

LC-MS residue analysis of antibiotics

What selectivity is adequate?



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Bjorn J.A. Berendsen

Thesis

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Abstract

In residue analysis of antibiotics quantitative and qualitative aspects are involved in declaring a sample non-compliant. The quantitative aspect regards the determination of the amount of the compound present in the sample. Validation procedures are available to determine the uncertainty of this result, which is taken into account in the decision making process. The qualitative aspect regards the confirmation of the identity of the compound present. In this, selectivity is the main parameter which is defined as the ability of a method to discriminate the analyte being measured from other substances. A trend observed in residue analysis is towards more generic methods for the detection of a broad range of compounds in a single run. As a result, by definition, selectivity is compromised. Procedures to determine the uncertainty of the qualitative aspect are lacking and, as a result, whether or not a method is adequately selective is a matter of experts' judgment.

In this thesis a method is presented for grading selectivity of methods using liquid chromatography coupled to tandem mass spectrometry. Based on the outcome it can be stated if selectivity is adequate and thus if a confirmatory result stands strong when challenged in a court case. If selectivity is found inadequate, additional measures can be taken like the selection of another product ion or the use of a third product ion to obtain adequate selectivity.

Furthermore, two examples of analyses are presented in which selectivity plays an important role. First, the analysis of the banned antibiotic chloramphenicol (CAP). CAP contains two chiral centers and the nitro-group can either be para- or meta-substituted. Therefore, eight different isomers of CAP occur of which only RR-p-CAP is antimicrobially active. In the analysis of CAP, extreme selectivity is needed to distinguish the antimicrobially active compound from its inactive isomers. A method applying chiral liquid chromatography with tandem mass spectrometry was developed to discriminate antimicrobially active CAP from its inactive isomers. Also the research for the possible natural occurrence of this drug is presented. It is shown that CAP can be produced in unamended soil by *Streptomyces venezuelae* in appreciable amounts and that crops can take up CAP from soils. Therefore, it is concluded that CAP can occur in crops and animal feed due to its natural production by soil bacteria.

Second, the development of a multi- β -lactam method is presented. In this method a derivatization is applied to be able to effectively detect off-label ceftiofur use. In this selectivity is intentionally compromised and no unequivocal confirmation can be carried out using this method. The developed method is applicable to a wide range of β -lactam antibiotics including penicillins, cephalosporins and carbapenems and is the best method available today for effective monitoring of off-label β -lactam usage in poultry breeding.

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Chapter 1

General introduction



Introduction

In animal breeding the use of antibiotics has become common practice. Antibiotics are used to treat bacterially infected animals but are also administered as a preventive measure. From an animal and human health perspective, responsible use of antibiotics is of importance and therefore extensive monitoring programs are in place within the European Union. The methods used for analysis of antibiotics aim for the detection, quantitation and confirmation of the antibiotic present in products of animal origin. In this chapter the use of antibiotics and its drawbacks are discussed followed by a description of the legal framework for antibiotic analysis and the methods employed. Finally some challenges are discussed that are subject of this thesis.

Antibiotics and their veterinary usage

Antibiotics

Antibiotics are used to treat infections caused by bacteria and other micro-organisms. Traditionally, the term “antibiotics” is used to describe any substance produced by a micro-organism that is effective against the growth of another micro-organism [1]. Nowadays the term “antibiotics” is used interchangeably with the term “antibacterials”, and includes synthetic substances like sulfonamides and quinolones as well.

Definition:

“Antibiotic ► noun, a medicine (such as penicillin or its derivatives) that inhibits the growth of or destroys micro-organisms.” [2]

In 1928, Alexander Flemming discovered the antibiotic action of the fungus of the genus '*Penicillium*' and attributed it to one of its constituents: penicillin [3,4]. In 1932, the first antibiotic substance, developed by Gerhard Domagk, became commercially available: prontosil. This was the first commercially available synthetic antibiotic belonging to the sulfonamide group and has a broad activity against Gram-positive bacteria, but not against enterobacteria (Gram negative) [5]. In 1939, Howard Florey and Ernst Chain continued the study on penicillin [6]

and showed its activity against a broad spectrum of bacteria and proved it to be safe for use in humans. Later many chemically altered semi-synthetic penicillins were developed. The penicillins derive their activity from the 6-aminopenicillanic acid nucleus which is effective against mainly Gram positive bacteria [4,7,8]. Amoxicillin, ampicillin, penicillin G (benzylpenicillin), penicillin V (phenoxymethylpenicillin), cloxacillin, dicloxacillin, oxacillin and nafcillin (figure 1.1) are nowadays registered for veterinary use [9].

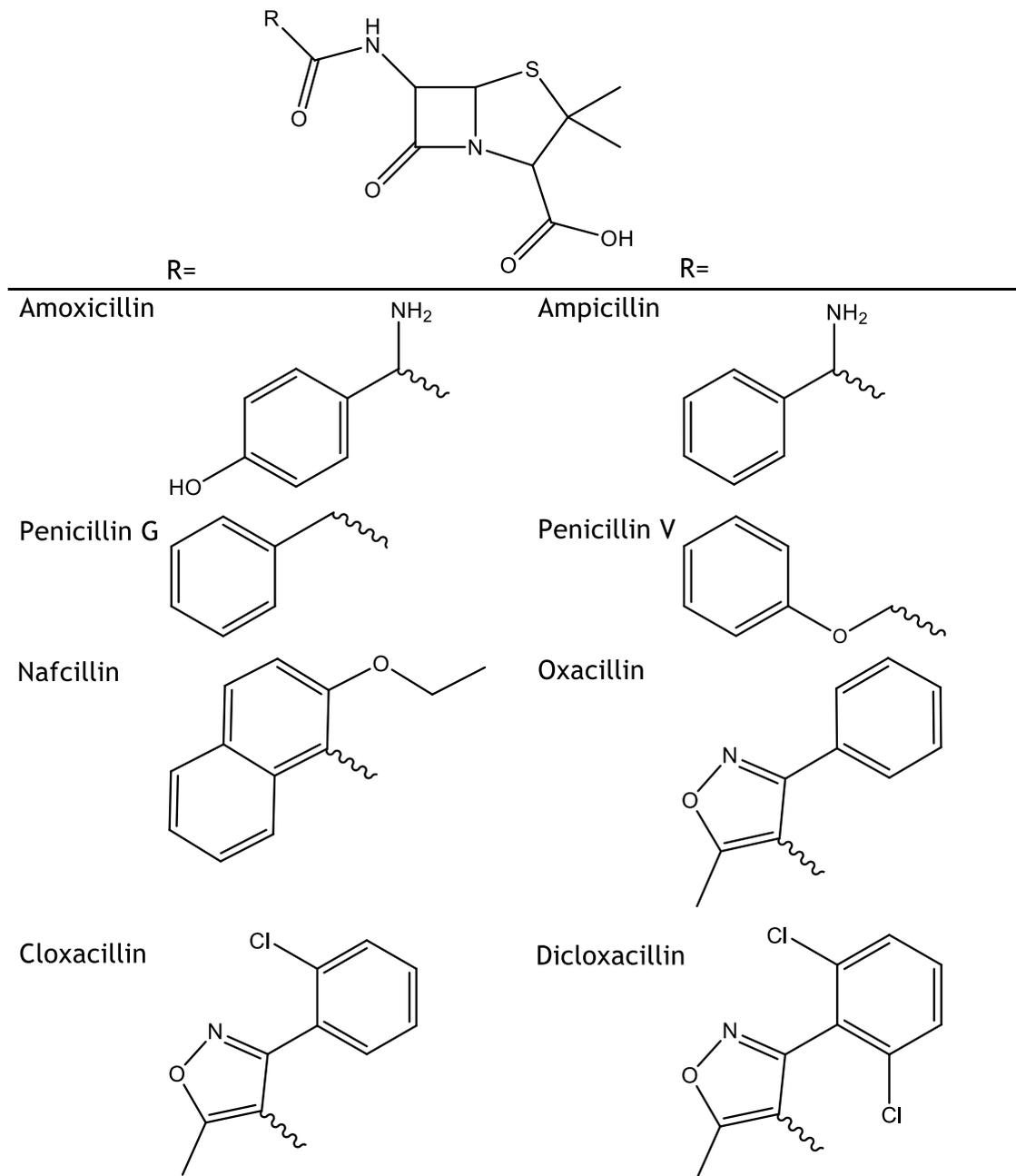


Figure 1.1. Molecular structure of the penicillins registered for use in animal practice.

