{abs}$ = $Blank_{abs}$ - 2.33SD, where $Blank_{abs}$ is the average signal of 20 blank milk samples and SD is its standard deviation. CCβ was calculated as $CC\beta_{abs}$ = $CC\alpha_{mean}$ - 1.64SD, where $CC\alpha_{mean}$ is the average of 20 milk samples spiked at CCα concentration and SD is its standard deviation. Analyses were performed thrice on different days.

(h) Ruggedness. The ruggedness of the method was tested using different milk samples from different brands. The samples were spiked with amoxicillin at the MRL level (4 µg L^{-1}) and analyses were performed three times.

Results and Discussion

The main objective of this work was the development of an immunoassay able to detect specifically low concentrations of penicillin-contaminated milk samples. In fact, during the last decade a lot of attempts have been made, but the antibodies obtained were never sufficiently good. Probably the explanation for such poor selectivity and sensitivity against these antibiotics is due to the chemical reactivity of the β-lactam ring (29). Several examples can be found in the literature, however, none of them reached the MRLs established by the EU. Lately, some authors thought to raise antibodies against the open-ringed form of the β-lactams. These compounds are the main degradation products of penicillins, and probably they must be found in milk when it has been spiked with β-lactamases. This strategy avoids the direct detection of penicillins, assuming that there is a direct correlation between the concentrations of the closed and the open-ringed forms. This methodology has been used by several authors, but it was Grubelnik *et al.* (29) who produced the first antibody against the penicillinoic acid with very good detection limits.

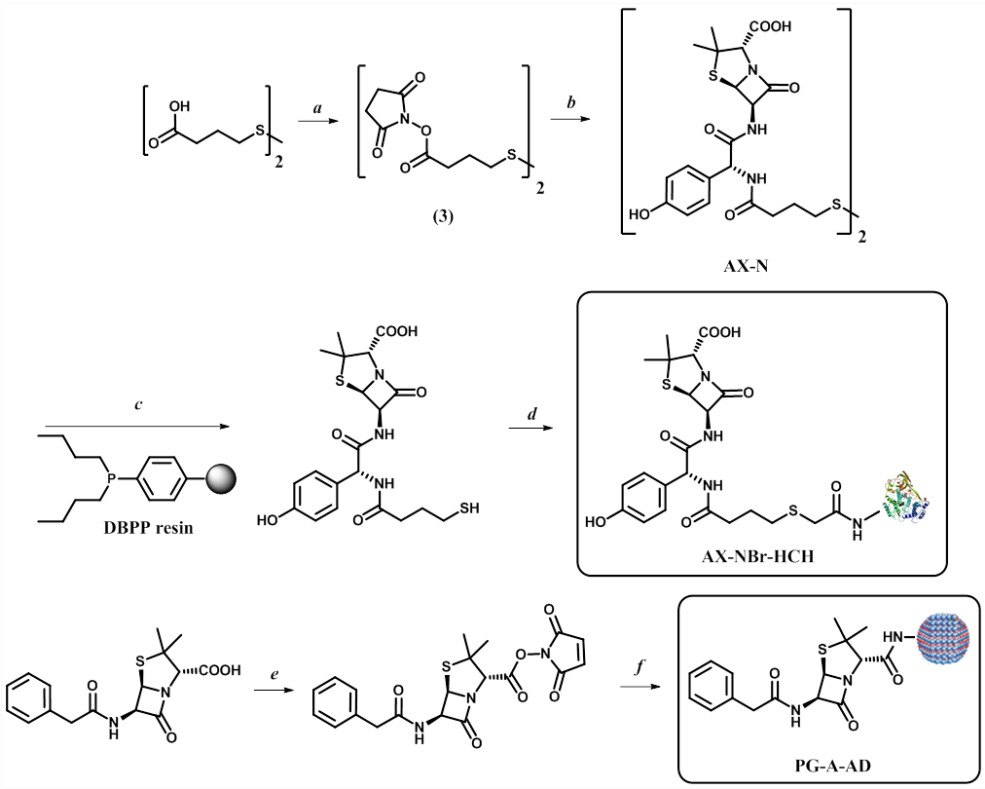


Figure 1. Synthetic pathway used to prepare the immunogen (AX-NBr-HCH) and competitor (PG-A-AD). (a) NHS, DCC, dioxane, 4h; (b) Amoxicillin, Et_3N, ACN, 2h, light protected; (c) H_2O, DMF, 2h; (d) NHS, DCC, CH_2Cl_2, 4°C, 3h; (e) DCC, NHS, DMF, 1h; (f) AD, PBS, 3h.

Preparation of the immunoreagents

Initially, we focussed our efforts to obtain antibodies against the common part of the penicillins, the β-lactam ring. In most cases, the production of amoxicillin-specific antibodies has been done using either a glutaraldehyde or a direct active ester strategy. However, it has been demonstrated that they do not provide well-characterized haptens nor highly specific antibodies (26). Our idea consisted on the design and synthesis of a new immunizing hapten able to maximize the exposure of the β-lactam structure to the immune system. Following chemical criteria, we decided to introduce the spacer arm of the hapten through the amino group of the amoxicillin, adding a thiol group at the end of the spacer in order to avoid possible side reactions in the conjugation step with the acid group of the penicillins.

The synthesis of the immunogen hapten AX-N is shown in Figure 1, following the procedure described by Estevez *et al*. (30). Thus, the strategy was based on the preparation of a dimer of amoxicillin through a disulfide bridge using the active ester of the 4,4'-dithiodibutyric acid. This methodology keeps the thiol group protected from oxidations, while their reduction under mild conditions allows obtaining two hapten molecules at the same time. The next step was the synthesis of the immunogen by coupling the hapten to hemocyanin HCH (Figure 1). First, the disulfide bridge was reduced with a phosphorous resin (DBPP resin). Then, the thiol groups obtained were able to react by nucleophilic substitution with the protein, which was activated with the heterobifunctional linker N-SBr, to obtain the final immunogen AX-NBr-HCH.

Immunoassays for low-molecular weight analytes work under competitive conditions. It has often been reported (31) that the feasibility and selectivity of the antibodies can be modulated with the competitor or coating antigen (Ag). Thus, a correct selection of the competitor is crucial for the final features of the assay. In our case, the selected competitor was prepared through the acid group of the penicillin G to aminodextran (AD) (Figure 1) using the active ester coupling strategy.

Immunoassay development

The conjugate AX-NBr-HCH was used for immunizing three New Zealand female rabbits, whose antisera were named As217, As218 and As219. Each antiserum was evaluated with the coating antigen (Ag), and the best combinations As-Ag were used in the competitive assays using amoxicillin as the analyte. However, in none of the combinations the inhibition of amoxicillin was observed. Again, the strategy to obtain antibodies against the β-lactam moiety failed. Two possible causes could explain this behaviour assuming the strategy selected was correct. Firstly, the immunogen hapten was not well characterized or conjugated, or the immunogen structure was transformed at some point.

Because the antisera showed affinity for almost all of the conjugates (data not shown), our hypothesis was focused on the possible degradation of the β-lactam ring of the immunogen, either in the conjugation step to the protein or in the host animal. In fact, it is described in the literature that at basic pHs the β-lactam ring can be hydrolyzed to the corresponding penicilloic acid (32). In order to check this assumption, all the immunoreagents were tested again using amoxicilloic acid as the target analyte. Several procedures have been reported in order to obtain the open-ringed forms of the penicillins. The most common is by alkaline hydrolysis, but this strategy produces a lot of byproducts difficult to separate (33). The other common procedure is by means of an enzymatic approach using a β-lactamase (*i.e*. from *Bacillus cereus*) (32).

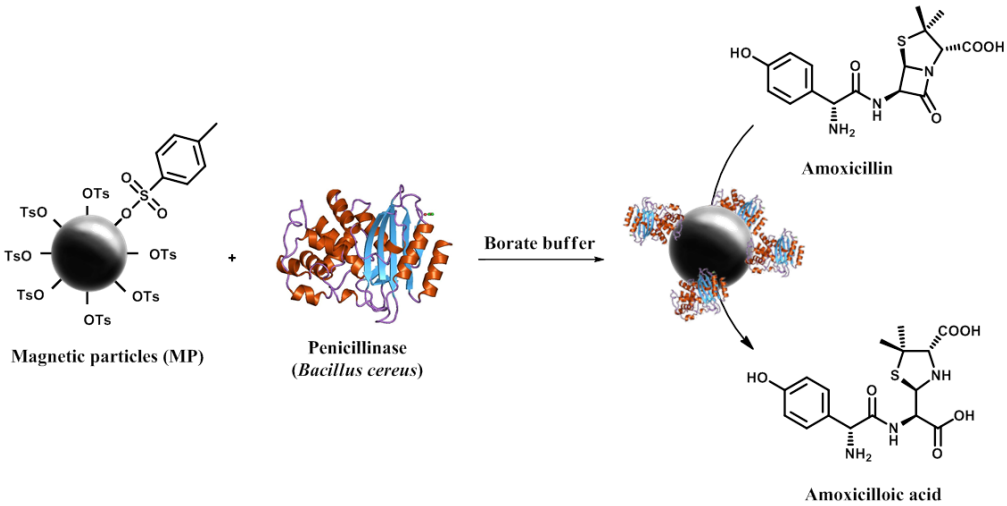
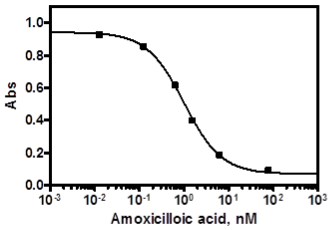


Figure 2. Preparation of the biohybrid MP using a β-lactamase enzyme and enzymatic hydrolysis of amoxicillin in carbonate buffer pH 10.

One of the singularities of this work was the immobilization of this enzyme to magnetic particles. The biohybrid particles allowed us to obtain pure concentrations of the penicilloic acids using only a magnetic rack for their separation (Figure 2). The results obtained demonstrate our hypothesis, since several combinations As-Ag showed highly selective inhibition curves against amoxicilloic acid, suggesting that the β-lactam epitope of the immunogen was hydrolysed.

The chosen combination used for further experiments was the conjugate PG-A-AD with As218. Some physicochemical parameters such as pH, ionic strength, tween 20 concentration and incubation times were tested in order to improve the features of the immunoassay. In general, changes in the conditions negatively affected the absorbance of the assay, while the IC_{50} remained more or less constant. In conclusion, incubation and competition times were better when longer, but a balance had to be found between good detectability and assay time. One hour for each parameter showed good results without increasing the overall time of the assay. The conductivity of the assay in the competition step also showed better results, the best one being at 2 mS cm^{-1}. The final features of the assay are enclosed in Figure 3.



Parameters of the assay (As218/PG-A-AD)			
[As]	1/4000	$Signal_{min}$	0.071 ± 0.002
[Competitor]	0.5 µg mL^{-1}	$Signal_{max}$	0.936 ± 0.018
Preincubation time	1h	Slope (m)	1.07 ± 0.03
Competition time	1h	R^2	0.992 ± 0.001
pH	7.5	IC_{50} (nM)	1.03 ± 0.05
Ionic strength	2.0 mS/cm	IC_{50}, µg L^{-1}	0.40 ± 0.02
Tween 20	0.001%	DR[a], µg L^{-1}	0.105 ± 0.008 to 1.501 ± 0.08
		CCα, µg L^{-1}	0.102
		CCβ, µg L^{-1}	0.191

Figure 3. Standard curve, conditions and features of the amoxicillin immunoassay. The data presented correspond to the average of eight assays performed on four days. Each assay was built using three well replicates. [a]DR corresponds to dynamic range, calculated as the concentration given between 20-80% of the maximum absorbance.

Sample treatment

Once the immunoassay was fully characterized and established, the next step was focused on the optimization of the sample treatment with the biohybrid particles. Because our assay was only able to detect exclusively the amoxicilloic acid, milk samples had to be previously hydrolysed. In summary, hydrolysis of amoxicillin was accomplished quantitatively in 10 min. On the other hand, the amount of the enzyme was also optimized, concluding that 10 µL of biohybrid particles (2.5 µg of β-lactamase, 3.7-7.4 U) was enough to quantitatively hydrolyse all the amoxicillin.

Table 1. Cross-reactivity of related compounds in the As218/PG-A-AD ELISA.

Group	Compound	IC_{50} (µg L^{-1})	MRL (µg L^{-1})	% CR[a]
Penicillins	Amoxicillin	> 0.73 × 10^3	4	>0.05
	Ampicillin	> 0.69 × 10^3	4	>0.05
	Penicillin G	> 0.75 × 10^3	4	>0.05
	Penicillin V	> 0.78 × 10^3	-	>0.05
Penicilloic acids	Amoxicilloic acid	0.49	-	100
	Ampicilloic acid	3.76	-	9.7
	Penicilloic G acid	2.71	-	14.4
	Penicilloic V acid	3.25	-	12.4
Cephalosporins	Cephalosporin C	> 0.96 × 10^3	-	>0.05
	Aminocephalosporanic acid	> 0.54 × 10^3	-	>0.05
Fluoroquinolones	Enrofloxacin	> 0.72 × 10^3	100	>0.05
	Flumequine	> 0.52 × 10^3	50	>0.05
Tetracyclines	Tetracycline	> 0.96 × 10^3	100	>0.05
	Doxycycline	> 1.03 × 10^3	-	>0.05
Sulfonamides	Sulfapyridine	> 0.50 × 10^3	100	>0.05
	Sulfametazine	> 0.60 × 10^3	100	>0.05

[a] Cross-reactivity is expressed as a percentage of the relation between the IC_{50} (nM) of the amoxicilloic acid and the IC_{50} (nM) of the other compounds tested.

Immunoassay evaluation.

The specificity of the immunoassay was evaluated for different penicillins, either the antibiotics themselves as well as their hydrolysed forms. Table 1 presents the cross-reactivity results. None of the parent drugs were detected by the assay while the open-ringed β-lactams showed high affinity for the antibodies. Interestingly, amoxicilloic acid was the most recognized structure, whereas the other penicilloic acids were similarly detected with worse detectabilities. Our hypothesis suggests that the antibodies recognize two different epitopes of the amoxicillin molecule, the hydrolysed β-lactam structure jointly

with the phenol group. Nevertheless, all the penicillins showed sensitivities below the MRLs established by the EU. We can assume that we have developed a broad-specific competitive immunoassay for the detection of the penicillin's family. Subsequently, other kinds of antibiotics such as cephalosporins, tetracyclines, fluoroquinolones and sulfonamides were also tested, but none of them showed assay inhibition.

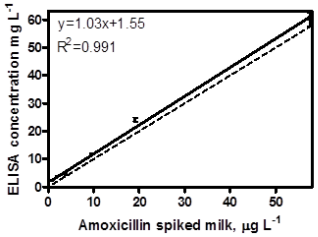
Matrix studies

The validation of the assay in milk was not as good as we expected. Diluting the milk 20 times was not enough to emulate the buffer curve. However, the unique parameter affected was the maximum absorbance, while the IC_{50} were similar for the different dilutions. It is well known that some components of the milk such as proteins and lipids can be absorbed non-specifically preventing the recognition of As-Ag. Sometimes, buffer standard curves are readjusted to mimic the milk curve by means of changing some immuno-reagent concentrations or using non-contaminated milk to build the curves (34,35).

It was the last approach used for our further experiments, using whole milk to prepare the calibration curves. Protocol conditions and immuno-reagent concentrations used were selected from the previous experiments made in buffer. Furthermore, microtiter plates were shaken during incubation and competition steps in order to minimize the formation of whey layers in the bottom of the wells. The features of the assay are enclosed in Figure 3. The assay shows an IC_{50} of 0.39 µg L^{-1}, 10 times lower than the MRL established by the EU, with an LOD around 100 ng L^{-1}.

Recovery assays were also carried out with blind samples. The results obtained are shown in Figure 4. They closely match the spiked values. The slope of the linear regression curve between the spiked and the calculated concentrations was close to the perfect correlation. According to Commission Decision 2002/657/EC, screening methods do not need to validate the decision limit. Notwithstanding, due to the high detection capability of our assay, both decision limit and detection capability were evaluated. The equations used correspond to analytes that have no MRLs since penicilloic acids have not been legislated yet. At least 20 blank samples were analysed on three days to calculate CC_{α}, which value was 0.102 µg L^{-1}. To calculate CC_{β}, 20 samples were spiked at the CC_{α} concentration and analysed on three days. The CC_{β} value for this assay was 0.191 µg L^{-1}.

Finally, the ruggedness of milk samples from different brands was evaluated, spiking them at the MRL levels. Figure 4 shows the recovery studies. In summary, no significant differences were observed for any of the brands. In addition, the recovery was 105 ± 7.2 % on average for all of the samples.



Robustness of the amoxicillin ELISA

Milk brand	spiked concentration, µg L^{-1}	ELISA values, µg L^{-1}	Recovery %
1	3.8	4.3 ± 0.49	112
2	3.8	4.2 ± 0.21	109
3	3.8	4.1 ± 0.39	106
4	3.8	3.6 ± 0.21	93

Figure 4. Results from the recovery study performed with milk. The graph shows correlation between the spiked and measured concentration values. The dotted line corresponds to a perfect correlation (m=1). The data correspond to the average of at least three-well replicates from three days. Whole milk samples from 4 brands were spiked with amoxicillin at the MRL (4 µg L^{-1}). Analytes were quantified using calibration curves prepared in milk. The data correspond to the average of at least 3 replicates.

Conclusions

We have developed an enzyme-linked immunosorbent assay that is able to detect the most common penicillins in milk samples without dilution at concentrations much lower than the MRL value. The strategy is based on the detection of the open-ringed forms of these penicillins, *i.e*. their penicilloic acid forms. The samples are easily treated by biohybrid magnetic particles, which are linked to a β-lactamase enzyme for the hydrolysis of the β-lactam structure. Within the same assay it is possible to analyse milk with and without any sample treatment. The difference between both results will be the concentration of the non-hydrolysed forms. And what is more important, this assay allows the detection of the two penicillin forms simultaneously. The ability to detect the open-ringed form is very important due to the illegal use of adding β-lactamase in the milk that the dairy producers commit in order to avoid fines with regard to the legislation on violating MRLs.

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CONFIRMATION OF B-AGONISTS RESIDUES IN *BOVINE* RETINA AND LIVER USING HPLC-MS/MS AND EVALUATION OF MATRIX-DEPENDENT PROBLEMS

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Abstract

β-Agonists or β-adrenergic agonists are synthetic compounds used as bronchodilators and tocolytic agents for therapeutic purposes in veterinary medicine. However, they also belong to a class of illegal growth promoters that decrease the fat content in farm animals, in favour of a higher percentage of lean mass. The accumulation of β-agonists in retinal tissue has been particularly useful in identifying residues of β-agonists at long withdrawal times (weeks to months). Liver has also been used as target tissue due to pharmacokinetic reasons. The purpose of this study was to set up an analytical confirmatory method for the detection of nine β-agonists in *bovine* retina and liver tissues. Additionally, the importance of matrix effect in these two tissues for the achievement of accurate detection of non-compliant samples has been studied and discussed. It could be concluded that the main characteristic of this phenomena is its considerable variability from one sample/animal to another. Also, the total amount of matrix in the final extract would provide differences in the matrix-matched calibration line.

Introduction

The most frequently used β-agonists or β-adrenergic agonists in veterinary medicine are clenbuterol, salbutamol and ractopamine. Several cases of human intoxication after consumption of meat containing residues of β-agonists have been reported, especially in the case of clenbuterol, including food poisoning (related to the presence of residues in the liver) giving cardiovascular and central nervous diseases. Moreover, the inappropriate use of these substances as growth-promoters has led to their prohibition, as it was manifested in Council Directive 96/22/EC (EEC, 1996). At the same time, in Commission Regulation (EU) N◦ 37/2010 maximum residue limits (MRLs) have been fixed exclusively for a single β-agonist for specific animal species and tissues, namely clenbuterol (EU, 2010). In the case of substances without MRLs, minimum required performance limits (MRPLs) have been established according to Commission Decision 2002/657/EC in a guidance paper from the Community Reference Laboratories released in 2007.

Since β-agonists are commonly used in veterinary medicine to treat disease, and sometimes in a fraudulent manner for increasing the weight of animals, highly sensitive analytical methods are required for quantification and confirmation of residues in livestock. While screening of animal tissue residues can be done relatively cheap using rapid immunochemical tests, accurate quantification and confirmation of banned substances require the sensitivity and specificity of mass spectrometry coupled with a chromatographic procedure (Stolker & Brinkman, 2005). In the literature, several confirmation methods have been described for different matrices such as milk (Li *et al*., 2010), urine and animal feed (Nielen *et al*., 2008) and edible animal tissues (Sai *et al*., 2012) using liquid chromatography coupled to mass spectrometry. Edible tissues such as liver appear also as interesting options to trace these compounds, especially when focusing in the prevention of possible human intoxications. Also, the accumulation of β-agonists in retinal tissue has been particularly useful in identifying residues of β-agonists long after treatment (weeks to months) after administration of the drug (Cristino *et al*., 2003). However, few confirmatory methods for determining β-agonists in retina have been found in the literature.

The purpose of this study was to set up an analytical confirmatory method for the detection of nine β-agonists in *bovine* retina and liver tissues. The method was based on SPE clean-up followed by HPLC-MS/MS analysis, following Commission Decision 2002/657/EC criteria (EC, 2002). Additionally, the importance of matrix effect in these two tissues and the differences between animals for the achievement of accurate residue quantifications has been studied.

Materials and Methods

Reagents and chemicals

Formic acid, methanol, acetonitrile, water tert-butylmethyleter, ortho-phosphoric acid 85%, sodium hydroxide solution (2N) and phosphate buffer (pH 7.2) were supplied by Merck (Darmstadt, Germany). β-glucuronidase from *Helix pomatia* was supplied by Sigma-Aldrich (St Louis, Mo, USA). All reagents were from analytical grade. β-agonist compounds used in this study were purchased from Sigma-Aldrich (St Louis, Mo, USA). The deuterium labelled internal standards clenbuterol-D_9 and salbutamol-D_3 were supplied by Dr. Ehrenstorfer GmBH (Augsburg, Germany). Stock standard solutions for each β-agonist (clenbuterol, clenproperol, clenpenterol, mabuterol, mapenterol, salbutamol, cimaterol, ractopamine and terbutaline) and internal standards (clenbuterol-D_9 and salbutamol-D_3) were prepared using methanol at concentrations of 100 µg mL^{-1}, and stored in the dark at -20°C.

HPLC-MS/MS analysis

The high pressure-liquid chromatography (HPLC) system consisted on a quaternary pump, degasser and auto-sampler from Agilent Technologies, model 1100 (Minnesota, USA). A Qtrap 2000 mass spectrometer with Ion Source Turbo Spray from AB Sciex (Toronto, Canada) was used. Nitrogen was produced by a high-purity nitrogen generator from PEAK Scientific Instruments Ltd (Chicago, Ill) and it was used as curtain, nebulizer and collision gas.

Aliquots of calibrators or sample extracts were separated using a Synergi 2.5 µm MAX-RP 100A (100 × 2 mm) column and pre-column from Phenomenex (Torrance, CA, USA). The mobile phase was acetonitrile (A) mixed on a gradient mode with water 0.2% of formic acid (B), at a flow rate of 250 µL min^{-1}. After the first 2 min with very aqueous mobile phase at 90% B, binary gradient mixing was initiated as follows: (B) 90 % to 30 % for 8 min and 30% to 90% for 2 min. Total run time was 12 min. The initial conditions 90% B – 10% A were set for 3 min for column re-equilibration between injections. Multiple reaction monitoring (MRM) in ESI positive mode was used. Table 1 shows the MRM transitions monitored for the selected β-agonists. Data were collected and processed with the Analyst 1.4.1 software package (MDS SCIEX).

Table 1. MRM transitions monitored for the selected β-agonists.

β-agonist	RT	MRM (Q1,Q3)
Clenbuterol	7.98	277 / 203
		277 / 259
Clenproperol	8.62	291/ 203
		291/ 168
Clenpenterol	7.14	263/ 245
		263 / 132
Mabuterol	8.72	311 / 237
		311 / 217
Mapenterol	9.25	325 / 237
		325 / 217
Ractopamine	7.58	302 / 284
		302 / 164
Salbutamol	1.79	240 / 148
		240 / 222
Cimaterol	2.02	220 / 202
		220/ 160
Terbutaline	1.83	226 / 152
		226 / 107

Samples

Bovine liver and eyeball (retina) samples were collected from cattle from different farms in Galicia (Spain) and stored at -20°C until analysis. All the samples were analysed with another validated method to ensure that they were compliant for all the assayed analytes.

Sample preparation

Liver samples. After cutting sample into smaller pieces in a blender, 2.5 g of homogenized *bovine* liver were transferred to a 50 mL tube and then 10 mL of phosphate buffer (pH 4.8-5.2) were added.

Retina samples. Eyeballs were thawed at room temperature just that the outside tissues can be manipulated but the aqueous and vitreous humours are still frozen. The eyeball was slowly incised across the cornea horizontally and vertically with a scalpel. A sample of 0.25 g of retina was placed into a 50 mL tube and an aliquot of 10 mL phosphate buffer (pH 4.8-5.2) was added.

Then, 100 µL of internal standard (deuterium labelled analytes at 0.02 µg mL^{-1} in methanol) were added to all samples and controls; 10 µL of β-glucuronidase were added to liver samples and controls. For validation purposes, spiked samples were prepared by adding 50 µL of working solution of β-agonists (0.01 µg mL^{-1} for clenbuterol, mabuterol and mapenterol, 0.025 µg mL^{-1} for clenproperol, clenpeterol and cimaterol and 0.05 µg mL^{-1} for terbutaline, ractopamine and salbutamol) according to the chosen validation levels (1.0*VL) for retina and liver, following the recommended European levels. For retina, the VLs were: 2 µg kg^{-1} for clenbuterol, mabuterol and mapenterol, 5 µg kg^{-1} for clenproperol, clenpeterol and cimaterol, 10 µg kg^{-1} for ractopamine, terbutaline and salbutamol. For liver, the VLs were: 0.2 µg kg^{-1} for clenbuterol, mabuterol and mapenterol, 0.5 µg kg^{-1} for clenproperol, clenpeterol and cimaterol, 1 µg kg^{-1} for ractopamine, terbutaline and salbutamol. In

the same way, samples spiked at 0.5*VL and 1.5*VL were prepared by adding 25 and 75 µL of working solution to retinal and liver samples. The mixture was shaken and introduced in an ultrasonic bath for 15 min; incubated at 55°C for 90 min in the case of liver samples. Afterwards, all samples were adjusted at pH 10-11 using NaOH and centrifuged (2,500 rpm at room temperature for 15 min).

Purification procedure
After sample preparation, samples were purified onto HLB 3 mL SPE cartridges which were activated with 5 mL methanol followed by 5 mL water. The cartridge was washed with 5 mL of water and 5 mL methanol/water (30:70, v/v). The analytes were eluted with 3 mL of methanol/tert-butylmethylether (10:90, v/v). The eluate was evaporated to dryness under a nitrogen stream at 35°C. The residue was dissolved in 100 µL of a mixture of 0.2% aqueous formic acid and acetonitrile 90:10 v/v and transferred into vials for the LC-MS/MS analysis.

Method validation
The method was validated on the basis of the criteria of European Commission Decision 2002/657/EC. A homogeneous mixture of blank retina (15 g) was divided in 57 subsamples of 0.250 g. In parallel, a homogeneous mixture of blank liver (150 g) was divided in 57 subsamples of 2.5 g. Each day (over the course of 3 days), 19 fortified samples at different levels were analysed: one non-spiked sample, six samples spiked at 0.5*VL, six samples at 1*VL and 6 samples at 1.5*VL. Each day, a calibration graph was built using standard solutions ranging from 0 to 1.5*VL. The concentration of the analyte in the validation and incurred samples was interpolated from calibration curves determined by calculating the area ratios of analyte peak area/IS peak area versus analyte concentration. The calibration graph was described by the equation $y = mx + b$.

Additionally, 20 blank *bovine* retina samples and 20 blank liver samples were analysed. Decision limit (CCα), detection capability (CCβ), selectivity, specificity, precision, trueness (as corrected percentage recovery), and applicability/ruggedness were evaluated.

Matrix effect experiments
Various sets of matrix-matched calibration curves were prepared to evaluate the presence of matrix effect in liver and retina samples. Standard lines were also constructed in mobile phase. Samples from different animals were used, to assess inter-individual variations. Also, a pull of samples collected from different animals was prepared for each matrix. In the case of liver samples, different amounts of matrix were tested as well. Post-extraction experiments were performed.

Results and discussion

Analytical methodology
Tissue and urine are commonly chosen as analytical matrices to monitor β-agonists in cattle and they are often analysed with methods based on LC-MS/MS. Urine is an interesting option to trace the abuse of veterinary residues because it is collected *in vivo*. However, its great variability during the course of the day in terms of dilution plus a great variability among animals, make this matrix a difficult. On the other hand, edible tissues such as liver, kidney and muscle are especially interesting due to the potential human consumption of these animal products and they are first-choice analytical matrices in many cases. Also, previous studies have shown the persistent accumulation of clenbuterol and other β-agonists in *bovine* retinal tissue. However, very few multi-residue confirmatory methods have been found in the literature for determining β-agonist compounds in retina and the existing methods are mainly focused on clenbuterol only (Williams *et al*., 2004).

In this case, *bovine* liver has been selected as post-mortem biological matrix along with eyeball (retina). Retina is not an abundant matrix (only around 1 g of retina available per eyeball) but it was selected basing on its accumulative properties for β-agonists and also due to the simplicity of collection in the slaughterhouse. Although no clear evidence of the necessity of enzymatic hydrolysis during animal tissue analysis have been found in the literature (Sai *et al*., 2012), some papers have reported its use (Williams *et al*., 2004). Unlike urine or liver samples, the no need of enzymatic hydrolysis during retinal tissue analysis is a true fact, and it simplifies the methodology when using this kind of matrix. In this study, glucuronidase was used in liver samples to release β-agonists conjugates in order to assure method accuracy. After sample hydrolysis and pH adjustment, all samples were centrifuged and the extract was purified using a SPE optimized procedure. This type of clean-up procedure has been previously reported by other authors for the determination of β-agonists in complicated biosamples to decrease the matrix effect in LC-MS/MS. In this case, the application of a simple purification onto HLB cartridges after sample preparation enabled the simultaneous extraction of nine β-agonists in *bovine* retina and liver, resulting in simple and fast confirmatory LC-MS/MS method.

With regard to the chromatographic separation, different columns with various sorbents, mainly reversed phased and C_{18}, have been applied to the analysis of β-agonists. In this study, a Synergi MAX-RP column from Phenomenex was used and it provided sufficient separation and good peak shape for all the selected analytes. The total run time (12 min) was comparable to the separation provided by UHPLC instruments (Nielen *et al*., 2008).

Validation process and method performance

Confirmatory methods are methods that provide full or complementary information enabling the unequivocal identification of the analyte at its level of interest. These methods are specifically designed to provide unequivocal identification of unauthorized substances and substances with an established maximum residue limit (MRL) and quantification of analytes (MRL compounds) and, wherever possible, have a detection capability (CCβ) similar to the detection capability of the screening method. According to the 2002/657/EC Decision, the performance of the analytical method was evaluated by checking the identification criteria in the spiked samples: the signal-to-noise ratio ≥ 3, the relative retention time of the analyte within the tolerance limit of 2.5% and the relative ion intensities within the criteria laid down in the Decision. All the validation parameters were in compliance with the Decision. An overview of validation results for CCα and CCβ is shown in Table 2.

Table 2. Validation levels obtained for determining β-agonists in retina and liver.

β-agonist	Retina (μg kg^{-1})		Liver (μg kg^{-1})	
	CCα	CCβ	CCα	CCβ
Clenbuterol	0.41	0.70	0.03	0.05
Clenproperol	0.83	1.42	0.05	0.09
Clenpenterol	0.79	1.34	0.07	0.13
Mabuterol	0.59	1.00	0.04	0.07
Mapenterol	0.29	0.50	0.04	0.07
Ractopamine	1.41	2.40	0.14	0.25
Salbutamol	1.23	2.10	0.16	0.27
Cimaterol	0.70	1.19	0.09	0.16
Terbutaline	1.54	2.63	0.18	0.30

Matrix effect

An important issue to be addressed during the development and validation of HPLC-MS/MS methods is matrix effect. Matrix effect is an alteration or disturbance in the ionization efficiency caused by the presence in the sample of substances that co-elute with the analytes of interest. These alterations may be difficult to see in the chromatograms but they cause problems in accuracy and sensitivity. Matrix effect is both matrix and compound dependent, and it can result in an increased signal or ionization (ion enhancement), or in a decrease (ion suppression). Two common ways to assess matrix effects are post-extraction addition of the analytes in the sample matrix and post-column infusion. The post-extraction addition technique consists on the addition of the analytes in sample extracts and their comparison with pure solutions prepared in mobile phase.

The slopes (m) and y-intercepts (b) of the different calibration lines obtained during the matrix effect experiments are shown in Table 3 for retina and Table 4 for liver. A translational effect is a signal produced not by the analyte but by concomitant substances present in the matrix, and it results in changes in the intercept of the calibration line but not in the slope. On the other hand, a rotational effect arises when the size of the signal of a fixed concentration of the analyte is affected by other constituents of the test solution, and it is usually proportional to the signal, resulting in changes of the slope of the calibration curve (Ellison & Thompson, 2008). In retina, the existence of rotational effect is clear, and it may be originated in the extraction procedure, mainly in the SPE step. However, the intercept of the curve is hardly affected. The problem seems to disappear when using a pull of samples, but a pull is not representative of individual matrices as they clearly vary amongst animals. With regard to the liver, both problems appear, and they could not be related with the amount of sample. Apparently, liver is a more difficult one, making it difficult to find a representative option to use as reference matrix.

Conclusions

The developed method was successfully validated according to the European criteria to determine β-agonists in *bovine* retina and liver. In this kind of approaches, matrix-matched calibration is commonly used for quantitation, but there are disadvantages associated with this approach. It is hard to collect blank matrix for a particular matrix, as inter-individual variations (sample or animal-dependent) are frequent, especially for liver. Also method-dependent variations/effects are unavoidable. These problems are clearly reflected in the parameters defining the calibration lines constructed with the selected animal matrices.

Table 3. Calibration curves prepared for studying matrix effect in β-agonists determination in bovine retina (m: slope; b: y-intercept; R^2>0.95).

Matrix/analyte		Clenbuterol		Clenpenterol		Clenproperol		Mabuterol		Mapenterol		Ractopamine		Salbutamol		Cimaterol		Terbutaline	
		m	b	M	B	M	B	m	b	m	b	m	b	m	b	m	b	m	b
Individual	Retina A	0.166	0.011	0.159	-0.014	0.149	0.039	0.056	0.000	0.158	0.024	0.080	0.010	0.197	0.010	0.113	-0.032	0.180	0.012
	Retina B	0.150	0.026	0.206	-0.053	0.208	-0.069	0.227	-0.012	0.256	-0.028	0.124	-0.061	0.193	0.084	0.115	0.007	0.226	-0.038
	Retina C	0.138	0.017	0.163	0.019	0.162	-0.022	0.208	-0.021	0.179	-0.004	0.111	-0.028	0.217	-0.055	0.199	0.047	0.162	0.001
	Retina D	0.174	-0.012	0.142	-0.057	0.143	-0.059	0.181	-0.045	0.173	-0.015	0.097	0.027	0.173	0.021	0.091	-0.015	0.111	0.140
Pull	Portion 1	0.012	0.124	-0.065	0.169	-0.023	0.178	0.081	0.190	0.002	0.269	0.016	0.080	0.105	0.187	0.020	0.216	0.037	0.201
	Portion 2	0.003	0.144	-0.004	0.135	0.028	0.134	0.053	0.154	0.005	0.296	0.021	0.094	0.023	0.198	-0.002	0.186	0.136	0.161
	Portion 3	0.010	0.125	-0.018	0.151	-0.046	0.158	-0.037	0.179	-0.009	0.281	0.050	0.072	0.021	0.185	-0.034	0.201	0.028	0.187
	Portion 4	0.018	0.121	-0.018	0.155	0.033	0.156	0.056	0.156	0.000	0.296	-0.017	0.081	-0.046	0.204	-0.049	0.231	-0.029	0.186
Standard	1	0.014	0.146	-0.081	0.222	-0.049	0.128	0.007	0.029	-0.027	0.206	-0.023	0.083	0.037	0.189	0.008	0.089	0.070	0.151
	2	0.009	0.120	0.000	0.162	0.016	0.117	0.006	0.173	0.018	0.230	-0.007	0.044	0.028	0.160	0.043	0.171	0.058	0.132

Table 4. Calibration curves prepared for studying matrix effect in β-agonists determination in bovine liver (m: slope; b: y-intercept; R^2>0.95).

Matrix/analyte		Clenbuterol		Clenpenterol		Clenproperol		Mabuterol		Mapenterol		Ractopamine		Salbutamol		Cimaterol		Terbutaline	
		m	b	M	B	M	B	m	b	m	b	m	b	m	b	m	b	m	b
	Liver A	1.461	0.040	1.372	0.011	1.666	0.038	1.838	0.043	2.073	0.051	0.707	0.050	1.854	0.066	2.487	-0.041	1.798	0.031
Individual 2 G	Liver B	1.169	0.016	1.575	-0.039	1.737	0.040	2.268	0.015	2.459	0.037	0.817	0.030	1.797	-0.015	1.780	0.006	1.548	-0.009
	Liver C	1.208	0.007	1.392	0.013	1.741	0.026	1.859	0.040	2.176	0.024	0.629	0.100	1.647	0.125	1.337	0.208	1.483	0.124
	Liver A	1.398	0.004	1.796	-0.034	1.295	0.016	2.259	0.008	2.453	-0.006	0.730	-0.004	1.879	0.051	2.228	0.050	1.665	0.004
Individual 0.25 G	Liver B	1.185	0.017	1.627	0.032	1.322	0.027	1.590	0.036	1.987	0.012	0.762	-0.012	1.848	0.028	2.487	0.020	1.794	0.018
	Liver C	1.166	0.025	1.531	0.047	1.352	0.024	2.003	0.014	2.180	0.025	0.616	0.018	1.691	0.098	2.201	0.047	1.473	0.122
Pull	0.125 g	1.263	0.004	1.568	-0.002	1.266	-0.001	2.369	0.006	2.330	0.013	0.593	-0.025	1.737	0.003	2.274	0.027	1.702	0.024
	0.25g	1.604	0.021	1.896	-0.006	1.404	0.062	2.562	-0.015	3.002	0.014	0.624	0.008	1.789	-0.024	1.956	0.030	1.709	0.094
	2 g	2.345	0.012	2.630	-0.074	2.600	-0.027	3.846	-0.009	7.857	-0.021	0.534	0.054	1.997	-0.075	3.378	-0.077	2.352	-0.121
	2 g	1.158	0.100	3.555	-0.049	3.250	0.099	3.794	-0.033	6.096	0.136	0.448	0.028	1.422	0.072	3.310	-0.062	1.767	-0.039
	2 g	1.690	-0.020	3.033	0.018	2.753	0.002	3.388	0.018	6.430	0.069	0.379	0.039	1.342	0.107	1.820	0.076	1.578	0.005
Standard	1	1.252	0.002	1.789	-0.002	1.289	0.004	1.787	0.003	2.274	-0.013	0.702	-0.032	1.621	0.049	2.124	0.041	1.493	0.019
	2	1.304	0.008	1.470	0.017	1.039	0.034	1.750	-0.009	2.709	0.005	0.640	0.004	1.784	0.121	1.939	0.076	1.605	0.073

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THE EFFECT OF PREGNANCY AND LACTATION STAGE ON THE FATTY ACID PROFILE OF *BOVINE* MILK

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Abstract

Milk is one of the most nutritionally complete foods. It can sustain the life of the human neonate and plays also an important role during adult life. Milk fat is the most variable component of milk, both in concentration and composition. The effects of the consumption of *bovine* milk on human health, particularly milk fat, have been studied in great depth. Sometimes associated with negative effects (heart diseases, weight gain and obesity), mostly related to its saturated fraction, milk has also several beneficial characteristics. Cancer prevention, antiviral activities, prevention of coronary heart disease, are some of the benefits related to milk consumption, mainly in relation to the fatty acids produced by rumen bacteria. With this regard, there has been great improvement in animal husbandry (management, feeding, care, breeding, etc.), enabling a relative control of the lipid profile in milk fat. Exogenous factors (nutrition, seasonal variations, animal health) or endogenous factors (breed and genotypes) are well-known modulators of milk composition. Pregnancy status is something less familiar but it can also alter the fatty acid profile of milk. In this study, the differences in the fatty acids composition of two groups of cows (pregnant and non-pregnant) have been studied.

Introduction

The consumption of milk and milk products has long traditions in human nutrition. Since very early times humans have domesticated hoofed animals to produce milk for food (Parodi, 2004). The consumption of this nutrient is an important part for a healthy well-balanced diet, especially in western societies (Haug, 2007). Fatty acids can be classified according to their length of their hydrocarbons chain into short chain (SC-FAs up to 6 carbons), medium chain (MC-FAs 8 to 12 carbons) or long chain (LC-FAs >12 carbons). Alternatively, they are categorized based on their saturation status: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs).

Bovine milk consumption has always been studied for its effects on human health. Lately milk and dairy products have been associated with negative effects for their contribution of saturated fat to the diet, related to heart diseases, weight gain and obesity. SFAs represent the most important portion of milk fat from a quantitative point of view (approx 75%). But in this milk there are also many fatty acids, partially produced by rumen bacteria, with beneficial effects on human health. The SC and MC-SFAs (C4:0-C12:0) are for example involved cancer prevention, antiviral activities and delay tumour growth (German, 1999; Thormar *et al*., 1994). It is important to note that these fatty acids are not present in other kinds of fat. Lauric acid (C12:0) may have antiviral and antibacterial functions (Hayes, 1984). Stearic acid (C18:0), monounsaturated fatty acids and SC-FAs, appear to have no effect on serum cholesterol levels (Grundy, 1994; Iggman & Risérus, 2011). Oleic acid (OA; C18:1n-9) is the unsaturated fatty acid with the highest concentration in milk. The effects of a diet rich in MUFAs decrease the risk of cardiovascular disease, reducing LDL cholesterol, triglycerides and the ratio between total cholesterol and high-density lipoprotein (HDL) (Mensink *et al*., 2003).

The main PUFAs in milk are linoeic (LA; C18:2n–6) and α-linoleic acid (ALA; C18:2n–3) acid. These fatty acids are considered essential dietary nutrients because humans cannot synthesize them. Arachidonic acid (AA; C20:4n–6), is synthesized from the essential fatty acid LA, which is the precursor of long chain n-6 PUFAs family. ALA is the precursor of the n-3 PUFAs group, and it is used for synthesizing two important fatty acids in humans: docosahexaenoic acid (DHA; C22:6n–3) and eicosapentaenoic acid (EPA; C20:5n–3).

From quantitative point of view, milk from ruminants can be considered an important source of omega-3 fatty acids in the human diet (Mansson, 2008). These n-3 fatty acids have important roles in the modulation and prevention of human diseases, particularly coronary heart disease and correct retinal and brain development in infancy (Connor, 2000). *Bovine* milk is the main dietary source of conjugated linoleic acid (CLA), a mixture of free fatty acid isomers produced by base-catalysed isomerization of LA. CLA benefits have been largely studied in the last two decades; reduces body fat, cardiovascular diseases and cancer, antidiabetic effects, antilipogenic effects and modulates immune and inflammatory responses as well as improves bone mass (Dilzer & Park, 2012; Khanal, 2004; Nagao & Yanagita, 2005; Rainer & Heiss, 2004).