The discovery of penicillin led to renewed interest in the search for antibiotic compounds with similar efficacy and safety. In 1956, the first cephalosporin antibiotic, closely related to the penicillins, was isolated from the *Acremonium* fungus species [10-12]. The six membered dihydrothiazine ring fused with a four membered β -lactam ring (figure 1.2) is responsible for the biological activity of this group of compounds. Cephalosporins are highly effective antibiotics in the treatment of bacterial infections of the respiratory tract [4,13]. As for the penicillins, many semi-synthetic cephalosporins were developed which are nowadays distinguished in several generations based upon their time of discovery and their range of activity [14]. Cefacetril, cefalonium, cefazolin, cefalexin and cefapirin (all 1st generation), cefoperazone and ceftiofur (3rd generation), and cefquinome (4th generation) are all approved for veterinary use (figure 1.2) [9].

Another β -lactam group consists of the carbapenems of which the first compound was isolated from *Streptomyces cattleyain* in 1971 [15]. The carbapenems are structurally very similar to the penicillins: the sulfur atom has been replaced by a carbon atom and an unsaturation has been introduced (figure 1.3) [16]. As a result the carbapenems possess the broadest antimicrobial activity amongst the β -lactams [16]. The most common carbapenems are imipenem, meropenem, ertapenem, doripenem and biapenem (figure 1.3). The carbapenems are not registered for use in food-producing animals and are used off-label in companion animals [8].

Another important group of antibiotics was introduced by Benjamin Duggar: the tetracyclines, of which chlortetracycline, isolated from the soil bacteria *Streptomyces aureofaciens*, was the first [17]. In the same year, David Gottlieb reported the isolation of a new broad spectrum antibiotic from the soil bacterium *Streptomyces venezuelae* called chloramphenicol [18] (figure 1.4).

Although, as these examples show, many antibiotics were first isolated from a natural source, most of them are now produced synthetically and new antibiotics are usually semisynthetic modifications thereof [19].

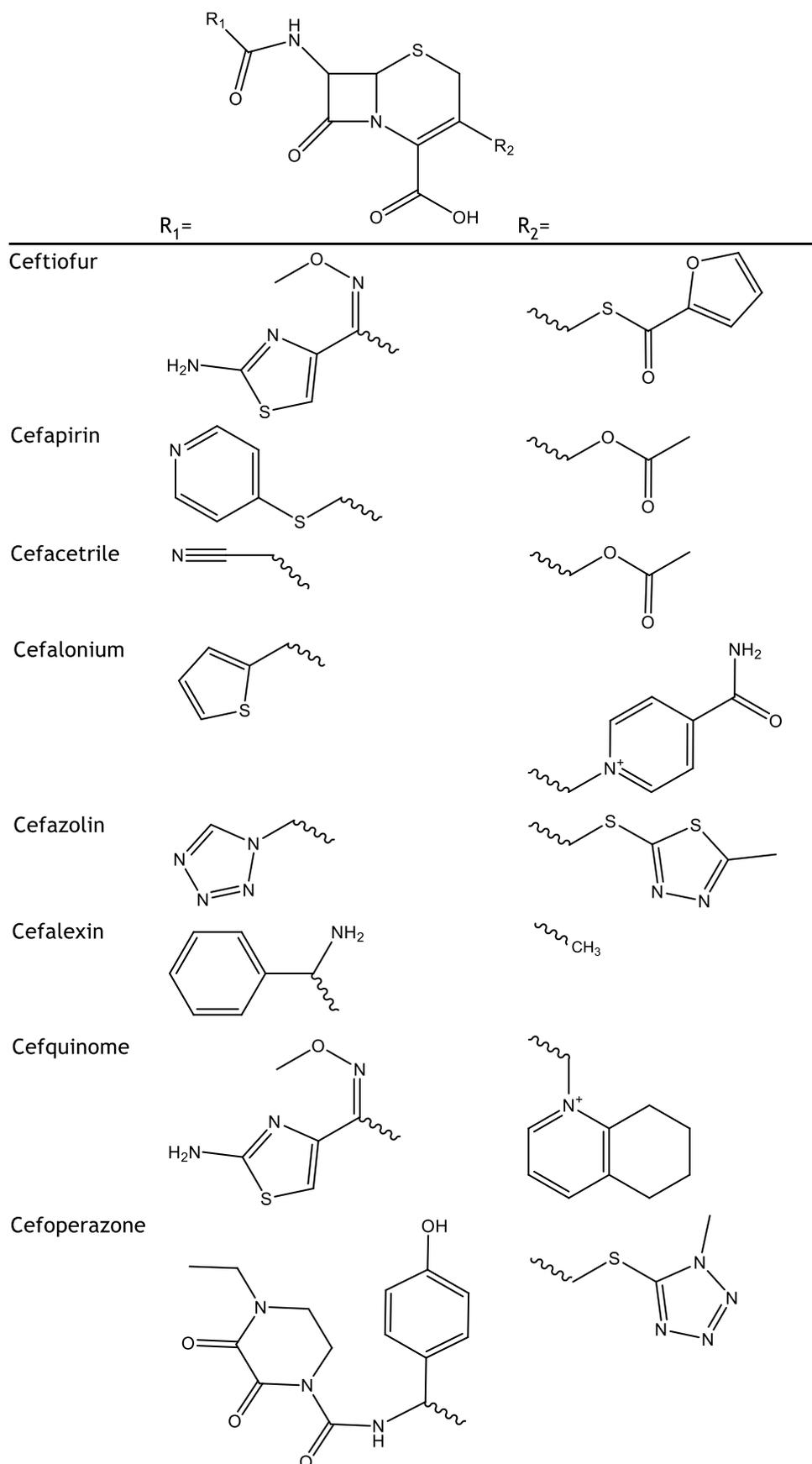


Figure 1.2. Molecular structure of the cephalosporins registered for use in animal practice.