There are a wide variety of factors which affect milk production and composition (quantity and quality) in dairy castle. These factors can be divided into endogenous factors, those that originate from within an organism (breeds and genotypes, stage of lactation, hormonal levels, sex of the foetus). And exogenous factors, which depend on the environment or human hand

(season of the year, climatic factors, nutritional modifications, management aspects, milking). Management aspects may alter significantly milk composition, in particular, milk fat content and fatty acid profiles. Based on the intrinsic factor of pregnancy, the aim of this study was to compare the lipid profile of two groups of cows, pregnant and non-pregnant, and to determine whether there are differences between them in terms of fatty acids composition and other lactation characteristics as nutrition.

Materials and Methods

Reagents and standard solution

For lipid extraction, sulphuric acid, methanol, *n*-hexane, isooctane and sodium sulphate anhydrous were purchased from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used. Fatty acid methyl esters (FAMEs) standard mixture (C4:0 to C24:0), methylated PUFA No.1 from marine source, individual FAMEs (C18:2n-6 9-12t, C18:1n-7 and C22:5n-3) and tricosanoic acid (C23:0, internal standard) were purchased from Sigma Aldrich (Madrid, Spain). To prepare the stock solution, the standards were diluted in isooctane and subsequent calibrators for GC-FID in *n*-hexane.

Animals and sampling

The samples were collected from a single dairy farm located in Galicia (Chantada, Lugo), one region in Northwest Spain. The farm had 300 cows and the herd was in a semi-intensive state; the feed is based on fresh grass, and is completed with forage and concentrate in the farm. The study was performed with 20 Holstein cows. The samples were divided in two groups, ten cows were pregnant and the remaining animals were taken as control group (non-pregnant). Date of insemination, days post-partum and the age of de animal were noted. The cows were milked twice a day (morning and evening) and the samples were collected in the second milking. Approximately 20 mL milk were introduced via manual, after discarding the first stream of milk from the udder. The samples were collected into a plastic tube by a veterinary surgeon. The raw milk samples were transported under refrigeration (10°C) to the laboratory and immediately analysed.

Extraction of total lipids and GC analysis

The lipid extraction and the qualitative and quantitative determination of the fatty acids composition in raw milk samples was preformed as follows. Briefly, total lipid was extracted from an aliquot of milk (10 µL) with 2 mL H_2SO_4/ methanol (2.5%). For Gas Chromatography (GC) analysis, fatty acid methyl esters (FAMEs) were generated by methyl esterification; methylation was carried out in a water bath for 2 h at 60°C. FAMEs were dissolved in a final volume of 1 mL *n*-hexane before injection for GC analysis. Chromatographic analysis was performed with a 6850 Gas Chromatography system (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a DB-Wax capillary column (60 m x 0.25 mm ID x 0.25 µm film thickness; Chrom Tech, Richmond, CA, USA). Fatty acids with the same chemical composition were grouped into SFAs, MUFAs and PUFAs, as it was reflected in Table 1. The data were recorded by integrator Software GC ChemStation version B.03.02 (Agilent Technologies).

Statistical analysis

Univariate statistics were performed using PASW Statistics 18.0 (SPSS Ibérica, Madrid, Spain). Breakdown analyses are represented by the mean and standard deviation of fatty acid levels (%wt/wt of total fatty acids) in milk for pregnant and no-pregnant cows (Galicia, Spain). These values are shown in Table 1. Discrimination test was applied to search for any possible statistically significant differences in FA levels. Differences between groups were evaluated by Student test at the $p < 0.05$ and $p < 0.01$ levels. Multivariate analysis was performed to visualize and compare the milk fatty acid profiles of pregnant and no-pregnant cows, by means of SIMCA-P+12.0 (Umetrics AB, Sweden) software. All variables were log transformed and sealed (pareto) to become uniform and suitable for multivariate analysis. Orthogonal partial least square discriminant analysis (OPLS-DA) was used.

Results

Table 1 shows the fatty acid composition (%*wt*/*wt* of all FAs) of the milk, and other characteristics of these animals obtained from pregnant and non-pregnant cows. Results are presented as mean level and standard deviation of each fatty and for the other parameters. All samples were analysed in duplicate and two samples of non-pregnant cows were eliminated because they were outliers. Statistically significant variations were indicated in Table 1 using superscript symbols. Five fatty acids differed significantly between groups at $p < 0.05$ level as follows: C10:0, C13:0, C15:0, C18:2n–6 and C20:1n–9. Ten fatty acids differed significantly at $p < 0.01$ level as follows: C17:0, C14:1n–5, C16:1n–5, C17:1n–9, C18:1 n–7, C18:2n–6 9t, 12t, C20:1n-11, C20:2n-6, C20:3n-6 and rumenic acid (RA; C18:2n–6 9c, 11t). The rest of fatty acids remained relatively constant or the differences were not statistically significant.

*Table 1. Fatty acid composition (%wt/wt of total fatty acids) of milk collected from pregnant and non-pregnant cows. Significance is indicated when the median in pregnant cows is statistically different from the median in non-pregnant cows by Student test: * p < 0.01 and ł p < 0.05.*

		Pregnant		**Non-Pregnant**	
No. Of cows		*N=10*		*N=8*	
n=samples		*n=20*		*n=16*	
		Mean	± SD	Mean	± SD
Age of cow (days)		1368	± 445	1604	± 464
Lactation days		216	± 63	34	± 17
Pregnant days		116	± 54		
%wt/wt of all FAs	Fatty acids				
Saturated FA					
C6:0	Caproic acid	2.814	± 0.458	2.693	± 0.303
C8:0	Caprylic acid	2.187	± 0.367	1.923	± 0.319
C10:0ł	Capric acid	3.938	± 1.189	2.676	± 0.858
C11:0	Undecylenic acid	0.310	± 0.146	0.177	± 0.118
C12:0	Lauric acid	4.469	± 0.620	4.288	± 0.647
C13:0ł	Tridecanoic acid	0.250	± 0.074	0.167	± 0.079
C14:0	Myristic acid	13.15	± 1.623	12.85	± 1.927
C15:0ł	Pentadecanoic acid	1.624	± 0.258	1.252	± 0.425
C16:0	Palmitic acid	28.69	± 4.042	31.04	± 3.346
C17:0*	Margaric acid	0.731	± 0.093	0.951	± 0.178
C18:0	Stearic acid	8.087	± 0.952	8.744	± 1.685
C20:0	Arachidic acid	0.093	± 0.015	0.089	± 0.012
C22:0	Behenic acid	0.039	± 0.006	0.035	± 0.004
C24:0	Lignoceric acid	0.011	± 0.006	0.013	± 0.005
Total SFA		65.22	± 1.677	65.84	± 4.292
Monounsaturated FA					
C14:1 n-5*	Myristoleic acid	2.207	± 0.500	1.447	± 0.298
C16:1 n-9c		0.305	± 0.059	0.363	± 0.074
C16:1 n-7c	Palmitoleic acid	2.756	± 0.445	2.671	± 0.812
C16:1 n-5*		0.062	± 0.011	0.132	± 0.063
C17:1 n-9*		0.357	± 0.042	0.587	± 0.225
C18:1 n-9c	Oleic acid	21.78	± 0.894	21.78	± 3.290
C18:1 n-7c*	Vaccenic acid	0.438	± 0.060	0.652	± 0.157
C20:1 n-11*		0.144	± 0.025	0.115	± 0.010
C20:1 n-9cł	Gondoic acid	0.060	± 0.011	0.088	± 0.031
C22:1(n-11)		0.022	± 0.007	0.017	± 0.003
C22:1(n-9)	Erucic acid	0.017	± 0.007	0.014	± 0.005
isomeros 18:1		1.088	± 0.574	0.873	± 0.806
Total MUFAs		29.23	± 1.384	28.73	± 4.111
n-6 Poliunsaturated FA					
C18:2 n-6cł	Linoleic acid	2.452	± 0.258	2.825	± 0.369
C18:2(n-6) 9.12t		0.283	± 0.042	0.230	± 0.066
C18:2(n-6) 9t.12t*		0.037	± 0.011	0.065	± 0.027
C18:3 n-6	γ-linolenic acid (GLA)	0.042	± 0.015	0.035	± 0.011
C20:2 n-6*	Eicosadienoic acid	0.373	± 0.068	0.045	± 0.014
C20:3 n-6*	Dihomo-γ-linolenic acid	0.220	± 0.051	0.144	± 0.035
C20:4 n-6	Arachidonic acid	0.328	± 0.074	0.278	± 0.068
Total n-6PUFAs		3.421	± 0.395	3.626	± 0.430
n-3 Poliunsaturated FA					
C18:3 n-3	α-linolenic acid (ALA)	0.423	± 0.062	0.450	± 0.133
C18:4 n-3	Stearidonic acid	0.076	± 0.017	0.070	± 0.028
C20:5 n-3	EPA	0.066	± 0.008	0.073	± 0.015
C22:5 n-3c	Clupanodonic acid (DPA)	0.086	± 0.020	0.083	± 0.038
C22:6 n-3	DHA	0.031	± 0.010	0.031	± 0.010
Total n-3PUFAs		0.682	± 0.070	0.702	± 0.167
CLA c9.t11*		1.304	± 0.173	0.971	± 0.090
CLA t10.c12		0.121	± 0.022	0.111	± 0.024
Total PUFAs		5.516	± 0.586	5.394	± 0.624

Figure 1 describes the multivariate regression analysis in terms of orthogonal partial least-squares discriminated analysis (OPLS-DA) that was applied to extract the systematic variation in the quantified milk profiles (X matrix) related to a response (y) and revealed the class separation. In the present study, the response y is a dummy vector describing the animal´s state: pregnant or non-pregnant. The two groups of cows were located in opposite sides of the plot. The pregnant group was situated on the left; the group was more homogeneous because cows are located more clustered. On the right side, non-pregnant cows are plotted more separately reflecting a more heterogeneous group in terms of fatty acid profiles. The corresponding loadings S- plot is shown below the OPLS-DA plot, representing the influence or relative contribution of each fatty acid to the separation of the classes for the DA. The fatty acids that were more abundant in non-pregnant cows were located on the top-right corner of the S-plot, while the most abundant fatty acids in pregnant cows are on the left-bottom of the plot. These fatty acids were the same that showed statistically significant differences in the univariate statistics.

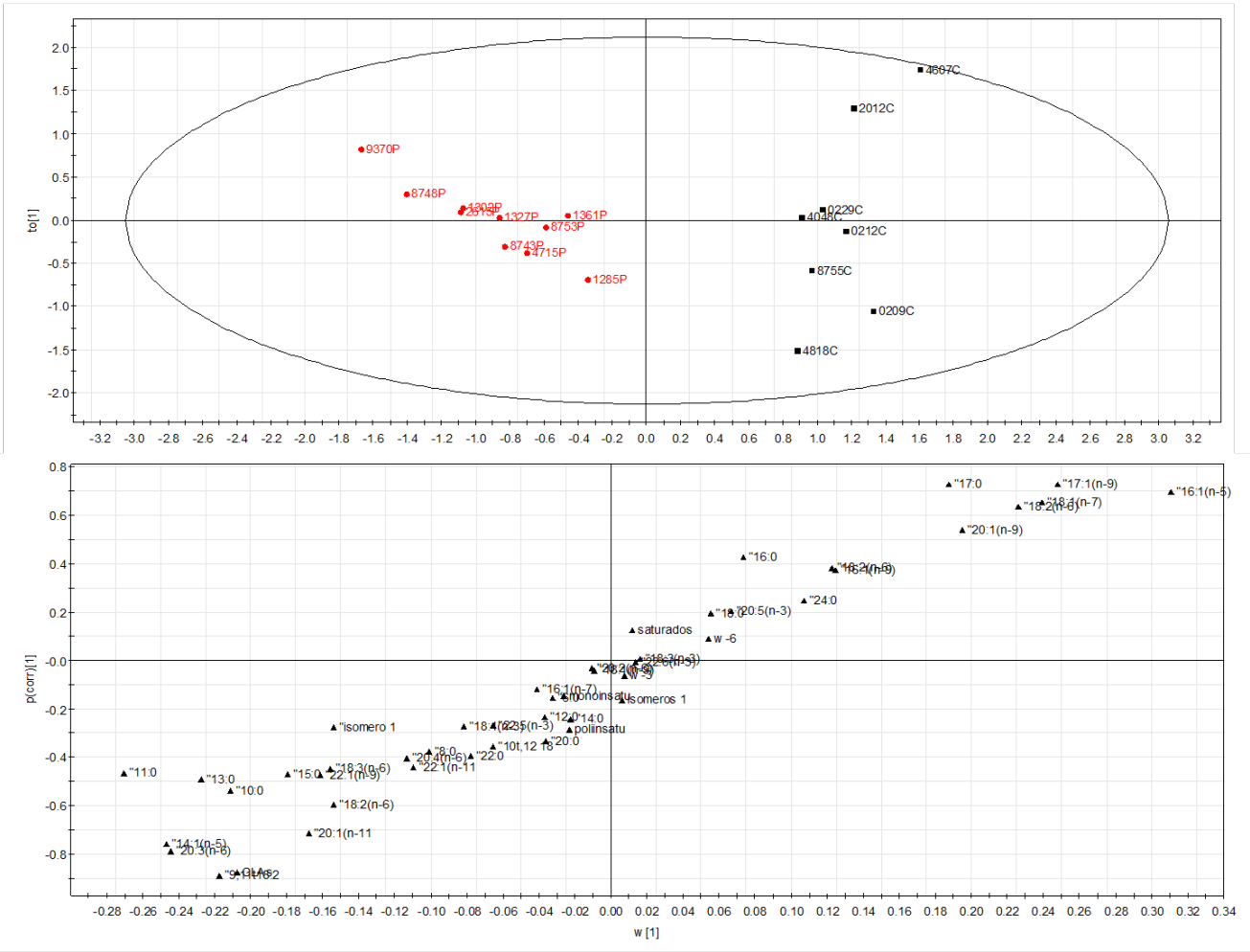

Figure 1. OPLS-DA plot showing the discrimination of milk collected from pregnant and non-pregnant cows, based on their fatty acid composition. The loadings S-plot shows the relative contribution of each fatty acid to the separation of the two classes.

Differences between pregnant and non-pregnant cows were not very pronounced except for those fatty acids that showed significant differences. The saturated fraction of cow milk was very similar in both states, (65.21 for pregnant and 65.84% wt/wt for non-pregnant) being lower in pregnant cows. The four most important fatty acids in terms of abundance in cow milk are C12:0, C14:0 C16:0 and C18:0. In the case of non-pregnant cows, short and medium chain fatty acids (C6:0-C12:0) decreased, as also C14:0. However, for C16:0 and C18:0 the opposite behaviour could be glimpsed. The C10:0, C13:0 and C15:0 were significantly different at $p < 0.05$ level, while C17:0 was significantly different at $p < 0.01$ level.

The total MUFA levels were higher in pregnant cows. The most important MUFAs from a quantitative point of view are oleic acid (OA, C18:1n–9), palmitoleic acid (C16:1n–7), myristoleic acid (C14:1n–5) and vaccenic acid (C18:1n–7). The most important MUFA, OA showed the same value for pregnant and non-pregnant cows (22% in both cases). Palmitoleic and myristoleic acid, decreased in milk from non-pregnant cows. In the case of vaccenic acid, clearly increased its value in non-pregnant animals. Statistically significant variations were observed for five MUFAs at $p < 0.01$ level: C14:1n–5, C16:1n–5, C17:1n–9, C18:1n–7, C20:1n–11 and, for gondoic acid (C20:1n–9) at $p < 0.05$ level.

With regard to PUFAs, the total amount was lower in no-pregnant cows. However, the total amount of n-3 and n-6 was slightly higher in non- pregnant cows for both cases. The variation of rumenic acid (RA; CLA *c*9,*t*11) was statistically significant at $p < 0.01$ level, being 1.30 in pregnant cows in contrast to 0.97 in non-pregnant. This value affects the total amount of PUFAs, being higher in pregnant cows. The most important PUFAs are linoleic acid (LA; C18:2n–6) and α-linoleic acid (ALA; C18:3n–3) essential dietary fatty acids. In both cases, the highest levels were observed for non-pregnant cows. Docosahexaenoic acid (DHA; C22:6n–3) and eicosapentaenoic acid (EPA; C20:5n–3) were more abundant in non-pregnant cows; while the value for arachidonic acid (AA; C20:4n–6) was lower in this state. Statistically significant variations were observed for four PUFAs C18:2n-6 9t-12t, C20:2n-6 and C20:3n-6 at $p < 0.01$. The LA was statistically significant at $p < 0.05$ level.

Discussion and Conclusions

There were no statistically significant differences between the total of SFAs, MUFAs or PUFAs, however, studying milk fatty acids individually some differences between pregnant and non-pregnant cows were observed. Certain fatty acids are important for calculation of two indexes closely related to human health: atherogenic (IA) and thrombogenic (IT) index. The IA indicates the relationship between the sum of the main saturated fatty acids (pro-atherogenic C12:0, C14:0 and C16:0) and the main classes of unsaturated fatty acids (those considered anti-atherogenic compounds). Briefly, the IT is defined as the relationships between the pro-thrombogenic fatty acids (C14:0, C16:0 and C18:0) and the anti-thrombogenic fatty acids (MUFAs and n–6 and n–3 PUFAs) (Garaffo *et al*., 2011). These two indexes were slightly higher in the milk obtained from non-pregnant animals, because these cows had lower levels of C12:0 and C14:0 and higher levels for C16:0 and C18:0 in their milk. Total saturated fraction is lower in pregnant cows, and this fact is beneficial for human health as it is associated with a lower risk of heart diseases, lower weight-gain and reduced obesity risk. Obviously, this drop in the total SFAs would be compensated by an increase in the unsaturated fraction.

The oleic acid (OA), the most abundant monounsaturated fatty acid in *bovine* milk, is closely related to the animal feed. Since animals where fed with the same type of ration/feed, one could expect that oleic acid will remain constant independently of the pregnancy status of the animal, as it was confirmed during the statistical analysis. Vaccenic acid (VA) was significantly higher in non-pregnant cows but myristoleic acid was lower in their milk. These two MUFAs are quantitatively important in the total MUFA fraction, and for this reason the total amount was lower in non-pregnant animals. Nutritionally, a decreased level of MUFAs is not recommended, because the benefits of a diet rich in MUFAs are well known. MUFAs decrease the risk of cardiovascular disease, and lower LDL-cholesterol and triglycerides in human blood. VA is the only known dietary precursor of c9,t11 conjugated linoleic acid (CLA). Despite being a *trans* fatty acid, recent studies has suggested that the consumption of this natural *trans* fatty acid may bring health benefits beyond those associated with CLA (Lock & Bauman, 2004).

Milk collected from non-pregnant cows was slightly richer in the essential fatty acids (LA and ALA), and also in the long chain PUFAs EPA and DHA. The benefits of n–3 fatty acids have been extensively reviewed. They reduce the risk of cardiovascular disease, type 2 diabetes, hypertension, cancer, and certain disruptive neurological functions, among other benefits. Milk and dairy products are a good source of this type of fatty acids, and as such they are important in human diet. The value for AA was slightly lower in non-pregnant cows, following the opposite trend of the omega 3 counterparts. The CLA levels were clearly higher in pregnant cows; this fact resulted in an increase of the total-PUFAs in milk from pregnant cows. However, if one only considers the n–6 and n–3 to calculate total-PUFAs, the polyunsaturated fraction is more elevated in milk from non-pregnant cows. It is important to consider the different impact of introducing n–6 or n–3 in human daily diet. Modern societies tend to introduce higher amount of n–6, and with this respect milk with higher amounts of n–3 is more recommendable.

In conclusion, the fatty acid composition of milk clearly varies as a result of pregnancy in the cow. In some aspects, milk from pregnant animals seems more adequate for human consumption. However, some fatty acids may lead us to the opposite conclusion. In addition to this, other studies have already demonstrated the effect of pregnancy in *bovine* milk composition, including steroid hormone levels (Regal *et al*., 2012). Because milk from pregnant animals contains considerable quantities of natural hormones, more abundant than in non-pregnant cows, it seems pertinent to consider if it is better to consume milk from non-pregnant animals. In the case of fatty acids, the non-pregnant milk is characterized by a high level of total SFAs, low level of total MUFAs and CLA but rich in essential fatty acids, EPA and DHA. However, other components of milk must be assessed to draw a clear conclusion. Leaving aside the possible negative aspects of milk, the fact that is a product full of important nutrients for humans should not be obviated.

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DETERMINATION OF IMIDACLOPRID AND METABOLITES RESIDUES IN LIVER, KIDNEY AND MUSCLE BY LC-MS/MS

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Abstract

Imidacloprid is a new and potent nitromethylene insecticide with high insecticidal activity at very low application rates. It is the first highly effective insecticide that, like nicotine, acts on the central nervous system, causing blockage of postsynaptic nicotinergic acetylcholine receptors. This analyte has a residue maximum limit by the Codex of 300 µg kg^{-1} for liver and kidney and 100 µg kg^{-1} for muscle. An LC-MS/MS with electrospray method was developed for measuring imidacloprid and its metabolites (5-hydroxy-imidacloprid, olefin-imidacloprid, 6-chloronicotinic acid, imidacloprid-urea) in animal tissue samples using ethirimol as internal standard. Imidacloprid and its metabolites were extracted from samples by a liquid–liquid extraction procedure with methanol and a subsequent HLB solid-phase extraction. The chromatographic separation was achieved by a reverse-phase column C 18 (2.1 mm i.d. 50 mm, 1.8 µm) with water and acetonitrile (90:10) with 0.1% formic acid as the mobile phase run at a 0.3 mL min^{-1}. The analytes were detected by a triple quadrupole detector in MRM mode.

Introduction

Imidacloprid belongs to a new chemical class of active ingredients, the chloronicotinyls (neonicotinoids). First it was designed as an agricultural pesticide and then used as an animal antiparasite (Adams, 2003). Some advantages are that this substance has a new mode of action, outstanding biological efficacy, a broad spectrum activity, low toxicity to warm-blooded animals and a good plant compatibility (Ishaaya and Degheele, 1998).

More than 90% of the intravenously administered dose is eliminated within 24 hours (Klein, 1987a). The distribution is carried out quickly into the tissues of higher perfusion. Except adipose tissue, central nervous system and the mineral part of bones, imidacloprid has been found in all tissues five min after intravenous administration and one hour after oral administration, using the technique of radiolabels and revealed with autoradiography X-ray (Klein, 1987b).

The high rate of elimination suggests that imidacloprid does not generate bioaccumulation of this drug or its metabolites. Imidacloprid is biotransformed first by oxidation after which the major metabolite found is 6-chloronicotinic acid. Imidacloprid is a substance with very low toxicity in mammals with Dl 380-650 mg kg^{-1} in rats and 120 to 170 mg kg^{-1} LD50 in mice when administered orally.

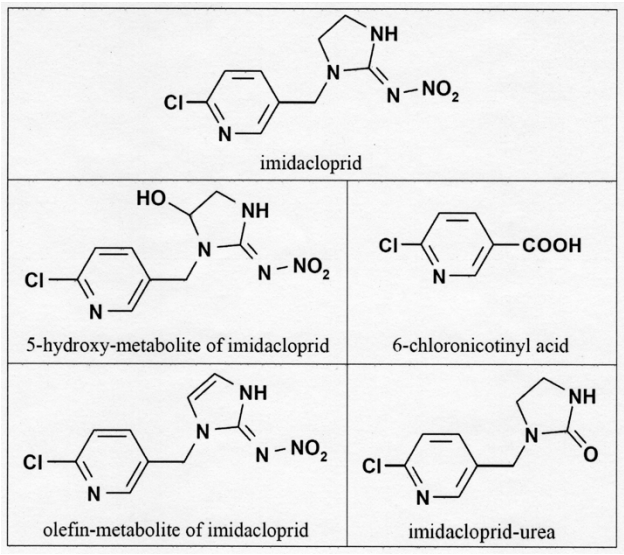


Figure 1. Structures of imidacloprid and metabolites.

Materials and Methods

Chemicals and reagents

Imidacloprid was purchased from Sigma-Aldrich; oleofinic metabolite and 5-hydroxyimidacloprid were obtained from Bayer CorpScience AG and 6-chloronicotinic acid, urea imidacloprid and ethirimol were purchased from Dr. Ehrenstorfer GmbH. Each standard compound was dissolved in acetonitrile (1 mg mL^{-1}) and stored at -18°C.

Acetonitrile (HPLC-grade) and water (HPLC-grade) were filtered through a 0.22 µm nylon filter before use. Hexane, acetonitrile, ethyl acetate and methanol ACS grade were used in the process of extraction and purification and solid phase extraction (SPE) columns HLB (200 mg, 6 mL) were used for the purification.

Instrumentation

The chromatographic system used was a UHPLC Agilent Technologies 1290 Infinity with a reverse-phase column C18 (2.1 mm i.d. 50 mm, 1.8 µm) using the following conditions: injection volume 10 µL, oven temperature 40°C and a flow rate of 0.3 mL min^{-1}. Stop time is 6 min and post-time is 4 min. The gradient used in the UHPLC method is listed in Table 1.

Table 1. Gradient used in the UHPLC method.

Time (min)	Solvent B % [a]
0.00	10
0.50	10
0.75	15
4.00	15
4.01	70
5.50	70
5.51	10

[a] *Solvent B: acetonitrile and 0.1 % formic acid and Solvent A: water and 0.1% formic acid.*

Mass spectrometry (MS) detection was carried out by an Agilent Technologies 6490 triple quadrupole mass spectrometer with electrospray ionization (ESI) performed in positive and negative mode in MRM (Multiple Reaction Monitoring Mode) with the following conditions: capillary voltage: 3.5 kV; gas temperature: 200°C; sheath gas heater: 400°C; gas flow: 14 L min^{-1}; nebulizer 20 psi; collision gas: nitrogen 5.0.

Table 2. MS/MS-Transitions for Imidacloprid and Imidacloprid metabolites.

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (ms)	Collision Energy (eV)
Imidacloprid	256	209	50	11
Olefin-imidacloprid	252	205	50	7
5-hydroxy-imidacloprid	272	191	50	15
6-chloronicotinyl acid	158	122	50	19
Urea-imidacloprid	212	128	50	23
Ethirimol	210	55	50	43

Sample preparation

In each propylene centrifuge tube containing 1 g of sample material, 200 µL of internal standard (IS) solution were added and mixed in a vortex for 30 s. For the sample extraction, 5 mL of methanol were added. Afterwards the tubes were shaken for 15 min using a vortex mixer. Then the extract was centrifuged for 15 min at 3,000 rpm at 10°C and supernatant was transferred to a 10 mL glass centrifuge tube and evaporated to dryness under a slow stream of air at 50°C. The dried extracts were reconstituted in 1,000 µL of a mixture hexane (60):ethyl acetate(40), and transferred onto an OASIS HLB cartridge, which was preconditioned with 6 mL of mixture hexane(60):ethyl acetate(40). The residues were eluted with 6 mL acetonitrile and evaporated to dryness under a slow stream of air at 50°C. The dried extracts were reconstituted in 1,000 µL of mobile phase, filtered through 0.22 µm nylon filter and were injected into the LC-MS/MS system.

Internal Standard ethirimol along with the mix of the five analytes were used to prepare the matrix calibration curve. All matrix calibration curves were constructed at the concentrations of 75, 150, 300, 450 and 600 µg kg^{-1} in blank *bovine* samples of liver and kidney and 25, 50, 100, 150 and 200 µg kg^{-1} in blank *bovine* muscle samples.

Results and Discussion

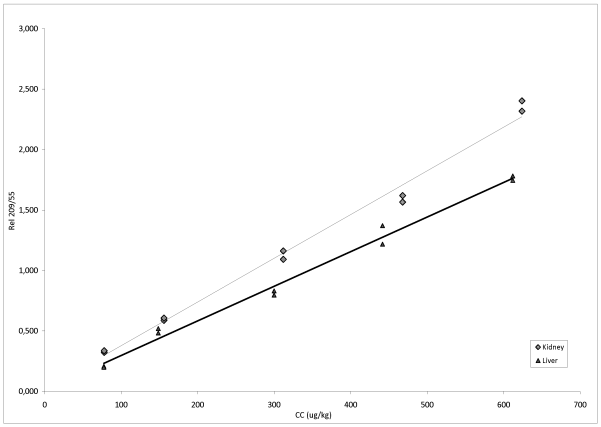
The MRM transitions and associated acquisition parameters were optimized for the maximum abundance of fragment ions under ESI positive and negative mode conditions by infusing standard solution of the target compounds into the tandem mass spectrometer and were analysed using the MassHunter Workstation Software Optimizer by Agilent Technologies.

Figure 2. Typical MRM chromatograms obtained from a bovine muscle sample spiked at 100 µg kg^{-1} and internal standard at 50 µg kg^{-1}.

Specificity was found to be satisfactory, with no chromatographic interference being observed around the retention time of the target compound. The Identification was done with the relation between the two most abundant ion transitions of each analyte and with the retention time.

All matrix calibration curves for imidacloprid and metabolites were linear from 75 µg kg^{-1} to 600 µg kg^{-1} for kidney and liver and from 25 µg kg^{-1} to 200 µg kg^{-1} for muscle. The correlation coefficient values (R) were all above 0.985 and linear regression results are shown in Figures 3 and 4, and Table 3.

Analytical recovery was tested though out analytical range and was determined by comparing the representative peak areas of the analytes extracted from drug-free tissues spiked with the target compounds with the peak area of matrix calibration curves. The average recovery is shown in the Table 4.



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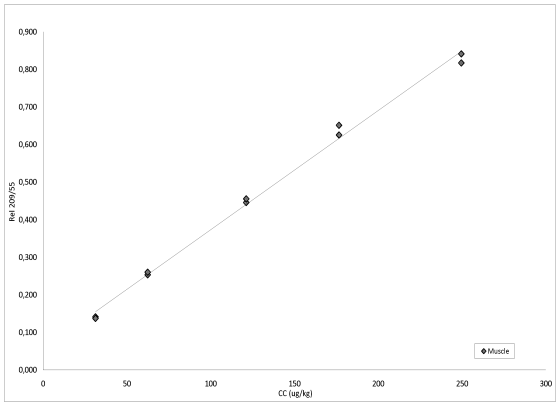

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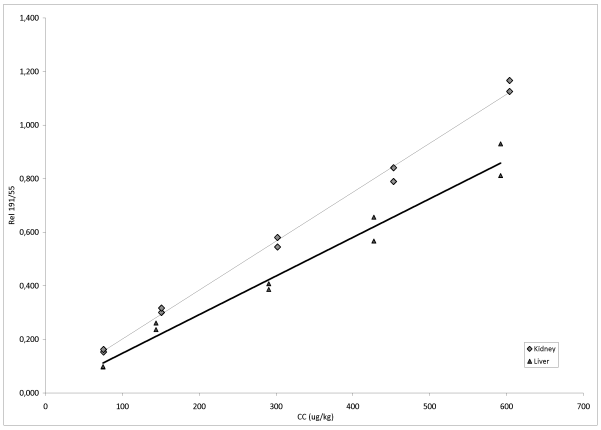

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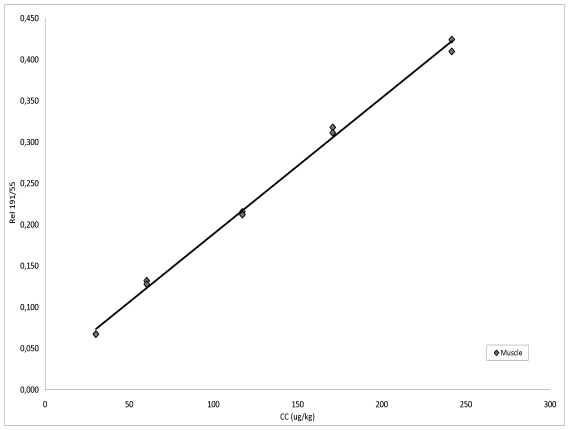

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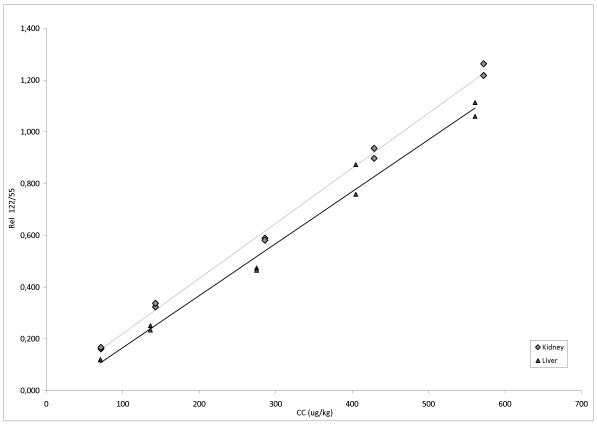

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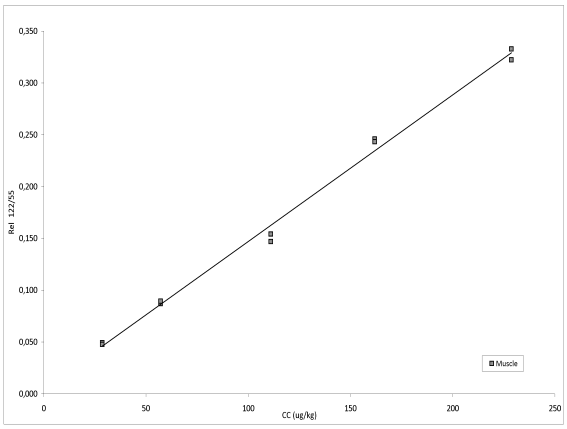

(f)

Figure 3. Matrix calibration curves for: (a) imidacloprid in liver and kidney; (b) imidacloprid in muscle; (c) 5-hydroxy-imidacloprid in liver and kidney;, (d) 5-hidroxi-imidacloprid in muscle; (e) 6-chloronicotinyl acid in liver and kidney and (f) 6-chloronicotinyl acid in muscle.

Table 3. The correlation coefficient values (R) obtained in the linear regression for imidacloprid and metabolites.

Compound	Liver	Kidney	Muscle
Imidacloprid	0.9950	0.9949	0.9975
Olefin-imidacloprid	0.9931	0.9858	0.9951
5-hydroxy-imidacloprid	0.9890	0.9973	0.9983
6-chloronicotinyl acid	0.9938	0.9983	0.9971
Urea-imidacloprid	0.9852	0.9981	0.9951

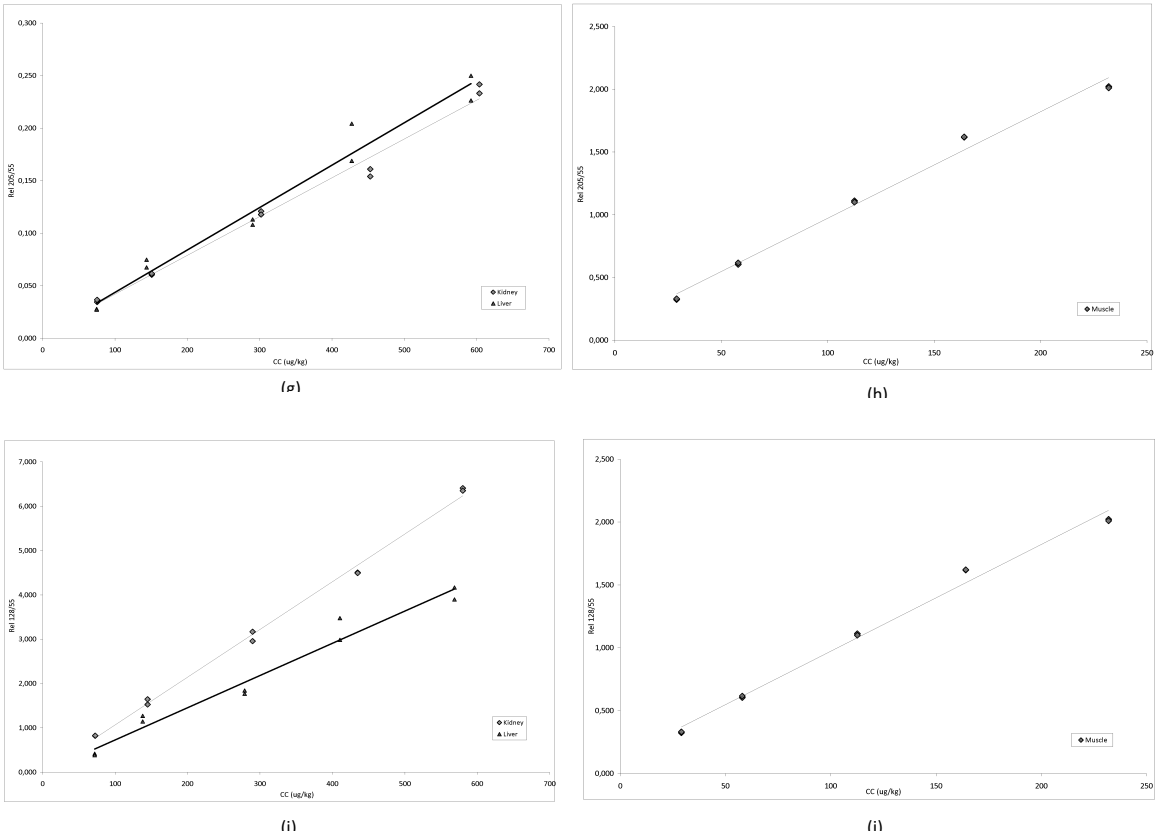

Figure 4. Matrix calibration curves for: (g) olefin-imidacloprid in liver and kidney, (h) olefin-imidacloprid in Muscle, (i) urea-imidacloprid in liver and kidney, and (j) urea-imidacloprid in muscle.

Table 4. Mean recovery for all of the compounds in the linear range.

Compound	Liver		Kidney		Muscle	
	R %	RSD %	R %	RSD %	R %	RSD %
Imidacloprid	100	8.5	101	16.2	103	10.6
Olefin-imidacloprid	99	12.2	100	17.8	107	12.6
5-hydroxy-imidacloprid	99	10.8	100	20.5	105	13.6
6-chloronicotinyl acid	97	12.2	102	17.4	107	8.7
Urea-imidacloprid	100	11.2	102	13.1	102	7.5

Conclusions

A rapid and sensitive multi-residue method based on an SPE extraction procedure in HPLC-MS/MS analysis, has been developed for imidacloprid and its four metabolites in *bovine* muscle, liver and kidney samples.

The most notable characteristic of this method is the run time of only 6 min with a flow rate of 0.3 mL min^{-1}, using only 1.8 mL of mobile phase per run achieving an environmental-friendly method.

The method obtained good recoveries, accuracy and precision according to the Commission Decision 2002/657/EC requirements.

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DETERMINATION OF ANDROGENIC STEROIDS IN URINE BY GC-MS

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Abstract

Androgenic steroids are prohibited for use in food-producing animals according to Resolution N° 447/2004 by SENASA (Argentina) and Council Directive 96/23/EC. These substances have no MRLs and follow the MRPL recommendations of CRL guidance paper (7 December 2007). A GC-MS (Single Quad) method was validated to measure 17β-19 Nortestosterone (β-NTT), 17α-19 Nortestosterone (α-NTT), 17β-Boldenone (β-BLD) and Methyltestosterone (MTST) in animal urine samples using 19-d3-Testosterone and d3-Boldenone as internal standards. The validation method was performed according to the Commission Decision 2002/657/EC requirements and regulations of SENASA, obtaining linear calibration curves in urine for α-NTT and MTST from 1 to 8 μg L^{-1} and for β-NTT and β-BLD from 0.6 to 5 μg L^{-1}. The method includes an enzymatic hydrolysis step using β-glucuronidase type H-1 and, subsequently, HLB solid-phase extraction. The eluate obtained with methanol was evaporated to dryness and the residue was reconstituted in phosphate buffer. The purification was continued with C18 solid-phase extraction. The cartridge was eluted with ethyl ether and the eluate was further purified by a liquid - liquid extraction using a NaOH solution. Finally, the extract is derivatised with a mixture of acetone/HFAA, reconstituted in isooctane and analysed by a GC-MS with electron impact (EI) at 70eV in SIM mode for qualification and quantification.

Introduction

Androgenic anabolics are steroids derived from testosterone, which is an endogenous human male hormone. These steroids act in different parts and tissues in the organism. The term anabolic refers to the growth-promoting effect of these substances in muscle, while the term androgenic refers to the promotion and maintenance of the male sex characteristics. The chemical structures of the androgenic steroids mentioned in this study are depicted in Figure 1.

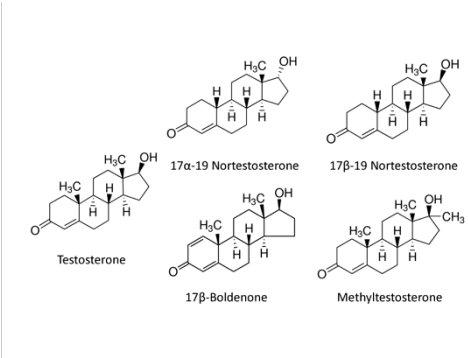

Figure 1. Chemical structures of studied androgens.

The steroid 17β-19 Nortestosterone, also known as nandrolone, has been used for a long period of time in humans and in more recent times in veterinary medicine. The finding of this substance in urine was considered proof of exogenous administration. In 1984 endogenous occurrence was proven in stallions and later on in boars. A few years later, natural production of β-NTT was found in swine as well as α-NTT in pregnant cows and in neo-natal calves.

The studied substances have no maximum residue level (MRL), therefore the guidelines of CRL GUIDANCE PAPER (7 December 2007) for the EU Laboratories referring to the minimum performance limits were followed during this method validation.

Materials and Methods

Androgenic Steroids

Methyltestosterone was purchased from Dr. Ehrenstorfer GmbH. α-Nortestosterone, β-nortestosterone glucuronide (potassium salt) and β-boldenone glucuronide (potassium salt) were obtained from the National Measurement Institute of Australia (NMI). 19-d3-Testosterone (d3-TST) and d3-boldenone (d3-BLD) which were used as internal standards, were also obtained from NMI.

Chemicals

Sodium acetate trihydrate was purchased from BioPack. β-Glucuronidase enzyme derived from *Helix pomatia* (Type H-1) partially purified powder (≥300,000 units g^{-1} solid) and heptafluorobutyric acid anhydride (HFAA, ≥98%) were purchased from Sigma-Aldrich. Methanol, acetone and ethyl-ether were purchased from Sintorgan. Hexane and sodium hydroxide were purchased from Anedra. 2,2,4-Trimetilpentane (isooctane) pesticide grade was purchased from U.V.E. Bidistilled water was used with conductivity less than 2 µS cm^{-1} and total dissolved solids less than 0.2 µg mL^{-1}. All solvents and reagents were analytical grade. Solid-phase extraction (SPE) Oasis HLB (200 mg, 6 mL) and C18 (500 mg, 3 mL) columns were purchased from Waters and Phenomenex, respectively.

Standards

Stock solutions and working solutions for each standard were prepared in methanol. The working solution mix contained approximately 400 ng mL^{-1} of α-NTT and MTST and 200 ng mL^{-1} of β-NTT and β-BLD (as free substance). The internal standard solution (IS) contained 800 ng mL^{-1} d3-TST and 200 ng mL^{-1} of d3-BLD. Stock solutions are kept for 5 years at -18°C and working solutions are kept for 2 years at -18°C.