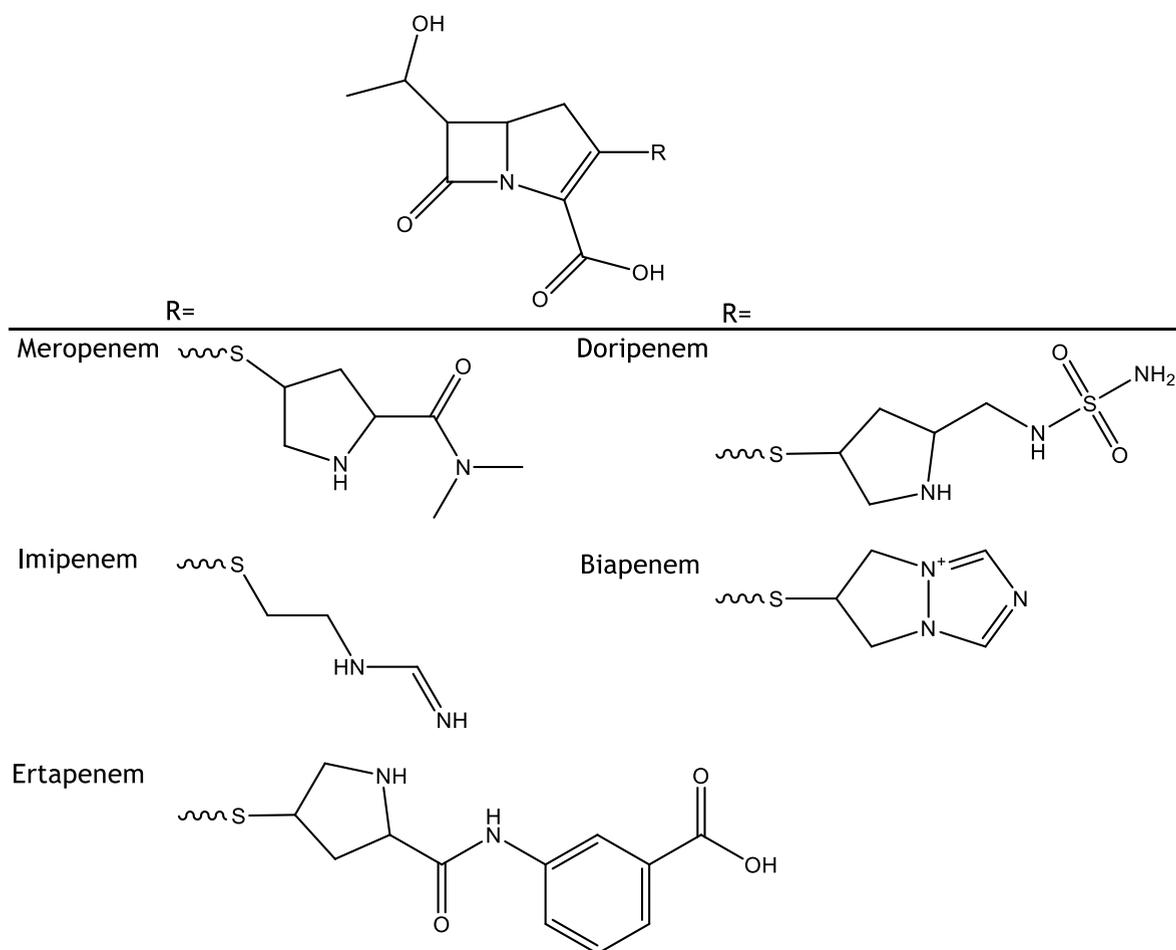


Figure 1.3. Molecular structure of the carbapenems: meropenem, imipenem, ertapenem, doripenem and biapenem.

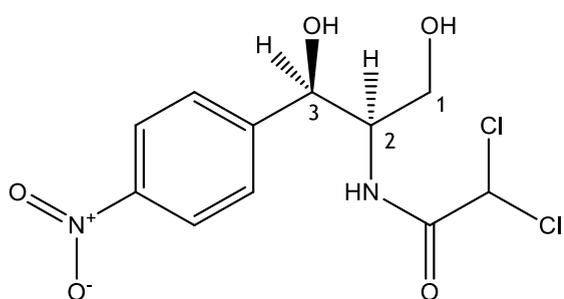


Figure 1.4. Molecular structure of chloramphenicol.

Different antibiotics have different antibacterial mechanisms of activity. The main mechanisms of antimicrobial action are presented in appendix 1.1. β -lactams inhibit the bacteria cell wall synthesis by covalently binding with penicillin-binding proteins (PBP), which catalyse the synthesis of peptidoglycan, the major component of the cell wall [20]. Chloramphenicol binds to the active site of transfer ribonucleic acid (tRNA) and thus inhibits the protein synthesis at the ribosome [21].

Antibiotic usage

Nowadays, the use of antibiotic agents in animal breeding for food production is general practice. Antibiotics are used to treat bacterially infected animals but are also administered as a preventive measure. Furthermore, administration of antibiotics at sub-therapeutic doses has a growth promoting effects, making its use economically advantageous [22]. This is especially of interest since the ban of antimicrobial growth promoting substances in animal feed since 2006 [23,24].

Antibiotic usage in veterinary practice in the Netherlands is monitored to obtain insight in the exposure of farm animals to antibiotics. One way of monitoring antibiotic usage is registering antibiotic sales for therapeutic use. The antibiotic sales from 1999 to 2009 are presented in figure 1.5 [25].

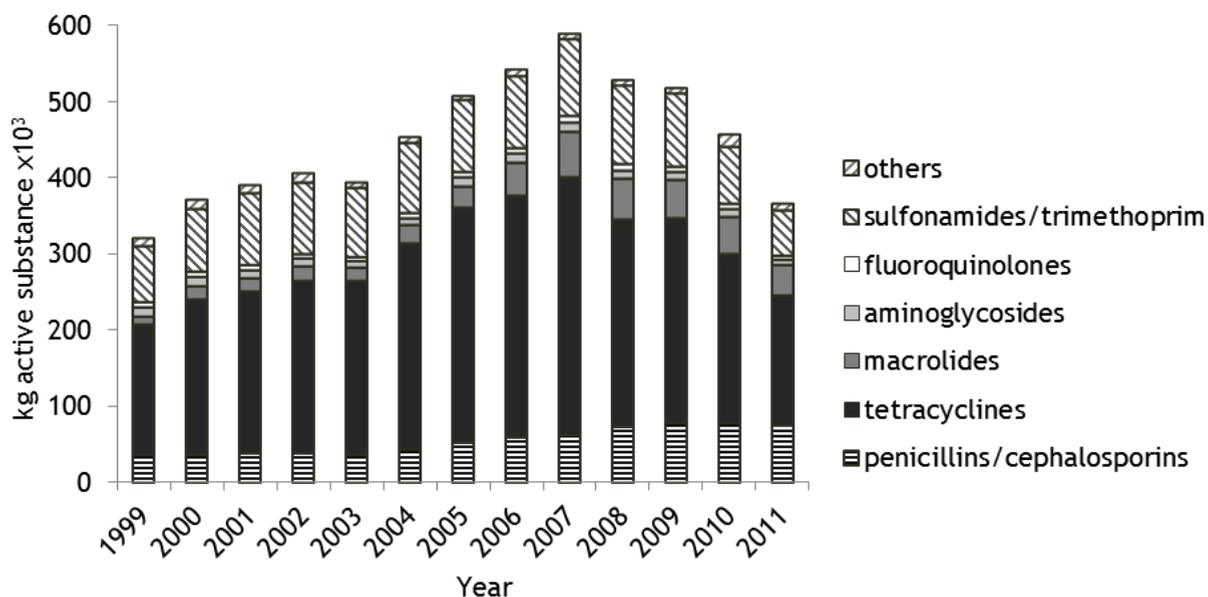


Figure 1.5. Total veterinary therapeutic antibiotic sales 1999 - 2011. Reproduced with permission from [25].

The livestock population remained roughly constant over the years [25] and thus is concluded that sales by the pharmaceutical industry of antibiotics for veterinary therapeutic use increased from 1999 to 2007. An antibiotic sales decrease of over 30 % was observed in 2011 compared to 2007. In all years monitored, tetracyclines are sold the most followed by sulfonamides/trimethoprim and penicillins/cephalosporins.

The therapeutic antibiotic sales were determined for several species: pigs, broilers, veal calves and dairy cows. In 2009, for pigs a tendency to reintroduce traditional antibiotics like tetracyclines and sulfonamides/trimethoprim was observed, whereas for veal calves and dairy cows, besides the traditional antibiotics, newer antibiotics like 3rd and 4th generation cephalosporins and fluoroquinolones were more frequently used. For broilers a severe increase of the use of penicillin antibiotics was observed in 2009 compared to previous years, possibly because penicillin administration results in the enhancement of the feed conversion and growth rate [26].