Samples Preparation

The validation was performed with *bovine* urine free of androgenic steroids and of suspended solids. The samples were kept at -15°C. Ten mL of urine in a Falcon tube were adjusted to pH 5.2 using glacial acetic acid before the addition of 1 mL of β-glucuronidase enzyme solution in 2 M sodium acetate buffer pH 5.2 (1,400 U mL^{-1}). After 30 s of vortex shaking, the urine was incubated overnight at 37°C. HLB cartridges were conditioned prior to use by the sequential addition of 5 mL methanol and 5 mL water.

The cooled sample was quantitatively applied to the cartridge. It was washed with 10 mL water and 10 mL methanol:water (40:60) and then left to dry for 3 min under vacuum. The final washing step was with 10 mL hexane and left to dry again for 3 min under vacuum. The sample was eluted into a glass tube (15-mL capacity) with 6 mL methanol and evaporated to dryness at 55°C under a stream of air. The pellet was suspended in 0.2 mL methanol, shaken for 1 min and 3 mL 2 M acetate buffer pH 5.2 were added prior to purification with a C18 SPE column. This column was conditioned prior to use sequentially with 10 mL methanol and 10 mL water. The sample was applied quantitatively to the cartridge, which was then washed with 10 mL water, 10 mL methanol:water (50:50) and left to dry for 3 min under vacuum. The column was then washed with 10 mL hexane and left to dry again for 3 min under vacuum. The eluate was collected with 4 mL of ethyl ether in a glass tube (15 mL capacity) and purified by two liquid-liquid extractions adding 1 mL 1 M sodium hydroxide solution. Each time after shaking, the aqueous layer was removed. After evaporating the glass tube to dryness at 40°C under a stream of air, 500 µL of methanol are added, shaken with vortex for 1 min to transfer into a glass vial and then dried again. The sample was then reconstituted and derivatised in 50 µL acetone/HFAA (4:1), vortexed for 1 min, left in a water bath for 60 min at 60°C, then evaporated to dryness at 55°C under a stream of air before being reconstituted in 30 µL of isooctane to be injected in the GC for further analysis.

GC-MS conditions

An Agilent Technologies Model 5975C mass-spectrometer coupled to a gas chromatograph (Agilent Technologies Model 7890) equipped with an automatic injector and an HP-5MS column of 30 m longitude and 0.25 µm film thickness was used. The injector was set to 220°C. Temperature programming started from 100°C (1 min), the first ramp increased 10°C min^{-1} up to 205°C (5 min), the next ramp increased 8°C min^{-1} up to 260°C (5 min) and the last ramp increased 30°C min^{-1} up to 280°C (5 min), with a flow rate of 0.7 mL min^{-1}. The interface temperature was 290°C, MS Source/ MS Quad temperatures were 230°C/150°C with EMV Delta of 506 V and ionization energy of 70 eV.

For the selected ion monitoring (SIM mode) shown in Table 1, a scan of each analyte at 1 µg mL^{-1} was carried out to select the most abundant ions. Figure 2 shows the SCAN for β-BLD.

Table 1. Monitored Ions for the analysis of each androgenic steroid.

Analyte	Quantifier Ion (*m/z*)	Qualifiers Ion (*m/z*)
α/β-NTT	666	453, 318, 306
β-BLD	464	678, 369, 343
MTST	465	480, 369, 355
d3-TST	683	
d3-BLD	681	

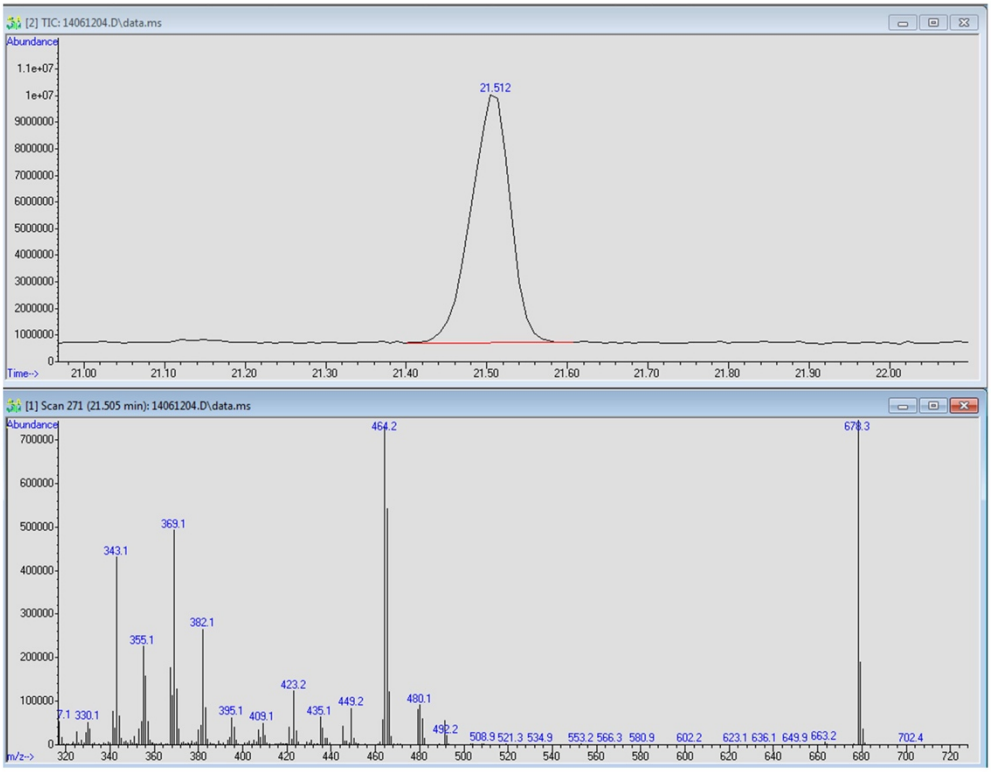

Figure 1. β-BLD SCAN at concentration of 1 µg mL^{-1}.

Results

During method development, different options were evaluated in order to optimize chromatographic separation and detection parameters as well as sample extraction.

Linearity

The calibration curve was done in spiked matrix and treated as samples. The analytical range was selected considering the first level of the curve as well as the lowest spiked samples to achieve the Minimum Performance Level Required (MPLR) and taking into account that this point must comply with all qualification criteria in GC-MS (according to Ratio Area: Qualifier Ion /Quantifier Ion). Spike volumes for the matrix calibration curve were 0, 25, 50, 100, 150 and 200 µL of androgenic solution and 50 µL of IS solution in all samples. Each level was injected thrice. Linearity curves are depicted in Figures 3 and 4.

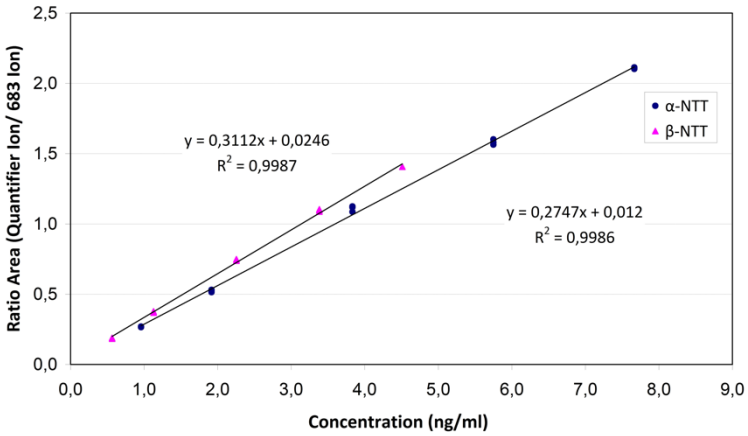

Figure 3. Linearity of α-NTT and β-NTT with d3-TST.

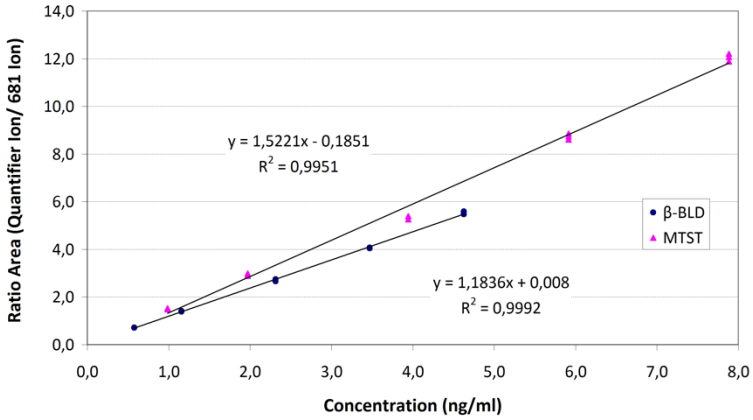

Figure 4. Linearity of MTST and β-BLD with d3-BLD.

Repeatability and Reproducibility

Figure 5 shows selected ion monitoring (SIM) for each analyte corresponding to 25 µL androgenic mix solution. Two assays were performed by two analysts in different weeks with matrix calibration curves at five concentration levels (Table 2). Table 3 shows reproducibility values for each concentration level, global reproducibility of the method and expanded uncertainty using a factor of k=2 to obtain a 95% confidence level.

Table 2. Validation parameters obtained for each androgenic steroid.

Analyte	Analytical range (µg L^{-1})	Average recovery %	LD o CCα (µg L^{-1})	CCβ (µg L^{-1})
α-NTT	0.96 – 7.97	96.2	0.32	0.55
β-NTT	0.56 – 4.51	95.8	0.12	0.26
β-BLD	0.58 – 4.63	100.4	0.11	0.13
MTST	0.99 – 7.88	99.5	0.19	0.34

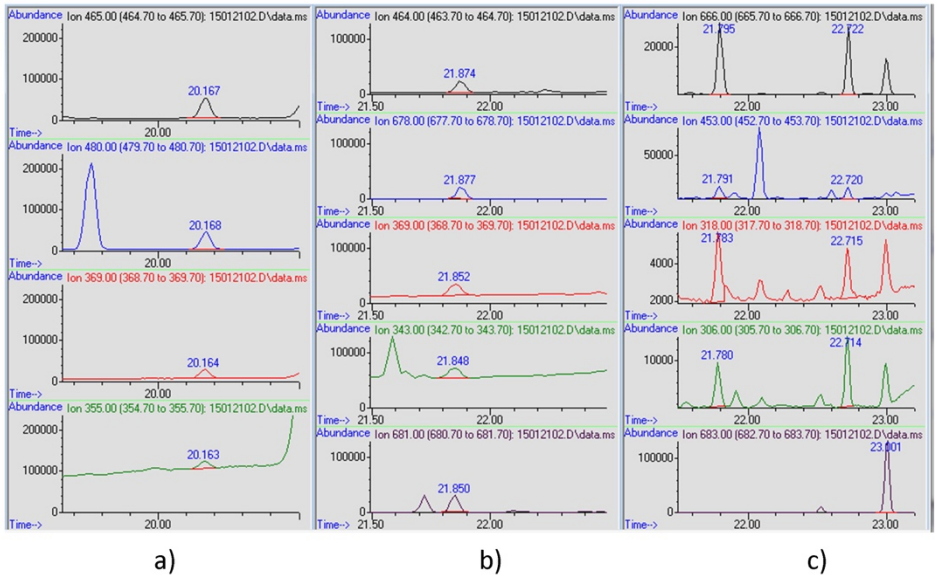

Figure 5. a) Chromatogram of MTST (RT: 20.167 min); b) Chromatogram of β-BLD and d3-BLD (RT: 21.874 min); c) Chromatogram of α-NTT/ β-NTT and d3-TST (RT: 21.795/22.722 and 23.001).

Verification of minimum confirmation level

A matrix calibration curve was performed along with twenty samples spiked at the lowest level of the analytical range, in order to demonstrate that this concentration is detected and to ensure correct identification by qualifier ions. Results show a 100% of identified samples as well as correct quantification within acceptance criteria.

Table 3. Total reproducibility and expanded uncertainty of the method through the analysis of five concentration levels for four compounds by two analysts.

		Level 1 (CV%)	Level 2 (CV%)	Level 3 (CV%)	Level 4 (CV%)	Level 5 (CV%)	CV% Global	Uncertainty (%)
α-NTT	Analyst 1	10.2	9.7	12.3	1.4	6.1		
	Analyst 2	2.1	3.2	5.3	1.9	7.9	9.62	19.24
	Total	14.4	6.7	9.1	5.3	6.4		
β-NTT	Analyst 1	7.6	8.4	9.4	0.78	3.2		
	Analyst 2	1.0	2.9	3.8	3.1	4.0	9.01	18.03
	Total	15.7	5.7	6.7	4.6	3.2		
β-BLD	Analyst 1	2.3	2.3	2.8	2.9	3.8		
	Analyst 2	0.95	1.3	3.9	2.6	1.1	5.47	10.94
	Total	2.3	4.1	3.4	2.8	2.6		
MTST	Analyst 1	5.8	5.3	9.7	3.2	5.6		
	Analyst 2	6.5	14.2	2.5	8.6	5.9	7.73	15.45
	Total	9.2	9.7	6.5	6.2	5.9		

Selectivity

A matrix calibration curve was performed along with twenty *bovine* urine samples from different origin spiked with internal standard, in order to find possible interferences in the chosen method. None of the tested samples showed any interference for the selected ions at the retention time of each studied analyte.

Ruggedness

Ruggedness was studied using eight samples simultaneously with a matrix calibration curve. Five parameters were modified in the clean-up process according to Youden and Steiner Test. Selected parameters are shown in Table 4.

Table 4. Ruggedness parameters.

Factor Value F	Modifying factors in sample preparation
A	10 mL *bovine* urine
a	10 mL *equine* urine
B	Wash column HLB with 10 mL methanol:water (40:60)
b	Wash column HLB with 10 mL methanol:water (60:40)
C	Elute column HLB with 6 mL methanol
C	Elute column HLB with 4 mL methanol
D	Resuspended in 3 mL buffer pH 5.2
d	Resuspended in 2 mL buffer pH 5.2
E	Wash column C18 with 12 mL methanol:water (50:50)
e	Wash column C18 with 8 mL methanol:water (50:50)

Results indicate that there are no critical points for α-NTT. For β-NTT, the critical points are the reconstitution in buffer pH 5.2 and the C18 column wash with methanol:water (50:50). β-BLD the only not critical point is the HLB column wash with methanol:water (60:40), while the critical points for MTST are urine type and C18 column wash with methanol:water (50:50).

Discussion

Some assays prior to this validation were performed using C18 SPE columns (1000 mg, 6 mL) These were replaced by HLB SPE columns (200 mg, 6 mL) adding the washing step with methanol:water (40:60) in order to achieve cleaner extracts and lesser chromatographic interferences. At the beginning of the validation the methylboldenone standard (MBLD) was not in stock so it could not be included in this study. Once it was acquired, a matrix calibration curve was carried out using the same internal standards mentioned in this work, in an analytical range of 1 ng mL^{-1} to 8 ng mL^{-1}, monitoring ions 367 as quantifier and ions 478, 463 and 435 as qualifiers. Figure 6 shows results for MBLD with d3-TST and d3-BLD.

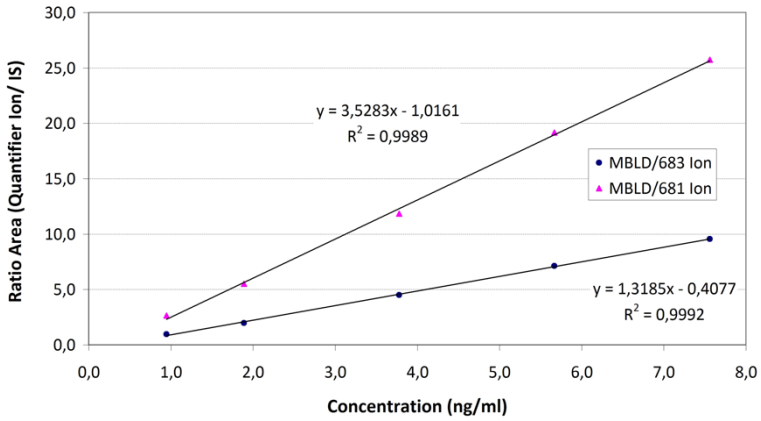

Figure 6. Matrix calibration curve for MBLD with d3-TST and d3-BLD in urine matrix.

Conclusions

All results obtained for the studied parameters are in accordance with SENASA Resolution N° 138/2002 and its modifying dispositions Nº 125/2006 and Nº 06/2004, as well as EU Council Directive 2002/657/EC.

As for the analytes found to be critical points in the ruggedness study for type of urine (*bovine* or *equine*) a further analysis is necessary evaluating matrix calibration curves of both species simultaneously in order to examine the observed differences more closely.

This method also demonstrates its effectiveness for the identification and quantification of methylboldenone in urine by GC-MS analysis.

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ENHANCING FOOD SAFETY LABORATORY CAPABILITIES AND ESTABLISHING A NETWORK IN ASIA TO CONTROL VETERINARY DRUG RESIDUES AND RELATED CHEMICAL CONTAMINANTS

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Abstract
Many countries in Asia face competition for global food markets and must ensure a safe and quality supply of agricultural products. They must also meet importing-country food safety and animal health requirements. For net importers in the region, effective verification mechanisms are needed to ensure safe and quality food imports. Thus, functional laboratories that meet international and national standards are necessary. While some countries in Asia have been more successful meeting standards and strengthening residue monitoring programs, others require support to improve. Therefore, starting 2016, the IAEA's technical cooperation program and the Joint FAO/IAEA Division for Nuclear Techniques in Food and Agriculture will support a regional project to enhance food safety laboratory capabilities and help establish a network, to improve the control of veterinary drug residues and related contaminants in Asia. Countries at varying levels of development will have a platform to interact closely and identify solutions to common challenges. Bangladesh, Indonesia, Jordan, Lao P.D.R., Lebanon, Malaysia, Mongolia, Oman, Pakistan, Papua New Guinea, Philippines, Singapore, Sri Lanka, Thailand and Viet Nam are involved in the project. Others countries such as Bahrain, China and Kuwait have also expressed interest, while post-soviet states are also most welcome. Resource countries such as The Republic of Korea and Japan are also encouraged to support the project. Participants will leverage benefits of isotopic, nuclear and related analytical tools and techniques to control residues of veterinary drugs in food stuff. The project counterparts will coordinate analytical and collaborative activities at ERVIII.

Introduction
Many IAEA Member States in Asia require effective verification mechanisms to ensure safe foods are available for local or international consumers. The region contributes a large share of the growing aquaculture industry which supplies over 50% of the global animal protein (FAO, 2004). Functional food safety laboratories, that meet international standards are required to ensure food safety and safeguard consumers.

While some countries in Asia have strengthened their national residue monitoring programs, more require support to improve. A regional project brings together better-developed or equipped and less developed laboratories to complement each other. While international trade has been a major influence on country decisions to improve their laboratory capabilities and therefore food safety initiatives, the growing middle class and the process of urbanization have resulted in increasing demands for a safe and quality local food supply. Producers and regulatory programs cannot ignore this trend of events for the sake of public health and the economy. The growing aquaculture industry also implies that animal health including feed safety and quality is also increasingly appreciated as an integral part of food safety due to the inevitable use of agrochemicals to prevent or control diseases in the industry (Tacon and Metian, 2008; FAO, 2014). Residues of such chemicals end up in the food chain thus endangering consumers hence the need to monitor them.

This regional project will enhance capabilities of food/feed safety laboratories and also help establish a network of laboratories that control veterinary drug residues, pesticides, mycotoxins and related chemical contaminants in agricultural products including aquaculture. The laboratories and network will leverage the comparative advantages of nuclear and isotopic analytical techniques. The project presents an opportunity for potential collaborative or joint regional/sub-regional residue monitoring programs. The capacities built and enhanced performance based on ISO/IEC standards will enhance opportunities for countries to meet international market demands for effective control of various chemical contaminants. In the network, institutions will share expertise, resources and experiences. Countries with lesser capabilities will learn from those better off.

Objective analysis
Local public health and international markets demand safe and quality foods that may be ensured through national residue programs driven by a functional and efficient food safety laboratory of international repute. Use of isotopic and nuclear based analytical tools/techniques (such as radio receptor assays, stable isotopes, radio-immuno assays etc) will be promoted. More competent laboratories with better capabilities will be supported to provide greater leadership in the network/region.

Stakeholders and partnerships
The project will seek buy-ins from governments, the FAO region offices, WHO, OIE, WTO, UNIDO, Asia Development Bank and related organizations interested in public health, food safety or security and trade matters in the region. Farmers and producers who will also be instrumental in providing analytical samples and consumers in support for advocacy and awareness, will benefit. Food safety laboratories, whose capabilities will be advanced or strengthened through the project, will be active in collecting and analysing food/feed samples, disseminating findings and informing good agricultural practices and relevant policies as applicable. Institutional capabilities will be enhanced. National regulatory agencies and institutions, farmers and producers, food exporters, research institutions among others, will benefit as laboratories become more functional and gain greater reputation. Consumers will have access to safer and good quality foods and the competitiveness of food exports will be enhanced as a result of a stronger and sustainable laboratory network. There will be increased surveillance and levels of safer animal feeds.

Materials and Methods - Implementation

Participation
To-date food safety institutions associated with control of chemical residues (particularly veterinary drugs) in the following countries are taking part: Bangladesh, Indonesia, Jordan, Lao P.D.R., Lebanon, Malaysia, Mongolia, Oman, Pakistan, Papua New Guinea, Philippines, Singapore, Sri Lanka, Syria, Thailand and Viet Nam. Others interested include Bahrain, China and Kuwait while the project is also open to post-soviet states.

Routine application of isotopic/nuclear based analytical tools and techniques such as the use of radio-receptor assays, stable isotopes along with chromatographic and spectrometric techniques as well as radio-immuno assays among others will be promoted. The project will also involve elemental analyses with ICP-MS (including isotope dilution/ratio) and Atomic Absorption Spectroscopy/Spectrometry among others.

Training, Proficiency (PT) and inter-laboratory tests.
Group and individual training programmes will be undertaken using regional and international experts. International and IAEA experts will be involved in the capacity building process. Participants will undertake regional or international PT tests as well as inter laboratory studies to for instance support method development/validation and application of analytical techniques for residue monitoring. The project will also promote the twining of participants to facilitate exchange of knowledge and experiences.

Joint meetings, workshops and seminars
The project counterparts will participate in joint technical meetings to exchange knowledge and experience and coordination meetings to plan for, evaluate and review project activities. They will also participate in international scientific meetings/conferences such as the EuroResidue VIII conference to harmonize common activities and enhance knowledge. Activities such as the EuroResidue VIII pre-conference workshop will help participants acquire new knowledge relevant to the project.

Promoting excellence
The project will identify leaders in the region and enhance their capabilities so that they become regional centres of excellence, providing regional training among others. There will also be twinning of countries to promote sharing of expertise between experienced or better equipped institutions and those in greater need.

Results (Expected) and Discussion

Project outputs
Regional collaboration among food safety laboratories will be established to monitor veterinary drug residues and related chemical/natural contaminants in foods. The indicator will be the establishment of a web platform by the second quarter of 2016. This will be used to share information such as analytical methods and relevant literature among others. Evidence of interaction between food safety laboratories with non-technical stakeholders will also be used as an indicator. Through the collaborative network, laboratory practices will be harmonized in monitoring veterinary drug residues and related chemical/natural contaminants in foods.

Institutional and analytical capabilities will have been built or strengthened and rapid alert systems initiated to help countries urgently address food safety concerns. The capabilities and systems are critical in ensuring timely awareness and prevention of emerging chemical and biochemical food risks (Kleter *et al*., 2009). Human resource will be strengthened in the participating countries to support residue monitoring programs. Through joint meetings the project counterparts will plan and review relevant activities in the region. For instance, the group will meet at the EuroResidue VIII conference to discuss the implementation of common activities while enhancing analytical knowledge. Such meetings will also facilitate closer interactions

with peers and acquaintance with recent advances and emerging issues in food safety, public and environmental health as well as trade. Laboratory competence will be enhanced during and at the end of the project, through activities such as PT and inter-laboratory tests. These tests will support countries in their accreditation process.

Establishment of databases for contaminants and pro-active reconnaissance can facilitate the identification of contaminated products (Kleter *et al*., 2009) and this can be promoted through this regional project. Also for purposes of international trade, prevention and early identification of hazards supported by a combination of quality management systems, relevant legislation and systematic inspections - which are some of this project's goals - will enhance exports and protect consumers in Asia (Kleter *et al*., 2009). Various contaminants - including those associated with feed - addressed by the project will include mycotoxins, veterinary drug and pesticide residues, persistent organic pollutants, agrochemicals and metals among others. These are important to the region (Tacon and Metian, 2008).

Centre(s) of excellence
The project will also identify, promote and facilitate two to three suitable centre(s) of excellence that will continue to enhance institutional capacity building and proficiency. This will include hosting of individual and group training programmes.

Overall project outcome
Ultimately the project will enhance effective monitoring and control of veterinary drug residues and related contaminants in Asia including the formation or strengthening of a laboratory network. Milestones will include, a minimum of ten countries in the region collaborating and undertaking joint activities or using harmonized techniques by 2019 to support food control programs in the regions. National and sub-regional laboratory collaboration will also be in place as building blocks for the regional networks.

Conclusions
A technical cooperation project (2016-2019) supported by the Joint FAO/IAEA has been initiated to enhance food safety laboratory capabilities (including the establishment of a laboratory network) in Asia to facilitate effective monitoring and control of veterinary drug residues and related chemical contaminants in foods. The project will commence by participating at the EuroResidue VIII conference and then hold its first coordination meeting thereafter to ensure effective implementation. Bangladesh, China, Indonesia, Jordan, Lao P.D.R., Lebanon, Malaysia, Mongolia, Oman, Pakistan, Papua New Guinea, Philippines, Singapore, Sri Lanka, Thailand and Viet Nam will be involved. Interested participants from other countries such as Bahrain, China, Kuwait as well as post-soviet states will be welcome.

Acknowledgements
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MARKED HISTOLOGICAL CHANGES IN THYMUS GLAND OF DAIRY COWS TREATED WITH RBST; RESULTS OF THREE ANIMAL EXPERIMENTS

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Abstract
Bovine growth hormone, also called *bovine* somatotropin (bST), may be used for dairy cattle to induce a higher milk yield. In some countries it is an allowed practice to inject the cows with recombinant somatotropin (rbST). In the European Union its administration is forbidden. To get data on effects of rbST on levels of biomarkers in milk, serum and blood three animal experiments have been performed in 2008, 2011 and 2015. In total 16 animals have been treated with bST (Posilac and in the 2015 experiment also 2 animals with Boostin S) and 3 control animals were used. During the experiments, milk and serum was sampled (data not shown) and at slaughter thymus was sampled for histology.
In all our experiments thymus of treated cows showed regeneration of both cortex and medulla, whereas in the control animals the thymus was involuted and consisted of mostly fatty tissue with some remnants of thymic tissue. For comparison with more control animals, a number of thymus glands from slaughtered normal dairy cows were collected, which also showed involuted thymus tissue. From our findings it appeared that the thymus gland of the adult dairy cows changed due to the growth hormone injections and got histological features of calf thymus. To our knowledge this is the first time that thymus rejuvenation is described in dairy cows. For routine screening of illegal administration of rbST to adult cattle histological control of thymic tissue appears a promising method.

Introduction
Growth hormone or somatotropin (ST) is a naturally occurring hormone secreted by the pituitary gland in mammals. Growth hormone affects growth, production and body composition. The effects include anabolic action leading to increased lean body mass and decreased fat. These properties have led to abuse by athletes and in animal production (Courtheyn *et al.*, 2002). ST exerts is biological effects on target cells by binding to membrane receptors. It has both direct and indirect effects on peripheral tissues. Indirect effects are mediated by insulin-like growth factor-1 (IGF-1) which is primarily generated in the liver as response to GH. GH is increased by both oestrogens and androgens and decreased by β-agonists (Ehrnborg *et al.*, 2000).

Since the late 1980s, recombinant human, *bovine* and *porcine* ST have been used clinically (human) or for increased milk production (*bovine*) or performance (pigs). Recombinant *bovine* ST (rbST) is produced using recombinant technology and its structure is almost similar to the endogenous form. At present two major forms of rbST are commercially available. Posilac was developed by Monsanto and is sold by Eli Lilly – Elanco Animal Health division. Boostin-S is produced by LG Life Sciences. Posilac is the most commonly used formulation and shows a difference from the endogenous form (main variant) with the alanine in NH_2 terminal position (bST) replaced with a methionine (rbST) (Dervilly-Pinel *et al.*, 2014).

Effects of rbST in dairy cattle are an increased milk production with 11-15 % (Dohoo *et al.*, 2003a). But side effects are an increased incidence of health disorders such as mastitis, birth disorders, hoof problems, increased somatic cell count, etc. (CVMA, 1998; Dohoo *et al.*, 2003b; US FDA, 1993), which was the reason for Canada and the European Union to ban its use. An additional risk for human health is posed by the increased use of antibiotics in rbST treated cows, increasing the risk of development of antibiotic resistance (Zwald *et al.*, 2009). There is also concern about the potential effects of rbST on the development of cancer. This, because rbST raises the level of IGF-1 in cow' sera and milk and elevated levels of IGF-1 in humans are associated with higher rates of colon, breast and prostate cancer (Yu and Rohan, 2000).

Since the use of rbST is forbidden in Europe there is a need to develop methods to control illegal use (Dervilly-Pinel *et al.*, 2014). Except influencing the mammary function and growth of muscles, ST has a major function as activator of the thymus gland (Hirokawa *et al.*, 2016) to promote T cell differentiation in young animals. The thymus is large in young animals and is important for the development of the peripheral parts of the lymphoid system. When lymph nodes have achieved functional competence the thymus regresses.

The thymus gland has two parts, a cervical part that is placed ventral of the trachea, that is connected to a thoracic part situated above the hearth. In cattle it grows rapidly in the first 6-9 months of life and regression starts at puberty (Dyce and Wensing, 1971). With advancing age there is a loss of epithelial cells and thymocytes in the cortex and medulla, whereas the thymic space becomes filled with adipocytes (Taub *et al.*, 2010). For many years it was believed that post-adolescence the thymus becomes a fatty non-functional organ. Growth hormone levels decrease with age as thymic involution progresses. However, despite significant atrophy the aged thymus still retains the capacity to promote T cell differentiation and produce

de novo T cells (Taub and Longo, 2005). Regrowth of the thymus has been found to occur in rbST treated rats, mice and dogs (French *et al.*, 2002; Bar-Dayan and Small, 1994; Goff *et al.*, 1987), so in our experiments with rbST we sampled thymus of cows for histological evaluation of the thymus.

Material and Methods

Animal experiments

In the first experiment, three control cows and four treated cows were used. All cows were lactating Holstein Friesians, bought from the market. All cows were between four and six years of age, in their fourth or fifth lactation and had calved five months before the start of the experiment. The three control cows were treated with a subcutaneous injection with placebo and the four other cows were treated six times with two-weekly injections of Posilac (500 mg/ sometribove zinc suspension injection, Monsanto). The animals were slaughtered one month after the last treatment.

In the second experiment eight cows were treated for four times with two-weekly injections of Posilac (500 mg/ sometribove zinc suspension injection, Monsanto). All cows were lactating Holstein Frisians, not pregnant and varied in age (from 2 to 7 years) and varied in stage of lactation. All animals were slaughtered four weeks after the last injection.

In the third experiment, two cows were treated with Posilac (500 mg/ sometribove zinc suspension injection, Monsanto) and two cows were treated with Boostin S (500 mg, LG Live Sciences, Korea) thrice with two-weekly injections. The animals were slaughtered three weeks after the last injection. All experiments were approved by the animal ethical commission. The treatment scheme of all three experiments is listed in Table 1, different colours indicating different experiments.

Table 1. Treatment scheme: treatment, number of injections, and withdrawal time

Animal number	Treatment	Number injections	Withdrawal time (weeks)
9497	Vehicle	6	4
7513	Vehicle	6	4
8248	Vehicle	6	4
4005	Posilac	6	4
5319	Posilac	6	4
2982	Posilac	6	4
1966	Posilac	6	4
6957*	Posilac	4	3
7894	Posilac	4	4
6348	Posilac	4	4
6973	Posilac	4	4
7597	Posilac	4	4
7554	Posilac	4	4
6328	Posilac	4	4
7749	Posilac	4	4
6137	Boostin	3	3
8910	Posilac	3	3
1102	Boostin	3	3
8930	Posilac	3	3

**Animal died a week before scheduled slaughter.*

During all trials, milk and serum were collected and somatic cell count and milk production were measured (data not shown). At slaughter, gross pathology of the cows was assessed and thymus was weighed and sampled for histological investigation.

To increase the number of control cattle in 2015 we sampled the thymus of 20 other normal dairy cows at a nearby slaughterhouse.

Histology

Samples of the cervical part of the thymus were sampled and fixed in 4% phosphate buffered formaldehyde and routinely processed to paraffin sections. Sections (5 µm thick) were stained with haematoxylin-eosin (HE) according to Mayer (Bancroft and Stevens, 1990) and assessed microscopically.

Results

Gross pathology findings noted
First experiment: Cow 9497 was pregnant; cow 7513 showed inflammation of the vagina; cow 8248 was skinny; and in cows 4005, 2982 and 1966 hypertrophy of the cervical thymus was observed. In cow 1966 the cervical as well as thoracic thymus was enlarged.
Second experiment: cow 6957 died during the experiment due to an infection of a joint in a leg. It was brought to the Animal Health Service in Deventer (NL) for necropsy where they also sampled the thymus. Cow 7749 had a tumour on the vestibulum vaginae.
Third experiment: cow 1102 had multiple congenital cysts in the kidney.

Table 2. Treatment, weights of thymus and histology.

Animal number	Treatment	Weight thymus (g)	Histology
9497	Vehicle	27.9	Severe fatty infiltration, cortical atrophy
7513	Vehicle	59.2	Severe cortical atrophy, mainly fatty tissue left
8248	Vehicle	131.4	Cortical atrophy, fatty infiltration
4005	Posilac	198.9	Intact thymus, hyperplasia
5319	Posilac	108.9	Hyperplasia, small amount of fat
2982	Posilac	129.6	Relative intact thymus, hyperplasia, small amount of fat
1966	Posilac	296.2	Much thymus tissue left, hyperplasia
6957*	Posilac	n.d.	Only fat sampled
7894	Posilac	168	Much thymus tissue left, hyperplasia
6348	Posilac	150	Much thymus tissue left, hyperplasia
6973	Posilac	142	Hyperplasia, some fatty tissue
7597	Posilac	192	Hyperplasia, some fatty tissue
7554	Posilac	316	Hyperplasia, some fatty tissue
6328	Posilac	132	Hyperplasia, some fatty tissue
7749	Posilac	508	Hyperplasia, necrosis Hassall body
6137	Boostin	n.d.	Hyperplasia, some fat, small haemorrhage
8910	Posilac	n.d.	Intact thymus like a calf
1102	Boostin	n.d.	Intact thymus like a calf
8930	Posilac	n.d.	Intact thymus, like a calf, small haemorrhage

**Animal died*

Weights of thymus gland
The thymus gland of the first two experiments were weighed, but due to the small number of animals thymus gland was not weighed of the third experiment. Weights are listed together with the histological findings in Table 2. Only in the first experiment we weighed the cervical part of the thymus of both control animals and treated animals. In general, the thymus glands of treated animals were larger, but data are too few to conclude that the size of the thymus is enlarged due to bST treatment.

Histology
In general control adult cows from experiment 1 showed severe fatty infiltration in the thymus with cortical and medullary atrophy leaving only fat and remnants of cortex and medulla around the vessels (Figure 1).

Extra control cows showed the same features as the control cows with severe fatty infiltration and little thymic tissue left (Table 3). The bST treated cows showed regeneration of the thymus with varying degrees of lymphocytic hyperplasia (Figure 2) until the features of a normal calf thymus (Figure 3), with only a little fatty infiltration.

Table 3. Histology of thymus of control cows from the slaughterhouse

Animal number	Histology
51	Fat tissue with remnants of thymus
52	Fat tissue with remnants of thymus
55	Fat tissue with sparse remnants of thymus
57	Fat tissue
58	Fat tissue
59	Fat tissue with thymus remnants
60	Fat tissue
61	Fat tissue with thymus remnants
62	Fat tissue with thymus remnants
63	Fat tissue with thymus remnants
64	Fat tissue with thymus remnants
65	Fat tissue with thymus remnants
66	Fat tissue with thymus remnants
67	Fat tissue with thymus remnants
68	Fat tissue
69	Fat tissue with thymus remnants
71	Fat tissue
72	Fat tissue with thymus remnants
73	Fat tissue with thymus remnants
74	Fat tissue with inflammation

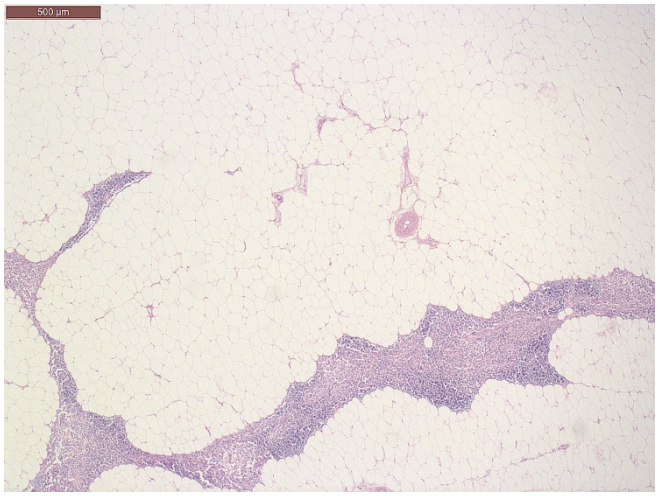

Figure 1. Thymus tissue of a control cow, showing severe fatty infiltration and only remnants of thymic tissue left.

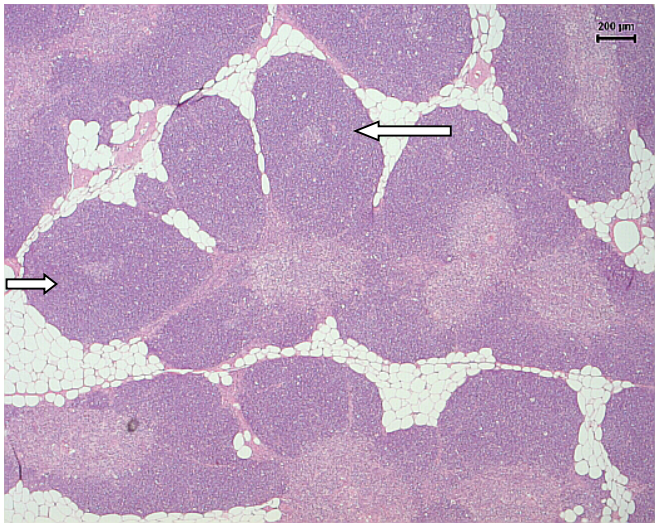

Figure 2. Thymus of a rbST treated cow showing hyperplasia of the thymic cortex (arrows) and about 10% interstitial fat tissue.

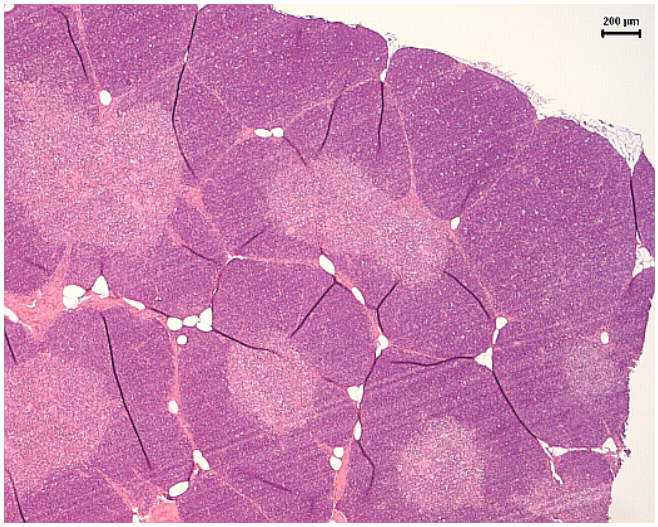

Figure 3. Thymus of a rbST treated cow showing normal tissue morphology like the thymus gland of a veal calf, with some sparse fatty infiltration.

Discussion

In these experiments with rbST in dairy cows, we observed regeneration of the thymus leading to marked histological differences between controls and treated cows after a withdrawal period of 3-4 weeks. It is not known if the thymus will involute again when observed after a longer withdrawal time. Commercial rbST products are recommended to be used till 4 weeks before the dry period.

Every mammalian thymus gland undergoes physiological involution which appears dependent on age, hormones and stress (Bodey *et al.*, 1997). Rejuvenation of thymus due to bST has been described for mice and dogs. Transgenic mice with high peripheral levels of *bovine* GH showed significant increases in the absolute weight of the thymus and the spleen (Dialynas et al, 1999). Age-related decline of the thymus in mice was reversed by repeated injections of *bovine* growth hormone (Bar-Dayan and Small, 1994). Also in middle aged dogs bST treatment resulted in morphological rejuvenation of thymic as determined with histomorphological procedures (Goff *et al.*, 1987). These findings were confirmed in another study (Monroe *et al.*, 1987) who reported that bST treated adult dog thymus tissue resembled the thymic tissue of young dogs. In the thymus gland of dairy cows, we found similar results.

Histological investigation of thymus glands of cattle and calves has been performed for the detection of illegal use of corticosteroids (Vascellari *et al.*, 2008; Cannizzo *et al.*, 2008; Bozetta *et al.*, 2011; Biolatti *et al.*, 2005). Following treatment with corticosteroids the thymus shows cortical atrophy and marked fatty infiltration. Thymus histology of adult cattle is not frequently described, because mostly veal calves (up to 6.5 months of age) or beef cattle (12-24 months of age) are used for experiments. Thymus histology of adult beef cattle (12-24 months) is described as having mild infiltration with fatty tissue

into the cortex and along the septa and multifocal atrophy of the outer cortex as sign of physiological thymus involution (Vascellari *et al.*, 2008). The latter authors found no differences between the mean weight of the thymus of adult cattle and that of veal calves. In our experiments the few control cows had a smaller thymus than the rbST treated cows, but histological assessment is needed to see if the thymus consists of mostly fat or functional thymus tissue, since macroscopically this cannot be easily distinguished. In an experiment with beef cattle (13-22 month of age) treated with dexamethasone for 40 days (0.7 or 1.4 mg/animal/day) and slaughtered after 6 or 26 days withdrawal time (Cannizzo *et al.*, 2010) the thymus was histologically investigated. Thymus of control animals showed some fatty infiltration, but was similar to young calves. After treatment with dexamethasone severe thymus atrophy with cortical atrophy and fatty infiltration was found, whereas after a withdrawal time of 26 days the thymus was regenerated to one with moderate fat infiltration or even the morphology of young calves. This shows that after corticosteroid induced regression regeneration can occur in beef cattle.

Thymus atrophy can also be caused by infectious disease, intoxication and chronic stress (Gruver and Sempowski, 2008). In dairy cows, stress occurs during calving, after removing the calf, during high production and when udder infections or other diseases occur. This might explain the differences between the histology of the thymus gland of beef cattle and the thymus of dairy cows we observed. Moreover, most of our cows were older than 24 months.