Adverse effects of antibiotic usage

Excessive antibiotic usage in veterinary practice in food producing animals can have adverse effects on human health [27-29]. Some antibiotics are banned for use in veterinary practice because of their negative effects on health, like bone marrow toxicity, aplastic anemia and carcinogenicity [28,30]. If these antibiotics are illegally administered, residues might occur in food products of animal origin. The adverse effects of the occurrence of these antibiotics in the food chain do not need any further elaboration.

Less obvious is that also the irresponsible and excessive use of regulated antibiotics is a risk to human health [4,27,29,31]. About six to eight percent of the population show a hypersensitive reaction to covalent penicillin-protein conjugates that can be present in food products from animals that have been treated with penicillin antibiotics [32]. Furthermore, the use of antibiotics in veterinary practice can result in the occurrence of resistant bacteria that can be disseminated throughout the food chain and the environment and thus possibly be transferred from animals to humans [31]. Furthermore, low levels of these antibiotics can end up in the human food chain or the environment and do contribute to the evolvement of bacterial resistance as well [29,33].

Recent examples of the occurrence of resistant bacteria are:

(1) methicillin-resistant *Staphylococcus aureus* (MRSA), a bacterium that is resistant to several β -lactam antibiotics. MRSA was found on swine in 2005 and transfer of the resistant bacteria from swine to man was observed [34,35].

(2) extended spectrum beta-lactamase (ESBL) producing bacteria, resistant to, among others, penicillins and 3rd and 4th generation cephalosporin antibiotics [36,37]. The emergence of ESBL is linked to the excessive antibiotic use in poultry breeding and it was found that a majority of animals carry these resistant bacteria [38].

(3) enterohaemorrhagic *Escherichia coli* (EHEC) is a toxin-producing bacteria of which certain strains have developed many antibiotic resistance genes [39-41]. An EHEC outbreak was reported by 9 countries in 2011 and cost the lives of at least 26 people [42].

Several mechanisms of bacterial resistance have been reported. The four main mechanisms of resistance are graphically presented in appendix 1.1 (page 52). Resistance development to β -lactam antibiotics is caused by the expression of β -lactamases, which are enzymes that hydrolyse the four-membered β -lactam ring and thus inactivates the antibiotics [4,43]. Widespread cephalosporin use is thought to have selected for ESBL resistance [44]. To prevent β -lactam hydrolysis due to ESBLs, β -lactam antibiotics are administered in combination with inhibitory substances like clavulanic acid and sulbactams that irreversibly bind to β -lactamases and thus inactivate them [45]. Also chloramphenicol resistance is the result of the expression of an enzyme: chloramphenicol acetyltransferase modifies the drug by acetylation at the 3-hydroxyl group, thereby inactivating the drug's affinity for bacterial ribosomes [46].

Bacterial resistance is steadily increasing [47]. As an example, in 2009 it was found that the resistance of *Escherichia coli* (*E. coli*) against β -lactam antibiotics was disturbing: resistance against 3rd and 4th generation cephalosporins had further increased compared to 2008 and cefotaxime resistance was detected in all animal species ranging from 1.5 % in dairy cattle to 17.9 % in broiler chicken [48]. Steadily increasing antibiotic resistance and the lack of the development of new still effective antibiotics appear to result in a period during which treatment of infections will become increasingly difficult [49,50]. Especially if one realises that many antibiotics applied in veterinary practice are the same antibiotics as used to treat bacterial infections in humans, it is clear that the occurrence of bacterial resistance is a serious healthcare issue [51-53].

Legal framework of antibiotic residue control

Monitoring programs

To detect off-label use of antibiotics and to prevent too high levels of antibiotics to occur in the food chain and thus to assure consumer safety, regulations were established within the European Union from 1986 starting with 86/469/EC [54], 89/610/EC [55] and 2377/90/EEC [56]. More recently 2002/657/EC [57], 2009/470/EC [58] and EU 2010/37 [9] have been established.

In 89/610/EC [55], national reference laboratories were assigned in each EU member state which were made responsible for the analyses of residues of veterinary drugs in products of animal origin. For that purpose, within 2377/90/EEC [56] veterinary medicinal products were divided into four categories which are generally referred to by their annex numbers:

- I: substances for which maximum residue levels (MRLs) have been established;
- II: substances not subject to maximum residue levels;
- III: substances for which provisional MRLs have been established;
- IV: substances for which no maximum levels can be established.

For simplification this was superseded by EU 2010/37 [9] which differentiates two groups: table 1 including all registered substances and table 2 including all banned substances. The established MRLs are included in this document and prohibition of substances for which no maximum levels can be established was laid down in 96/22/EC [59].

Definition:

“Maximum Residue Limit (MRL) ► the maximum concentration of residue resulting from the use of a veterinary medicinal product which may be accepted by the Community to be legally permitted or recognised as acceptable in or on a food. It is based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake (ADI), or on the basis of a temporary ADI that utilises an additional safety factor. It also takes into account other relevant public health risks as well as food technology aspects.” [56]

Guidelines to monitor food and feed products according to these regulations were stated in 86/469/EC [54], which was later repealed by 96/23/EC [60] in which two main groups of substances that should be monitored in food and animal products were distinguished:

Group A: substances having an anabolic effect and unauthorised substances as mentioned in table 2 of EU 2010/37 [9] respectively 96/22/EC [59];

Group B: Registered substances as mentioned in table 1 of EU 2010/37 [9]. An overview of substances per category is presented in table 1.1.

Table 1.1. Overview of substances in group A and B [60].

Group A	Group B
(1) Stilbenes, stilbene derivatives, and their salts and esters	(1) Antibacterial substances, including sulphonamides, quinolones
(2) Antithyroid agents	(2) Other veterinary drugs
(3) Steroids	(a) Anthelmintics
(4) Resorcylic acid lactones including zeranol	(b) Anticoccidials, including nitroimidazoles
(5) Beta-agonists	(c) Carbamates and pyrethroids
(6) Compounds included in Annex IV to Council Regulation (EEC) No 2377/90 [56]	(d) Sedatives
	(e) Non-steroidal anti-inflammatory drugs (NSAIDs)
	(f) Other pharmacologically active substances
	(3) Other substances and environmental contaminants
	(a) Organochlorine compounds including PCBs
	(b) Organophosphorus compounds
	(c) Chemical elements
	(d) Mycotoxins
	(e) Dyes
	(f) Others

Furthermore, 96/23/EC [60] further established the European laboratory infrastructure assigning, besides the national reference laboratories, community reference laboratories (CRL, nowadays called EU reference laboratories (EURL)) which were made responsible for development of methods and assisting NRLs in the analysis of specific substance groups. Last, this document very specifically describes requirements for monitoring plans for the detection of residues of the

mentioned substances in live animals, their excrements, body fluids, tissues, animal products, animal feed and drinking water. The establishment of 96/23/EC [60] has resulted in extensive control programs in each of the EU member states. In 1997, with the implementation of 97/78/EC [61] additional guidelines were established governing the veterinary control of products entering the EU from third countries.