We conclude that rbST-treated cows after a withdrawal time of 3-4 weeks showed marked histological regeneration of the thymus which showed a huge difference with the thymus of control dairy cows. Histological screening of thymus tissue of dairy cows can be a promising method to control illegal use of rbST.

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DEVELOPMENT AND VALIDATION OF MULTI-CLASS MULTI-RESIDUE ANALYTICAL METHOD FOR DETERMINATION OF VETERINARY DRUGS IN FISH BY LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY (LC-MSMS)

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Abstract
A multi-residue method for determination of different veterinary drugs classes in fish was developed. The targeted drugs included; sulfonamides, tetracyclines, macrolides, β-lactam, quinolones and unauthorized antibiotics such as chloramphenicol. Different extraction procedures were tested involving methanol/ Mcilvaine buffer, acetonitrile / Mcilvaine buffer, acetonitrile /acetate buffer at pH4 and acetonitrile/ acetate buffer at pH4 with 1% EDTA. The extraction with acetonitrile/ acetate buffer at pH4 with 1% EDTA was selected based on the satisfactory recovery and precision.
After extraction, the solvent was evaporated and the residue dissolved in methanol/10 mM ammonium formate buffer before injection into an LC–MS/MS system (API 4000QT from Applied Bio-systems). LC-MS/MS parameters (e.g declustering potential and collision energy) were optimized. All analytes were measured using positive mode electrospray ionization (ESI+), except chloramphenicol which was measured in negative mode (ESI-). Due to LC-MS/MS signal suppression, determination of veterinary drugs was based on matrix-matched standard curves.
The method was validated for twenty veterinary drugs in fish samples at different fortification levels. Fourteen analytes showed accepted performance criteria according to the European Commission 657/2002/EC. Decision limits and detection capabilities were estimated for all analytes. Method performance was tested by participation in different proficiency testing rounds (FAPAS, UK) and satisfactory z-scores were obtained.

Introduction
Aquaculture is the production of marine or freshwater food fish under controlled conditions (Cañada-Cañada et al., 2009). Most of the aquaculture systems in the world are based on intensive cultivation methods. This type of aquaculture is characterized by high stock density and volume use of antibiotics, antifungal and other pharmaceuticals that can cause serious health problems in consumers, such as allergic reactions in hypersensitive individuals and bacterial resistance (Wen et al., 2006; Cháfer-Pericás et al., 2010). To limit human exposure, many organizations, such as the Codex Alimentarius, European Union (EU) and US Food and Drug Administration (FDA) have established maximum residue limits (MRLs) for veterinary medicinal products in foodstuffs from animal origin foods to ensure food safety.

Sensitive and specific analytical methods are necessary for the determination of veterinary drugs in food matrices (Romero-González *et al*., 2007). Some analytical techniques such as microbiological and enzymatic assay (Khaldeeva *et al*., 2002) or immunochemical procedures such as enzyme-linked immunosorbent assay (ELISA) (Knecht *et al*., 2004; Aga *et al*., 2005), have been used because they are simple and very cost-effective. Nevertheless, they present poor selectivity and they are not able to differentiate among several types of drugs (Aghazadeh *et al*., 2001; Meng *et al*., 2005) providing only semi quantitative measures, which sometimes give rise to false positives.

However, in recent years, mass spectrometry (MS) has been selected as the most suitable technique for detection of veterinary drug residues in foodstuffs according to Public Health Agencies from many countries. It provides namely an unambiguous identification and a reliable confirmation as well as it reduces chromatographic interferences, especially when multiple reaction monitoring (MRM) mode is used (Vinci *et al*., 2005; Rocha *et al*., 2009). Thus, high performance liquid chromatography (HPLC) coupled to tandem mass-spectrometry (MS/MS) has become the predominant technique, combining analyte separation with structural information (Dubois *et al*., 2001). It is used for monitoring chemical residues in food matrices, such as fish, since this technique requires simple sample pre-treatment and increases sample throughput (Díaz-Cruz *et al*., 2006).

Actually more than 809 drugs compounds are used worldwide. To control local, imported and exported food, multi-residue analytical methods are preferred to reduce the workload. In this study, a simple and reliable multi-residue method of analysis for determination of veterinary drugs residues in fish was developed. In this method, the most commonly used drug groups, *i.e*. sulfonamides, tetracyclines, macrolides, β-lactams, quinolones, and unauthorized antibiotics such as chloramphenicol (CAP) was developed.

Materials and Methods

Reagents and standards

All reagents used were of analytical and HPLC grade. Deionized water was generated by a Milli-Q unit (Millipore Corporate, USA). Methanol (MeOH), acetonitrile (ACN) and formic acid 98–100% were from Merck (Darmstadt, Germany). Ammonia solution (30%) was from Riedel-de-Haën. A 10% solution was prepared by mixing 30.3 mL 30% ammonium hydroxide with 100 mL water. Sodium hydroxide (Riedel-deHaen, ≥99%) was used to prepare a 10 M solution by dissolving 40 g in 100 mL water. 1 M Citric acid was obtained by dissolving 21.14 g citric acid monohydrate (Riedel-de-Haën, ≥99%) in 100 mL water and pH was adjusted to pH 4.0 with 10 M sodium hydroxide. A 0.5 M Na_2-EDTA (Fluka, ≥99%) was prepared by dissolve 18.61g in 100 mL water and 10 N NaOH was used to facilitate the solubility of Na2-EDTA at pH 8-10. Mcilvaine buffer was prepared by mixing 0.2 M Na_2HPO_4 and 0.1 M citric acid. For preparation of buffer solutions in MeOH /water (10:90 by volume), formic acid 98–100%, 10 % ammonia solution and deionized water were used. A buffer stock solution of 50 mM ammonium formate (pH 2.75 ± 0.1) was prepared by drop-wise addition of 10% ammonia to 800 mL water containing 1.73 mL formic acid, and finally 200 mL MeOH was added. This stock buffer solution was diluted five times to 10 mM with MeOH /water (10:90) for preparation of the working mobile phase. The pH of the solution rose to finally 4.0-4.2. Dilution solvent for test portion: add 25 mL MeOH to 75 mL stock buffer to have MeOH/ buffer ratio equal to (25:75 by volume).

Veterinary drug standards of analytical grade (>95%) were purchased from Ehrensdorfer (Augsburg, Germany). Stock solution of 1,000 µg mL^{-1} of drugs standards were prepared by dissolving about 10 mg of active ingredient in 10 mL MeOH. Stock solutions were kept in the refrigerator at -18˚C in the dark covered with aluminium foil. Intermediate solution of 100 µg mL^{-1} for each drug standard, except for CAP at 1µg mL^{-1}, was prepared as a single mixture in MeOH. A working solution mixture at 4 µg mL^{-1} for all drugs, except 0.1 µg mL^{-1} for CAP, was prepared by diluting appropriate volumes of the intermediate solution with methanol and was store at 4˚C in the dark covered with aluminium foil. This mixture was used for spiking and recovery experiments and for the daily calibration solutions. Matrix-matched standard solutions were prepared with blank matrix daily (with each set of samples) to compensate for the matrix effect. Individual standard solutions were prepared at 0.25 µg mL^{-1} for each drug for LC-MS/MS optimization, including declustring potential (DP) and collision energy (CE).

Sample Extraction

A well homogenised sample of 2 g was weighed in a 50-mL PTFE centrifuge tube. The samples were left to stand for 15 min. After addition of 1 mL 1 M sodium citrate at pH 4, 1 mL 0.5 M Na_2EDTA and 10 mL CAN, the suspension was homogenized for 2-3 min using Ultra Turrax followed by shaking for 1 min. The mixture was centrifuged at 4,000 rpm for 10 min at 4˚C and the supernatant was transferred into a 100-mL round-bottomed flask. The extraction was repeated with another 10 mL ACN, followed by centrifugation as described and the supernatants were combined in the same 100-mL flask. The solvent of the combined extracts was evaporated using a rotary evaporator at 37˚C. At the half of evaporation step, *i.e*. as soon as aqueous bubbles appear, another 15 mL ACN was added to avoid back-suction and to help reaching just to dryness. The sample was diluted in 2 mL dilution solvent MeOH/buffer (25/75 by volume). The sample was filtered using an acrodisc (0.45 µm) and 25 µl of the sample was injected into the LC-MS/MS system. For recovery tests, blank samples were fortified by adding appropriate volume of the standard solution to the sample.

LC-MS/MS analysis

All measurements were performed using liquid-chromatography electrospray ionisation (ESI) tandem mass-spectrometry (LC–ESI-MS/MS). All analytes were measured in the positive mode (ESI^+), except CAP which was measured in the negative mode (ESI^-). LC instrument (Agilent 1200 Series) was coupled to an API 4000 Qtrap MS/MS (Applied Biosystems) equipped with an electrospray ionisation (ESI) interface. Chromatographic separation was performed on a C18 column (ZORBAX Eclipse XDB-C18; 4.6 x 150 mm, 5 µm particle size). The linear gradient program was: start at 20% B; 0–3 min from 20 to 90% B; 3–15 min 90% B; 15–15.5 min from 90 to 20% B; 15.5–20 min 20% B at a flow rate of 0.4 mL min^{-1}, with (A) 10 mM ammonium formate solution in MeOH-water (1:9), and (B) MeOH.

Results and Discussion

Optimizing LC–MS/MS condition

To achieve maximum sensitivity, the mass spectrometry parameters including ionization mode, declustering potential (DP) and the collision energy (CE) were first optimized by direct flow infusion of each standard. The results indicated that the positive mode was most favourable for all analytes except for CAP which was measured in the negative mode. The characteristic ions, DP and CE for each compound are listed in Table 1.

Veterinary drugs standard solutions (0.1 to 0.5 µg mL^{-1}) in MeOH/ ammonium formate buffer (1/1) were injected individually to optimize for parent ion by scanning at different DPs (MS1 scanning and MS2 static). The optimum DP, which gave the highest intensity, was used to optimise the CE for the daughter ion (MS1 static and MS2 scanning). The standard solutions were injected directly into mass spectrometer, the (de-)protonated ions were chosen in ESI+ (MW+1) or ESI- (MW-1) mode.

Table 1. LC-MSMS acquisition parameters.

Analyte	Group	Mode	Parent ion (*m/z*)	Daughter ion I (*m/z*)	Daughter ion II (*m/z*)	DP (V)	CE (ev) daughter ion II	CE (ev) daughter ion I
Chloramphenicol	Antibiotic	Negative	321.0	152.0	121.0	-65.0	-24.0	-44.0
Ciprofloxacin	Fluoroquinolones	Positive	332.1	231.3	288.2	61.0	53.0	27.0
Erythromycin	Fluoroquinolones	Positive	734.5	158.2	83.0	51.0	47.0	95.0
Flumequine	Fluoroquinolones	Positive	262.2	244.2	202.1	36.0	27.0	47.0
Trimethoprim	Inhibitor	Positive	291.2	230.3	261.2	71.0	33.0	35.0
Enrofloxacin	Macrolides	Positive	360.2	342.0	245.0	61.0	31.0	39.0
Tylosine tartarate	Macrolides	Positive	916.7	772.6	174.1	111.0	39.0	57.0
Sulfacetamide	Sulfonamides	Positive	215.1	156.0	92.0	51.0	15.0	33.0
Sulfadiazine	Sulfonamides	Positive	251.0	92.0	156.0	72.0	39.0	23.0
Sulfamerazine	Sulfonamides	Positive	265.0	156.0	172.0	64.0	27.0	25.0
Sulfamethazine	Sulfonamides	Positive	279.1	92.0	124.0	41.0	43.0	33.0
Sulfamethoxazole	Sulfonamides	Positive	254.1	65.1	92.1	41.0	63.0	33.0
Sulfapyridine	Sulfonamides	Positive	249.2	156.0	92.0	51.0	25.0	43.0
Sulfathiazole	Sulfonamides	Positive	256.0	92.1	156.0	46.0	39.0	21.0
Chlortetracycline	Tetracyclines	Positive	479.0	444.0	154.0	71.0	29.0	43.0
Doxycycline	Tetracyclines	Positive	445.0	428.0		46.0	39.0	
Oxytetracycline	Tetracyclines	Positive	461.2	426.1	443.1	56.0	25.0	17.0
Tetracycline	Tetracyclines	Positive	445.3	410.0	427.0	56.0	27.0	19.0
Ceftiofur	B- Lactam	Positive	523.9	241.1	125.0	76.0	27.0	77.0
Penicillin V	B- Lactam	Positive	351.0	160.0		91.0	23.0	

Optimization of extraction step

Various solvents were probed for the extraction of veterinary drugs from fish tissue: a) MeOH/ McIlvaine buffer, b) ACN/ McIlvaine buffer, c) ACN/ acetate buffer at pH 4 and d) ACN/ acetate buffer at pH4 with 1% EDTA. A mixture of 20 compounds was prepared and used in the primary experiments for optimization of the extraction. Six replicates were carried out with spiked blank fish samples at a concentration level of 50 µg kg^{-1} for all analytes, except CAP at 0.3 µg kg^{-1}. Blank samples, standard in solvent and standard in matrix were injected in parallel to spiked samples in the same run. Due to a suppression effect of fish matrix, standards prepared in blank matrix were used for recovery correction. The extraction with ACN/ acetate buffer at pH4 with 1% EDTA was selected finally based on the good recovery and CV% results (Table 2). McIlvaine buffer was not preferred due to its phosphates, which can precipitate in the ion sources corroding the ion spray needle.

Method performance

Linear range and calibration curves. Calibration curve was established by injecting five different levels, namely 25, 50, 100, 200 and 500 ng mL^{-1} in positive mode for nineteen analytes, and 0.1, 0.15, 0.3, 0.6 and 3 ng mL^{-1} for CAP in negative mode. The correlation coefficients of the standard calibration curves for all analytes were satisfactory greater than 0.999.

Method recovery. Six replicates of recovery tests were performed at different concentration levels (0.25, 0.50, 1.0, 1.5 and 2.0 x MRL) for authorized drugs and at the MRPL of the forbidden drug CAP. Table 3 shows the results of mean recovery, CV%, $Q_{typical}$ and pooled CV% performed on blank fish samples. The average recoveries of eighteen compounds were within 70-120% which is widely accepted in the residue analysis field. Fourteen compounds were within the accepted criteria (80-110%) of the European Commission 657/2002/EC. The recovery of erythromycin is too high at some levels and ranged between 75%-184% (average 125%) due to some interference peaks which requires an extra clean up step.

Decision limit (CCα) and the detection capability (CCβ). The method decision limit (CCα) and the detection capability (CCβ) were evaluated in accordance with the EU guidelines 2002/657/EC. The results are summarized in Table 4.

Table 2. Recovery tests and CV% (n=6) using different extraction reagents applied to fish matrix at concentration level of 50 µg kg^{-1} for all analytes except chloramphenicol at 0.3 µg kg^{-1}.

Analyte	MeOH/McIlvaine buffer at pH3			ACN /McIlvaine buffer at pH3			ACN acetate buffer at pH4			ACN acetate buffer at pH4 with 1% EDTA		
	Matrix effect%	Mean rec %	CV%	Matrix effect%	Mean rec%	CV%	Matrix effect%	Mean rec%	CV%	Matrix effect%	Mean rec%	CV%
Ceftiofur	63	56	11	72	64	11	81	75	10	81	88	9
Chloramphenicol	76	71	9	81	76	9	77	87	8	72	91	7
Chlortetracycline	61	49	14	69	56	13	77	65	12	75	77	10
Ciprofloxacin	62	55	12	71	63	11	83	74	10	85	87	9
Doxycycline	59	49	15	70	56	14	77	65	13	74	77	11
Enrofloxacin	60	58	11	69	66	10	80	77	9	82	91	8
Erythromycin	59	189	38	119	122	35	135	119	32	124	140	28
Flumequine	68	67	15	74	65	14	81	77	13	88	90	11
Oxytetracycline	58	51	12	71	58	11	77	68	10	81	80	9
Penicillin V	66	57	11	69	64	10	80	76	9	84	89	8
Sulfacetamide	67	55	15	70	63	14	76	74	13	79	87	11
Sulfadiazine	61	47	9	66	65	9	77	77	8	81	90	7
Sulfamerazine	70	55	16	75	63	15	79	74	14	77	87	12
Sulfamethazine	62	54	14	68	61	13	81	71	12	85	84	10
Sulfamethoxazole	66	58	15	72	66	14	86	77	13	88	91	11
Sulfapyridine	71	56	18	75	64	16	77	75	15	83	88	13
Sulfathiazole	63	50	14	68	57	13	82	67	12	88	79	10
Tetracycline	66	52	12	70	59	11	78	69	10	79	81	9
Trimethoprim	59	59	11	74	67	10	85	79	9	88	93	8
Tylosine	60	57	14	68	64	13	84	76	12	81	89	10

Table 3. Recovery tests and CV% (n=6) at different five concentration levels on fish blank samples.

Analyte	25 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}		150 µg kg^{-1}		300 µg kg^{-1}		Q_{typ}[a]	pooled CV%[a]
	Mean re-covery (%)	CV (%)	Mean re-covery (%)	CV (%)	Mean re-covery (%)	CV (%)	Mean re-covery (%)	CV (%)	Mean re-covery (%)	CV (%)		
Ceftiofur	62	11	81	9	72	8	78	7	81	10	75	9
Chloramphenicol[b]	89	9	99	7	106	3	94	3	108	3	99	3
Chlortetracycline	74	9	72	4	64	11	79	9	96	8	77	8
Ciprofloxacin	79	10	80	7	92	14	82	9	86	4	84	9
Doxycycline	109	14	74	6	82	8	77	5	83	4	85	8
Enrofloxacin	98	9	87	6	79	8	102	7	96	8	92	8
Erythromycin	89	10	184	21	75	32	183	18	91	10	124	20
Flumequine	81	11	106	4	73	8	112	4	91	3	93	7
Oxytetracycline	91	12	72	9	73	5	71	5	87	3	79	7
Penicillin V	75	15	86	8	78	10	79	3	78	4	79	9
Sulfacetamide	87	9	81	7	79	8	78	5	84	7	82	7
Sulfadiazine	84	10	88	7	94	9	121	9	88	8	95	9
Sulfamerazine	76	11	88	6	83	8	84	9	88	3	84	8
Sulfamethazine	80	11	93	7	83	6	83	10	92	2	86	8
Sulfamethoxazole	79	12	84	8	86	7	74	11	84	3	81	9
Sulfapyridine	77	11	91	7	86	9	95	4	85	4	87	8
Sulfathiazole	88	10	86	9	85	12	112	14	93	11	93	11
Tetracycline	86	9	71	8	81	14	81	7	84	10	81	10
Trimethoprim	73	6	94	5	79	8	82	3	87	8	83	6
Tylosine	73	5	74	13	74	10	77	6	78	12	75	10

[a] Q_{typ} and pooled CV% calculated from for all recovery tests at the five concentration levels; [b] Q_{typ} Concentration levels 25 µg kg^{-1}, 50 µg kg^{-1}, 150 µg kg^{-1} and 300 µg kg^{-1} for all analytes except for CAP which were 0.075 µg kg^{-1}, 0.15 µg kg^{-1}, 0.3 µg kg^{-1}, 0.45 µg kg^{-1} and 0.6 µg kg^{-1}.

Table 4. Calculated decision limit (CCα) and detection capability (CCβ).

Analyte	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	Analyte	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	Analyte	CCα (µg kg^{-1})	CCβ (µg kg^{-1})
Ceftiofur	117	132	Flumequine	214	225	Sulfamethoxazole	116	131
Chloramphenicol	0.03	0.07	Oxytetracycline	115	126	Sulfapyridine	115	127
Chlortetracyclin	116	130	Penicillin V	67	82	Sulfathiazole	120	139
Ciprofloxacin	117	133	Sulfacetamide	114	126	Tetracycline	118	135
Doxycycline	116	129	Sulfadiazine	116	131	Trimethoprim	63	73
Enrofloxacin	115	128	Sulfamerazine	115	128	Tylosine	118	134
Erythromycin	234	267	Sulfamethazine	115	128			

Proficiency testing results. The method performance was tested by participation in different PT rounds from FAPAS (Fera Science Ltd., York, UK). The results are summarized in Table 5. Satisfactory results were obtained for all detected analytes indicating that the developed method could be used successfully in routine analysis.

Table 5. Proficiency testing results.

FAPAS Round No	Matrix	Analyte	Assigned value (µg kg^{-1})	Result (µg kg^{-1})	Z-Score
2150	Prawns	Chloramphenicol	1.5	2.2	2.3
2154	Fish muscle	Oxytetracycline	350.0	330.0	-0.3
2253	Pig muscle	Sulfadiazine	124.0	135.0	0.4

Conclusions

In the present study, a multi-residue multi-class method for determination of twenty veterinary drugs in fish was developed.

The extraction with ACN/ acetate buffer at pH 4 with 1% EDTA was selected based on good recovery and CV% results.

The mass spectrometric parameters were optimized to give the best sensitivity, two MRM's were chosen for quantification and confirmation of most analytes. The selected MRM's were based on optimized DP and CE.

The proposed extraction method followed by LC-MS/MS determination is simple, rapid and reliable. Satisfactory recoveries and repeatability were observed. The described method requires little amount of solvents and sample and could be used in controlling levels of different classes of veterinary drugs in fish samples.

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MONITORING OF CORTICOSTEROIDS IN THE NATIONAL PLAN FOR CONTROL OF RESIDUES IN RUSSIA

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Abstract

In accordance with the Russian Federation regulations the residues of synthetic steroids, β-agonists, sedatives, thyreostats and of some groups of antibiotics are not allowed in food products. Therefore, the monitoring of these residues is necessary to ensure that food products are meeting the Russian Federation regulations. The aim of this study was to develop a rapid and sensitive method for the analysis of 4 corticosteroids (dexamethasone, prednisolone, methylprednisolone, and triamcinolone acetonide) in meat, milk and liver using LC-MS/MS. A binary solvent delivery system (Eksigent UltraLC-100, Eksigent, USA) and a triple quadrupole mass spectrometer (QTRAP 5500, AB SCIEX, Toronto, Canada) were used. Separation of analytes was carried out on a Pursuit 3 C18 column (150 mm× 2.1 mm, Agilent). The sample preparation includes liquid-liquid extraction with methyl tert-butyl ether and defatting with hexane. The method was validated in the range of 0.1 to 30 µg kg^{-1} for all analytes. The recovery lies in the range of 75 to 110%, the combined uncertainty was below 40 % for all analytes. The method was applied for the analyses of 119 samples in the National Residue Program.

Introduction

There are strict requirements for veterinary drug residues in the Russian Federation. The residues of some veterinary drugs (such as dexamethasone, prednisolone, methylprednisolone, azaperone, carazolol, trenbolone, zeranol, ractopamine) that are approved for use in many countries are not allowed in food products in the Russian Federation. In Table 1 we compare the Codex Alimentarius and the Russian Federation requirements (Customs Union 2010; Customs Union 2013) for veterinary drug residues in food.

Table 1. Comparison of the Codex Alimentarius and the Russian Federation regulations for veterinary drug residues in food of animal origin.

Drug class	Codex Alimentarius requirements	Russian Federation requirements
Sedatives	MRLs have been set for **azaperone** and **carazalol**	The residues are **not allowed**
Steroids	MRLs have been set for **dexamethasone, trenbolone** and **melengestrol acetate**	The residues are **not allowed**
β-Agonists	MRLs have been set for **clenbuterol** and **ractopamine**	The residues are **not allowed**
Resorcylic acid lactones	MRL has been set for **zeranol**	The residues are **not allowed**
NSAIDs	The residues are **not allowed**	The residues are **not allowed**
Nitroimidazoles	The residues are **not allowed**	The residues are **not allowed**
Nitrofurans	The residues are **not allowed**	The residues are **not allowed**
Dyes	The residues are **not allowed**	The residues are **not allowed**
Carbadox, olaquindox	The residues are **not allowed**	The residues are **not allowed**
Thyreostats	The residues are **not allowed**	The residues are **not allowed**

In this study we present a rapid and sensitive method for the analysis of dexamethasone, prednisolone, methylprednisolone, and triamcinolone acetonide in meat, milk and liver using LC-MS/MS. The method was applied for analysis of 41 *bovine* liver samples and 78 beef samples.

Prednisolone, dexamethasone, triamcinolone acetonide, and methylprednisolone are synthetic corticosteroids, structurally based on endogenous corticosteroid – cortisol (Figure 1).

However, recent studies reported on a potential endogenous origin of prednisolone. It is suggested that unlike other analogues, such as dexamethasone, prednisolone does not contain any halogen atom and its structure closely resembles that of other endogenous steroids, including cortisol. From this structural similarity arises the hypothesis that in meat cattle prednisolone could be generated by physiological metabolic processes, possibly under extremely stressful conditions, such as transport and slaughtering, or by faecal contamination (Leporati *et al*., 2013).

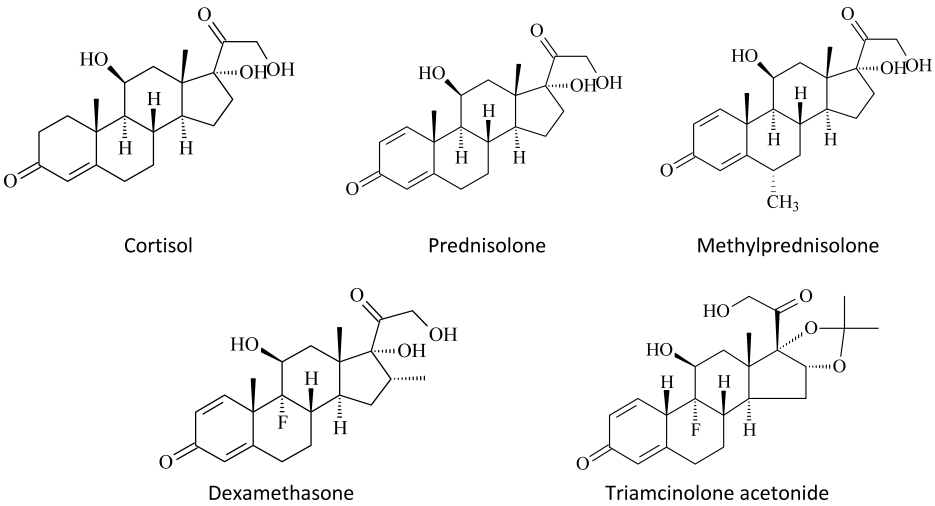

Figure 1. Chemical structures of corticosteroids.

Prednisolone, methylprednisolone, dexamethasone and its epimer beta-methasone are authorized for therapy in both human and veterinary practices. They affect glucose utilization, fat metabolism, and bone development and commonly used in the treatment of allergic reactions. They are also used to reduce inflammation (Tolgyesi *et al*., 2012).

Corticosteroids are also known to exert growth-promoting effects in cattle fattening and to show synergetic effects in combination with β-agonists and other anabolic steroids in cattle. Therefore, they might be used illegally as growth-promoting agents (Schmidt *et al*., 2009).

Materials and Methods

Sample preparation

Meat, milk or liver samples (5.0 g) were weighed into 50 mL centrifuge tubes. Samples were fortified with mixed internal standard at a level corresponding to 10 µg kg^{-1} by adding 50 µL of 1000 ng mL^{-1} internal standard solution (triamcinolone acetonide-D6). After fortification, samples were held for 15 min, then 6 mL of sodium acetate buffer (pH 5.2) was added and the samples were homogenized with a WiseTis homogenizer (Daihan, Seoul, Republic of Korea) for about 1 min. The pH of each mixture was readjusted to 5.2. Additionally, for liver samples an enzymatic hydrolysis (37°C, overnight) with glucuronidase/arylsulfatase from *Helix pomatia* was performed. The mixture was extracted with 15 mL of methyl tert-butyl ether (10 min rotating and centrifuged at 2,100 g). The organic layer was evaporated to dryness and the residue was dissolved in 1.5 mL of methanol/water (80/ 20, v/v). Next hexane (3 mL) was added, the sample was vortexed for 1 min and centrifuged at 3,000 g for 10 min. The hexane layer was discarded and 20 µL of extract was injected in the HPLC-MS/MS system.

LC conditions

A binary solvent delivery system (Eksigent UltraLC-100, Eksigent, USA), including a binary pump and a degasser, was used. Chromatographic separation was achieved by reversed phase chromatography and gradient elution. Separation of the analytes was carried out on a ACE Excel 3 Super C18 column (150 mm× 2.1 mm, particle size 3 µm, ACE), maintained at 40°C. The autosampler temperature was set to 4°C and the injection volume was 20 µL. Samples were injected in negative polarity mode. The mobile phases were water (phase A) and methanol (phase B). A linear gradient was applied at a flow rate of 150 µL min^{-1} starting at 20% B, increasing to 70% B within 20 min, and keeping 70% B for 10 min. Subsequently, the column was re-equilibrated for 10 min at 20% B.

MS/MS parameters

A triple quadrupole mass spectrometer (QTRAP 5500, AB SCIEX, Toronto, Canada) was used with electrospray ionization source. The transitions for each analyte as well as the corresponding collision energies are shown in Table 2. Declustering potential and collision cell exit potential were -110 V and -20 V in negative mode, respectively, for all analytes. The resolution of quadrupole 1 (Q1) and quadrupole 3 (Q3) was set to “unit”.

Table 2. HPLC-MS/MS conditions.

Compound	Retention time (min)	Parent ion (*m/z*)	Daughter ion (*m/z*)	Collision energy (eV)
Prednisolone	18.0	405.2	280.1[a], 295.1	-45, -50
Methylprednisolone	20.3	419.2	309.2, 343.2[a]	-43, -23
Dexamethasone	20.0	437.1	345.2, 361.2[a]	-34, --24
Triamcinolone acetonide	20.4	479.2	337.2, 413.2[a]	-32, -26
Triamcinolone acetonide-D6	20.4	485.2	337.2	-65

[a] *The ion used for quantitative analysis.*

Results and Discussion

Time-consuming procedures based on GC-MS with derivatisation have been used mainly in the past for the analysis of corticosteroids. More recently, LC-MS/MS procedures without a derivatisation step have been developed. The sample preparation in LC-MS/MS procedures usually involves two solid phase extractions on reversed and normal phases. In our study we compare two sample preparation procedures: A) liquid-liquid extraction with methyl tert-butyl ether and solid phase extraction on silica cartridges, and B) liquid-liquid extraction with methyl tert-butyl ether and defatting with hexane. Both schemes have resulted in high recoveries and low detection limits. As a result, the scheme with the defatting step was chosen as it is less time-consuming. The final optimized scheme of sample preparation is shown in Figure 2.

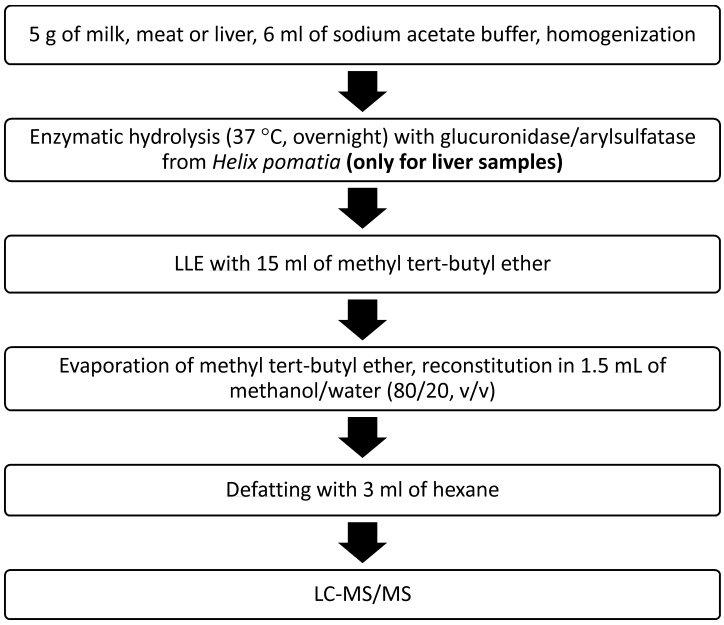

Figure 2. Sample preparation according to procedure B.

The validation experiment was based on full factorial design for two factors and consisted of four runs. A run contains blank samples of meat, milk and liver fortified at 0.1, 0.5, 2, 8 and 30 µg kg^{-1}, which were analysed in two replicates and matrix-matched calibration samples fortified at the same levels. Operator and storage of extract after sample preparation were selected as factors which cannot be controlled in routine analysis. The method was validated in the range of 0.1 to 30 µg kg^{-1} for all analytes. The recovery lies in the range of 75 to 110%, the combined uncertainty was below 40% for all analytes.

In order to prove the specificity and the lack of susceptibility to matrix interferences, several blank samples were additionally analysed in each runs. The specificity of the method was demonstrated as no interfering peaks were observed at the retention time of analytes in a variety of blanks (Figure 3).

A number of 41 *bovine* liver samples and 78 beef samples from Russia and South-America were analysed in the frame of the National Residue Programme. All samples were determined as compliant.

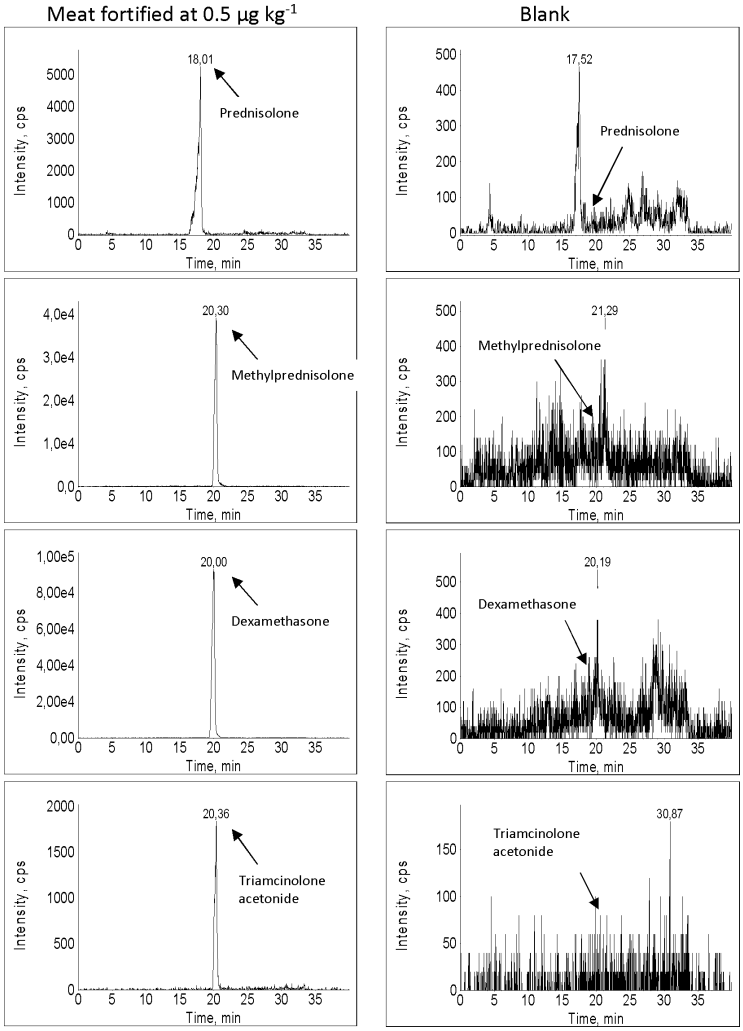

Figure 3. Selected reaction monitoring chromatograms obtained from blank meat and meat fortified at 0.5 µg kg^{-1} (SRM transitions used for quantitative analysis are shown).

Conclusions

A rapid and sensitive method with simple sample preparation for the determination of four corticosteroids was developed. The method was validated and applied for the analyses of 119 samples in the National Residue Program.

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RAPID MONITORING OF ANTIMICROBIALS IN THE ENVIRONMENT

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Abstract

Today 700,000 people die from resistant bacteria, of which around 25,000 are in Europe alone. By 2050 this number will increase to a staggering 10 million people, while costing the world an estimate of $100 trillion of economic losses if no immediate action is taken[1]. Misuse of antibiotics in human and animals, and the irresponsible production of antibiotics by the pharmaceutical industry lead to the increased risk of antibiotics in the environment causing antimicrobial resistance (AMR). In the meantime, easy-to-use tests to detect antimicrobial activity in water, soil and meat is lacking. This hampers evidence generation that is necessary to inform the public and policy makers about the high prevalence of antibiotics in the environment.

Introduction

Infections caused by resistant bacteria have claimed millions of lives worldwide. The overuse and misuse of antibiotics have been largely blamed for this problem. Surprisingly, high concentration of antibiotics in natural environments has been recorded in areas close to the food-producing animal facilities and manufacturing plants. External reports have disclosed current practices by the agriculture, food industries and pharmaceuticals releasing millions of gallons of untreated waste streams containing hazardous amount of antibiotics into lands, rivers and seas. Continuous exposure to multiple drugs will select for resistant bacteria and over time, will dominate the whole microbial population. Water loaded with antimicrobial residues and resistant bacteria can leach deep into the ground and ended up in drinking water. This situation has dire consequences to human health and the balance of environmental microbiota. Indirectly, resistant pathogens can also be transmitted to human via consumption of crops that have been irrigated with polluted water. In addition, resistant genes of clinical concerns are now being found in ecosystems not known for such contamination miles away.

Unfortunately, local authorities do not always control on the level of antibiotics in the environment due to unavailable information and user-friendly detection tools to mobilize to different sites. Data generation from such test can serve as a valuable input for various studies, government interventions and provide useful insight to making policy decision.

To date, rapid testing of antimicrobial residues in food, water and soil requires extensive laboratory equipment and experienced technicians. When a sample is taken, it needs special storage condition to avoid any biological and chemical degradation. Analytical test such as high performance liquid chromatography coupled with mass spectrometry is costly and therefore, not feasible for routine monitoring at multiple sites with hundreds of samples. A reliable and practical tool is therefore, needed to differentiate between samples contain antimicrobial residues with the ones that do not. If necessary, only a selection of positive samples will be sent to the laboratory for further analyses. This approach will not only save time and money but will speed up public health actions on the underlying danger of AMR in the surrounding areas.

DSM Sinochem Pharmaceutical (DSP) has developed an in-house test to detect the presence of β-lactams in a matter of hours. Multiple samples can be read at the same time and requires no sophisticated laboratory equipment. Our regular check has enable us to closely monitor contamination higher than the threshold limit in waste streams around the world. However, there is still an unmet need for such test targeting other classes of antibiotics such as tetracycline, macrolides or aminoglycosides. Although tetracycline is rarely used in humans, it is mixed in feed and water for animal consumption at factory farms.

Bacteria can develop cross-resistant toward other medically important antibiotics after a prolonged contact with a similar acting drug. Current practice of dumping or applying animal manure containing high concentration of antibiotics and resistant human pathogens into the environment or as fertilizer contributes to AMR closer to home. For this reason, we aim to develop a practical and robust detection tool to monitor the presence of various antibiotics in food products, drinking water, waste water streams and soil (Figure 1). The outcome can later be used to inform local authorities, regulatory bodies, public health officers, academics and industry leaders on the AMR “hot spots”, prepare risk mitigation assessment and action plan.

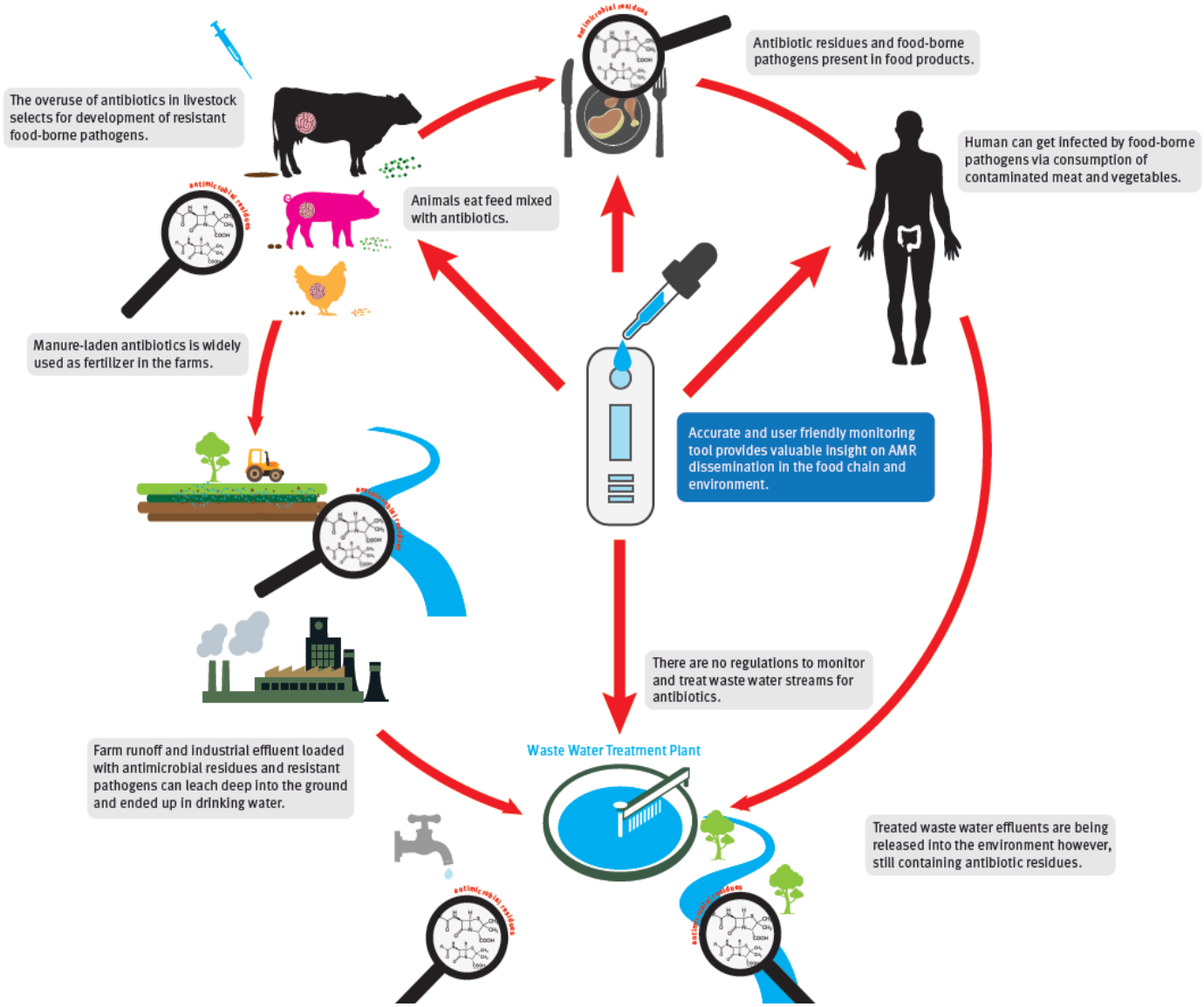

Figure 1. Rapid monitoring of antimicrobial in the environment.

Conclusions

Effective surveillance on AMR and the management of antimicrobial activity levels in water starts with the availability of reliable data on the presence of antibiotics in our water, and the environment. Current methods such as HPLC / LC-MS, used for the detection of antibiotics in samples from various origins are costly, time intensive, and only suitable to detect specific antibiotics and not the antimicrobial activity. To allow economic and effective routine sampling, we have developed and implemented a monitoring system for antimicrobial activity at all our waste water treatment plants around the world. Building on gained knowledge and experience, we are looking for potential partners in developing a user friendly tool for effective monitoring of antibiotics in the environment.

References

1. The AMR Review Team, headed by Jim O'Neill, 2015

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