Within the Netherlands, the legal framework of 2009/470/EC [58], 96/22/EC [59], 96/23/EC [60] and the general food law (178/2002/EC [62]) is included in 'Wet dieren' [63] which was effectuated on January 1st 2013 and is framework legislation integrating several other legal documents including 'Regeling diergeneesmiddelen' [64], 'Besluit diergeneesmiddelen' [65], 'Diergeneesmiddelenwet' [66] and 'Regeling verbod handel met bepaalde stoffen behandelde dieren en producten' [67]. In Dutch legislation the focus is on self-control in which producers are responsible for product quality. As surveillance, a 'national plan' has been established by the Dutch Food and Consumer Product Safety Authority (NVWA), part of the Ministry of Economic Affairs, describing the exact type and number of samples that are controlled annually for specified substances or substance groups. More recently, legislation has been established on registration and justification of antibiotic use in poultry breeding [68] aiming for a decrease in antibiotic usage. General legislation on the obligation of keeping an administration on antibiotic usage in veterinary practice is in preparation [69] as well as additional legislation to prevent the unnecessary use of third and fourth generation cephalosporins and fluoroquinolones in veterinary practice. In this it is stated that these antibiotics are only allowed after demonstration of resistance against other antibiotic substances [69].

Method criteria

In 89/610/EC [55] the concept of reference methods was established indicating which analysis techniques and methods are considered suitable for the detection of specific residues. In this, screening and confirmatory methods can be distinguished. This concept was superseded by a criteria based approach as laid down in Commission Decision 2002/657/EC [57]. In this document the minimum requirements of analytical methods used in the analysis of veterinary drug residues, be it screening or confirmatory methods, were described.

Definition:

Screening method ► *methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results. [57]*

Definition:

Confirmatory method ► *methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest. [57]*

This includes criteria for, amongst others, trueness, repeatability and within-laboratory reproducibility. Besides criteria for the performance of analytical methods, the concepts of the minimum required performance limit (MRPL), the concept of the decision limit ($CC\alpha$) and detection capability ($CC\beta$), and regulations on the confirmation of an analytical result were introduced.

Minimum required performance limit

The concept of the minimum required performance limit (MRPL) was introduced to harmonise the analytical performance of analytical methods focusing on group A substances. Official MRPLs were established for chloramphenicol, nitrofurans residues and medroxyprogesterone acetate [70] and later for the sum of malachite green and its metabolite leuco-malachite green [71]. Provisional MRPLs for many other banned substances have been established since, but these did not obtain a legal status yet.

Definition:

“Minimum required performance limit (MRPL) ► minimum content of an analyte in a sample, which at least has to be detected and confirmed. It is intended to harmonise the analytical performance of methods for substances for which no permitted limit has been established.” [57]

In 2005, the interpretation of the established MRPLs changed. According to 2005/34/EC [72] for all products entering the EU that are analysed for veterinary drugs and thus are within the scope of 97/78/EC [60], the MRPL should be regarded as a reference point of action. In other words, for banned substances, only concentration levels above the MRPL are considered being non-compliant results. If the result of an analytical test is below the MRPL “the competent authority has to retain a record of the findings in case of recurrence” [72] and appropriate measures should be taken in the case “results of analytical tests on products from the same origin show a recurrent pattern indicating a potential problem related to one or several prohibited or unauthorised substances” [72]. Due to the principle of equality this guideline is considered to apply not only for imported products but for all products tested [73,74].

Criteria for quantitative residue analysis

For quantitative results, the measurement uncertainty expresses the dispersion of the quantitative value of a single analytical result. Validation procedures to determine the measurement uncertainty of quantitative methods are described in 2002/657/EC [57]. In this document the concept of measurement uncertainty is incorporated by the parameters $CC\alpha$, the decision limit, and CCB , the detection capability, allowing this uncertainty to be applied in the decision making process. This concept was already stated in ISO 11843-1 [75] but was adopted in the analysis of veterinary drugs residues in products of animal origin in 2002. These parameters are discussed in detail elsewhere [57,76,77].

Definition:

“Decision limit($CC\alpha$) ► the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant.” [57]

Definition:

“Detection capability (CCB) ► the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of B . In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of $1 - B$. In the case of substances

with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1 - B.” [57]

Criteria for identification

In 1977 the Food and Drug Administration was the first to publish criteria for regulatory assays for carcinogenic residues [78]. For confirmatory analysis a requirement is that “the observed response must without question be due to the compound being measured and only this compound” [79].

In 1978, for the first time mass spectrometry guidelines for confirmatory analysis using electron impact ionisation (EI) were reported by Sphon [79]. Based on a database containing the empirical results of over 30.000 compounds, using diethylstilbestrol as a model compound, it was concluded that the number of ions in an EI mass spectrum needed for confirmatory analysis depends on their uniqueness, but that three ions of the proper ratio can be regarded as the minimum number [79,80]. Although this was specifically reported as being a guideline instead of a rule, this observation became known as the “three-ion criterion” [81] and was applied in practice. An update of Sphon’s experiment was reported in 1997 [82] and again in 2008 [83], showing that the choice of ions used for confirmatory purposes is critical to prevent false positive findings.

Sphon [79] only took the mass spectrometric detection into consideration and therefore in 1997 a method was suggested to determine the suitability of complete methods based on “selectivity indices” [82]. This was based on the fact that selectivity is directly related to the different steps of the method of analysis applied, including the extraction and the sample clean-up. In this approach different extraction, clean-up, and detection techniques were assigned a certain number of selectivity indices. By combining extraction, clean-up, separation (thin layer chromatography, LC or GC) and detection techniques to obtain a minimum number of selectivity indices, a method could be considered adequate for confirmatory analysis.

In 2002 the European Union established criteria concerning the performance of analytical methods and the interpretation of results [57]. According to this document, to obtain adequate identification power, confirmatory methods have to provide information on the chemical structure of the substance and thus only spectrometric detection techniques are considered to be sufficient, among which fluorescence detection (only for group B substances) and mass spectrometry, both in combination with chromatography. However, based on recent false positive findings, gas chromatography coupled to mass spectrometry (GC-MS) or liquid chromatography coupled to MS (LC-MS) are the preferred techniques for confirmatory analysis [84,85].

According to 2002/657/EC [57], if full scan single MS is used, a minimum number of four ions (group A substances) or three ions (group B substances) must be present in the spectrum having a relative intensity of > 10 % of the base peak. This is a more stringent criterion compared to the “three-ion criterion” [79]. If product ions are monitored using other than full scan techniques, the “identification points concept” applies, which was introduced in 2001 [86] and was later included in 2002/657/EC [57]. This approach is derived from the previously mentioned approach based on “selectivity indices”, but because selectivity was then predominantly considered a function of the measuring technique, the extraction and clean-up were not included.

When using GC- or LC-MS a method should earn a minimum number of identification points for confirmatory analysis. For the identification of group B substances, three identification points were considered adequate and for group A substances, this is four identification points [57,87]. The number of points earned for each mass spectrometric technique is presented in table 1.2. It is stated that “(1) each ion may only be counted once, (2) GC-MS using electron impact ionisation is regarded as being a different technique to GC-MS using chemical ionisation, (3) different chemical derivatives of an analyte can be used to increase the number of identification points only if the derivatives employ different reaction chemistries and (4) transition products include both daughter and granddaughter products” [57].

Table 1.2. Earned identification points per MS technique [57,87].

MS technique	Identification points
Low resolution mass spectrometry (LRMS)	1.0
LRMS ⁿ precursor ion	1.0
LRMS ⁿ transition products	1.5
High resolution mass spectrometry (HRMS)	2.0
HRMS ⁿ precursor ion	2.0
HRMS ⁿ transition products	2.5

As a result, when using GC or LC in combination with low resolution MSⁿ a combination of one precursor ion and two product ions results in four identification point which is sufficient for confirmatory analysis. Another option is the monitoring of two precursor ions, each with one product ion, resulting in five identification points. In all cases a minimum of one ion ratio (relative ion abundance) must be determined and all determined ion ratios must comply with the criteria as presented in table 1.3 before the result can be considered as being non-compliant. Furthermore, in LC, the difference of the retention time of the unknown analyte and the reference standard should not exceed 5 %. This is 2.5 % in the case a relative retention time is calculated based upon an internal standard.

Parallel to the EU criteria, other guidelines stating confirmatory analysis criteria were established [88-94]. All are in agreement with the EU guidelines with regard to the requirement to use chromatography in line with either full scan MS or MS/MS in selected ion monitoring mode [95,96]. Furthermore, all state that in the case of MS/MS at least two transitions from precursor to a product ion should be monitored to obtain adequate selectivity and according to all documents confirmation of the identity should be carried out by comparing the ion ratio of the unknown compound in the sample with a known reference standard permitting maximum tolerance levels. Even though the approaches are very similar, the criteria for accepting or rejecting a match differ significantly. The different criteria that apply to LC-MS(/MS) are presented in table 1.3. Furthermore, criteria for the (relative) retention time in LC differ (table 1.3).

Table 1.3. Maximum allowed tolerance levels of relative ion abundances and retention time for confirmation of the identity based upon LC-MS/MS according to different guidelines [57].

Guideline	Ion ratio (%)	Maximum tolerance levels relative ion abundance	Maximum tolerance levels for (relative) retention times
EU [57]	> 50	± 20 % relative	RT: 5 % RRT: 2.5 %
	> 20 - 50	± 25 % relative	
	> 10 - 20	± 30 % relative	
	≤ 10	± 50 % relative	
SANCO 12495 [88]	Equal to EU		
SOFT/AAFS [89]	All	± 20 - 30 % relative	“Slightly larger than” 2 %
AORC [91]	All	± 20 % absolute or ± 40 % relative, whichever is larger	RT: 2 % or 12 s, whichever is the larger RRT: 2 %
CVM [92]	All	± 10 % relative in case of two transitions ± 20 % relative in case of three transitions	5 %
IOC [90]	All	± 10 % absolute or ± 25 % relative, whichever is larger	RT: 1 % RRT: 1 %
UK Drug testing [93]	All	± 20 % relative	2 % or 3 s, whichever is the lower
WADA [94]	≥ 50 - 100	± 15 % absolute	RT: 2 % or 24 s, whichever is the lower
	≥ 25 and < 50	± 25 % relative	
	< 25	± 10 % absolute	

SANCO = Directorate General for Health and Consumer Affairs

SOFT/AAFS = Society of Forensic Toxicologists / American Academy of Forensic Sciences

AORC = Associating of Official Racing Chemists

CVM = Center for Veterinary Medicine

EU = European Union

IOC = International Olympic Committee

WADA = World Anti-Doping Agency

Confirmation versus identification

In ISO 17025 [97] general requirements for the competence of testing and calibration laboratories are laid down. This document states that “calibration certificates shall contain the measurement results including the measurement uncertainty”. Often, uncertainty is solely regarded as a parameter applicable to quantitative results. However, ‘uncertainty’ can be interpreted as a much broader concept which involves both the quantitative and the qualitative aspect.

Definition:

“Uncertainty (in statistics) ► noun, the estimated amount or percentage by which an observed or calculated value may differ from the true value.” [2]

“Uncertainty ► noun, the state of being uncertain -Uncertain ► adjective, not able to be relied on; not known or definite.” [2]

Although 2002/657/EC [57] does not specifically discuss the uncertainty of a qualitative result, it states that a result can only be considered non-compliant if “the decision limit of the confirmatory method for the analyte is exceeded”, indicating that the use of a confirmatory method is mandatory.

Furthermore, the document defines confirmatory methods as “a method that provides full or complementary information enabling the substance to be unequivocally identified (...)” [57]. To obtain a clear view on the implications of this statement, a brief discussion on the concept of confirmation versus identification is needed, which is discussed in more detail elsewhere [80,81,95,98].

No universally-accepted definitions for confirmation and identification exist. In this thesis the term ‘confirmation’ presumes knowledge on the identity of the compound present, e.g. from previous analysis or from other information. A positive confirmation indicates that the result is not against the presumptions made: the compound shows the same characteristics as the reference compound selected, but it does not exclude the possibility of the presence of another compound showing the same characteristics. From this Lehotay *et al.* [80] concluded that for confirmation a second method (preferably having orthogonal

selectivity to the first method) is mandatory. At least one of both methods should meet the identification criteria [80]. However, neither in 96/23/EC [59] nor in 2002/657/EC [57] a second method is prescribed.

Definition:

“Confirmation ► noun. the action of confirming something or the state of being confirmed.” [2]

“Confirm ► verb. Establish the truth or correctness of (something previously believed or suspected to)” [2]

In contrast, in ‘identification’ no a priori presumption on the substance present is made and thus identification requires that all other substances are excluded, so that the reported substance is the only possible candidate [95].

Definition:

“Identification ► noun, the action or process of identifying someone or something or the fact of being identified.” [2]

“Identify ► verb, establish or indicate who or what (someone or something) is.” [2]

In conclusion, although some uncertainty is allowed for a quantitative result, no uncertainty is specified for the qualitative result (the confirmation). When realising the indefinite number of substances that exist (also including substances not yet known to mankind), unequivocal identification is an impossible task [80] and therefore a degree of uncertainty of the qualitative result should be considered depending on the risks involved related to a false positive finding [81].

Selectivity

The identification power and therefore the qualitative uncertainty of the confirmatory method is expressed in its selectivity. This term is often interchangeably used with ‘specificity’ [99], which is actually the ultimate of selectivity [100]. In contrast to specificity, selectivity can be graded (e.g. being high, excellent or extreme) but it is not clear what each gradation exactly reflects [101,102].

Definition:

“Specific ► adjective, clearly defined or identified.” [2]

Definition:

“Selective ► adjective, relating or involving the selection of the most suitable or best qualified.” [2]

“Selectivity ► the ability of a method to distinguish between the analyte being measured and other substances.” [57]

“Selectivity ► the extent to which a method can determine particular analyte(s) in a complex mixture without interference from other components in the mixture.” [103]

Selectivity is a measure directly related to the chance of the occurrence of a false positive identification. In case of a false positive result the presence of a certain substance is reported whereas this specific substance is not present. The power of discrimination between the substance present and closely related substances (isomers, metabolites, degradation products, endogenous substances, matrix constituents, etc.) is key to prevent false positive results in confirmatory methods. When one realises that non-compliant analytical results in food product analysis can have a huge effect on personal lives and society as well as on economy this becomes more than evident. In case the presence of a banned substance is reported whereas it is not truly present in the sample, farmers may be falsely accused of using banned carcinogenic antibiotics and go bankrupt after prosecution; certain branches may get a bad reputation and people may choose to avoid buying certain products; shipments of perfect quality food products might be destroyed, affecting the economy, international relations and food security. From this it is clear that, especially for confirmatory methods, selectivity is a very important parameter.

None of the established MS and LC criteria [57,89-94] are based on scientific data, but are a result of experts' judgments and thus it cannot be concluded that one criterion is better than the other. This depends on the point of view taken: strict criteria result in an increased probability of false negative results whereas less strict criteria result in an increased possibility of obtaining a false positive result. This concerns respectively producer protection versus consumer safety.

A review on the fundamentals of the confirmation criteria was reported by Van Eenoo *et al.* [104] and Faber [105] who both observed that there is no fluent transition between the maximum allowed tolerances for the different ion ratio categories in the WADA and EU regulations. For instance, in the WADA regulations, in case the reference ion ratio is 24 %, the maximum allowed tolerance is 10 % (absolute), whereas for a reference ion ratio of 25 % the maximum allowed tolerance is 6.25 % (absolute).

Although all of the ion ratio criteria were established based on experts' judgment, it is not unlikely that incorrect identification takes place. Critical reviews of the ion ratio criteria were presented by Kaufmann *et al.* [76,106]. The influence of matrix constituents on the ion ratio was described and appointed as a possible cause of deviating ion abundances. In the described analysis of quinolone antibiotics in a marbofloxacin containing muscle sample, this resulted in deviating ion ratios and therefore the detected compound could not be identified as being marbofloxacin according to EU regulations [57] and would in practice result in a false negative result. Also, a case of a false positive identification was reported [107]. Sebuthylazine, a pesticide compound, was identified according to the EU criteria [57] in a tarragon (*Artemisia dranunculus*) sample whereas, after additional high resolution mass spectrometric analysis, the compound could be identified as the endogenous nepellitorine [107].

In summary, maximum tolerance limits for ion ratio and retention time as established in several legal documents are very useful tools for the confirmation but not necessarily for the identification of compounds. However, some aspects that are not explicitly stated in the guidelines should be taken into account:

- The selectivity of the whole procedure, including the sample preparation procedure.
- The importance of LC: low resolution and high resolution LC are still considered equally selective.
- The relative importance of certain transitions: EU legislation indicates that “the selected diagnostic ions should not exclusively originate from the same part of the molecule” [57], but the loss of water or ammonia during collision induced dissociation (CID) is considered equally selective to the loss of larger, more compound specific losses. The UK guidelines for urine drugs testing mention product ions that are

considered insufficiently selective, viz. $m/z < 50, 58, 86, 91$ and 105 [93] and the Society of Forensic Toxicology indicated the low selectivity of the loss of water [102].

An alternative to the use of a priori established tolerance limits as indicated by the legal framework was presented by Van de Voet *et al.* [108] for GC-MS analysis. The applicability of constructing a confidence interval for the ion ratio based on empirical data was demonstrated using a multi-variate approach. This approach was found very useful to replace a priori established tolerance limits, especially at very low concentration levels. A simpler uni-variate approach was pointed out by Faber [105]. Using these approaches, the confidence needed for determination of the identity can be easily set by adjusting the confidence interval parameters. But the chance on false positives and false negatives is set based on empirical data (e.g. from a validation) and it does not give information on the selectivity of the applied method.

Assessment of selectivity

According to 2002/657/EC [57] a confirmatory method shall be able to distinguish between the analyte and other substances under the experimental conditions chosen. Two approaches are recommended to demonstrate the selectivity. First, potentially interfering substances that are likely to be encountered shall be evaluated. Representative blank samples shall be fortified at a relevant concentration with these substances to test for interferences. Second, at least 20 representative blank samples shall be analysed under within-laboratory reproducibility conditions to detect the presence of possible interferences and to estimate the effect of these interferences.

According to the regulations, from the combination of the ‘identification points concept’ and the recommendations to test for interferences, optimal selectivity should be obtained, even though the guidelines do not seem to originate from statistically supported data. At the same time the document states: “An estimate to which extent this (the occurrence of a false positive result) is possible has to be provided.” However, no guidelines are given to do this. How to determine and express the grade of selectivity?

To express the grade of selectivity of chemical reactions, in 1965, the use of selectivity indices was suggested [102,109]. A selectivity number was assigned based on the number of compounds that would respond to the reaction in the most favorable circumstances possible. One compound would result in a selectivity index of I, two compounds in a selectivity index of II, three to five in a selectivity index of III, etc. It would not be necessary to have more than about six or seven groups because if several compounds respond in a reaction the term selectivity would become meaningless [102]. This approach was further improved in 1976 differentiating natural selectivity (under general conditions) and selectivity under the most favorable conditions [110].

An approach for evaluation of the certainty of analytical methods, among which chromatography and mass spectrometry, was reported in 1989 [111]. An uncertainty factor was introduced representing the reciprocal value of the number of possibilities for an open set and the ratio of the number of indistinguishable items to the total number of items for a closed set. As a result, an uncertainty factor of 50^{-1} was found for LC. However, this is based upon the number of peaks fitting in a chromatogram and is not correlated to any parameters (e.g. polarity) of the compound detected.

The same approach resulted in an uncertainty factor of 10^{-8} for low resolution MS. However, this is based upon equal probability of all ions in a mass spectrum. From 10,000 mass spectra, Van Marlen *et al.* [112], determined the probability of the presence of an ion in GC-MS. Binary coded mass spectra were used and thus the abundance of the ions was discarded. On this basis the information given by a specific ion was determined indicating that the presence of an ion at m/z 77 gave the most information and is therefore the most selective.

McLafferty *et al.* [113] and Pesyna *et al.* [114,115] carried out probability based mass spectra matching and regarded the probability of ions to occur in a mass spectrum. From a large collection of GC spectra, the occurrence of ions between m/z 29 - 400 were modeled indicating that ions at m/z 29, 39, 41, 43, 55, 57, 73, 91 and 105 have a high probability in contrast to ions at m/z 37, 38, 49 - 53 and 62 - 66, which have a low probability. Furthermore, a nearly linear correlation was found between the mass and the occurrence of ions above m/z 115. Based on this probability matching was carried out based upon the probability theory which states that if a number of events occur with a certain probability, the

probability of all these events to occur is the multiplication of all the individual probabilities. This overall probability is a measure for the uniqueness of a spectrum and thus for selectivity.

Only limited data are available on the probability of the occurrence of ions in MS using electrospray ionisation (ESI) as is the most commonly applied ionisation technique in veterinary drug residue analysis [116].

Methods of analysis

Methods are generally divided in screening and confirmatory methods. Screening methods are usually inexpensive, rapid and suitable for high-throughput analysis, but do not provide unequivocal identification and usually do not result in exact quantitative results. Confirmatory methods must be instrumental spectrometric techniques and therefore are more expensive and time-consuming, but are supposed to be highly selective in order to provide unequivocal identification. The combination of a bio-based screening method and an instrumental confirmatory method is very strong in residue analysis. With a bio-based screening a fast qualification (compliant or suspect) of samples can be made based on biological activity. Compliant samples can be reported right away and the usually few suspect samples can be subsequently analysed by a more elaborate confirmatory method based on chemical properties of the compound.

Bio-based screening methods

Several bio-based tests have been reported for the screening of antibiotic substances in different matrices. Bio-based screening methods used for the detection of antibiotics in products of animal origin have been reviewed recently [117-120]. The most commonly applied bio-based screening techniques for antibiotics are immunoassays, microbiological inhibition assays and reporter gene assays [120].

Immunoassay

Immunoassays are based upon a binding reaction between a compound and an antibody. The most commonly applied immunoassay in antibiotic analysis is the enzyme-linked immuno sorbent assay (ELISA) [117]. Different test formats exist in ELISA, but all tests are based upon the same principle. The sample that is screened for antibiotic content is incubated with antibodies, under the production of an analyte-antibody binding complex. Next, the degree of binding, which is related to the level of antibiotics present in the sample, is determined (e.g. by adding a fluorescent label) [117,118,121]. ELISA tests for analysis of β -lactam antibiotics have been reviewed recently [122] and immunoassay tests for the analysis of chloramphenicol were reported as well [123-128]. Note that apart from ELISA other platforms such as surface plasmon resonance (SPR) biosensors [121,129-136] and dipstick assays can be applied [137].

An important advantage of immunoassays is that they are able to detect the presence of antibiotics at very low levels, which makes them even useful for screening of banned substances but the main challenge of immunoassays is the production and supply of antibodies, which should be selective towards the aimed antibiotic compound or group.

Microbiological inhibition assays

Microbiological inhibition assays are based on a reaction between a bacteria and the antibiotic present in the sample. The tube and plate test are the most common formats for this type of screening assays.

The tube test consists of a growth medium inoculated with a bacterium, supplemented with a pH or redox indicator. If no specific antibiotics are present, the bacteria start to grow and produce acid, which will cause a detectable color change. If antibiotics are present that inhibit bacterial growth, no color change will occur [119,138]. Within Europe, the tube test is commonly applied for the analysis of milk.

The plate test consists of a layer of inoculated nutrient agar and samples are brought onto the surface. If no specific antibiotics are present, the bacteria start to grow throughout the plate. If a specific antibiotic is present, no bacterial growth will occur around the sample, which can be observed from the bacteria-

free inhibition zone. In Europe this has been the main test format since screening of slaughter animals for the presence of antibiotics started [119]. Many combinations of plates (up to seven within one test) containing different bacteria under varying environments are applied to cover the relevant spectrum of antibiotics at relevant levels [119,122]. A few critical comparisons of plate tests were reported in literature [139-141].

An important advantage, compared to immunoassays and instrumental methods is that microbiological tests can detect any antibiotic compound that shows antibacterial activity [142] and they have the potential to cover the entire antibiotic spectrum within one test [119]. The most important drawbacks of the microbiological tests are their lack of selectivity of especially the tube test, relatively high detection limits and the long incubation time. As a result microbiological inhibition assays are not suitable for detection of banned antibiotic compounds like chloramphenicol.

Reporter gene assays

Reporter gene assays consist of a genetically modified bacterium, containing an inducible promoter, responsive to a particular antibiotic, coupled to a reporter gene or operon [120]. Based on the presence or absence of responsive antibiotics, the reporter gene induces a fluorescent signal or the operon affects the transcription to produce or inhibit a signaling process.

An example is the Tet-Lux which is based on specific, tetracycline-controlled expression of bacterial luciferase genes that code for enzymes responsible for light emission [143]. When tetracyclines enter the genetically engineered cell, it releases a repressor protein from the luciferase operon allowing synthesis of the luciferase reporter genes, resulting in a luminescence signal [120,144,145]. A comparable assay has been reported for the screening of macrolide antibiotics [146].

The tetracycline cell-biosensor was compared with the microbial inhibition test [147]. The cell-bioassay was found to be more sensitive and faster than the microbial assay.

Instrumental methods

Until the last decade of the 20th century, the main instrumental techniques used for veterinary drug residue analysis were liquid chromatography (LC) using ultra violet detection (UV), diode array detection (DAD) and fluorescence detection (FLD), and gas chromatography (GC) using flame ionisation detection and electron capture detection [148].

Since 1992 LC coupled to mass spectrometry (MS) became more affordable and was gradually implemented for the analysis of compounds for which GC is unfavorable due to thermolability or insufficient volatility [149]. Since then numerous methods for the analysis of veterinary drug residues were developed using either LC or GC coupled to triple quadrupole (QqQ) MS [150,151] as can be concluded from the increase in peer-reviewed papers during the last decade (figure 1.6).

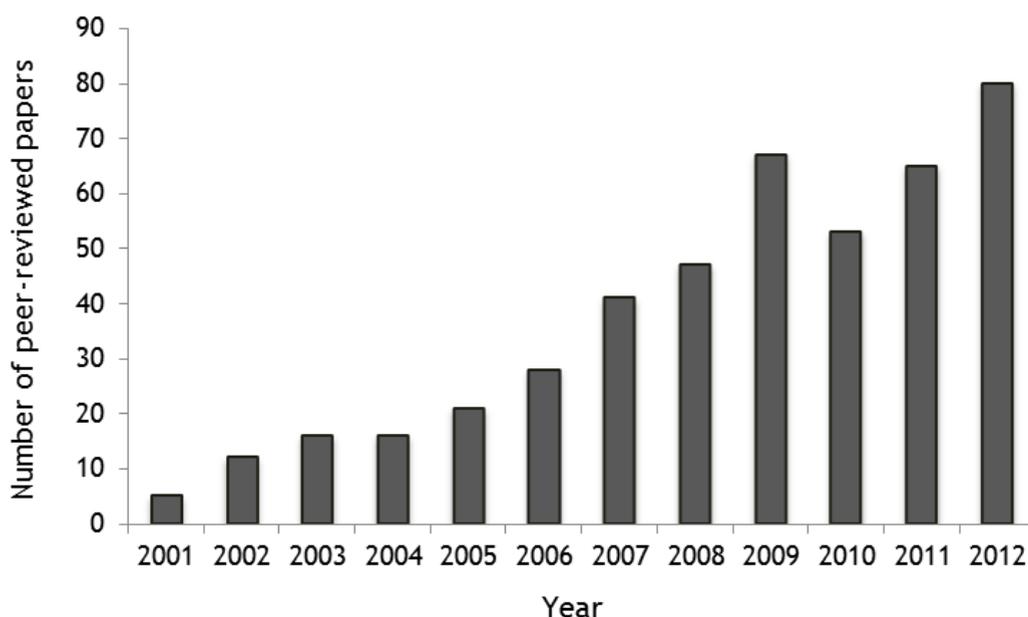


Figure 1.6. The number of peer-reviewed papers in the last decade (found in SciFinder [152], using the key words 'mass spectrometry' AND 'chromatography' AND 'residue' AND 'veterinary drugs').

The first generation QqQ-MS systems needed relatively long dwell times (> 100 ms) in the multiple reaction monitoring (MRM) acquisition mode to obtain a sufficient number of ions for accurate detection of an ion transition [153]. This dwell time in combination with the low resolution of the LC system limited the number of compounds that could be detected within one run [154]. As a result

single-compound methods and multi-methods that included antibiotics belonging to a single compound group, were developed [155].

In the last decade fast switching (< 10 ms) triple quadrupole instruments became available. This facilitated the development of high performance liquid chromatography (HPLC) MS/MS methods in which a larger number of compounds were detected within one run. These methods include up to 120 compounds belonging to different antibiotic groups [155-168] and even to different classes like veterinary drugs and pesticides [154].

The development of high resolution LC (HRLC) featuring sub-2 μm stationary phase particles delivered a higher chromatographic resolution and higher sample throughput, because separation can be carried out with the same or better chromatographic resolution within a shorter time frame [169]. This innovation resulted in further development of multi-compound and multi-class methods containing over 250 compounds belonging to different compound groups [168-177].

Meanwhile, with developments in the detection limits of time of flight mass spectrometry (TOF/MS) and the development of orbitrap instruments, highly selective, HR-MS detection techniques became routinely available [178]. Because HR-MS is a full scan technique, in theory, an indefinite number of compounds that can be ionised by the selected ionisation technique can be analysed simultaneously within a certain m/z range. Several methods using these techniques for the analysis of, among others, antibiotics have been published [179-186].

Challenges in antibiotic residue analysis

Mass spectrometry is considered a highly selective technique. It is often assumed that, when carrying out a confirmatory analysis according to the confirmation criteria, the identification is unequivocal. This suggests that the identity of the compound present is proven beyond any doubt. However, as discussed above, this depends on many factors including the measurement technique, sample preparation, and the number and nature of the (product) ions monitored. Therefore, the occurrence of false positive results cannot be entirely ruled out.

Challenge 1

Legitimate questions are:

- Is the reported substance truly the substance present?
- What are the chances the observed signal is a result of the presence of an interfering substance rather than the reported substance?

To be able to answer these questions, ***a method is needed to determine the uncertainty of the identification provided by confirmatory analysis.*** Whether or not the certainty of the identification of the compound present is adequate depends on the nature of the detected compound and the risks at stake.

Challenge 2

However, other aspects are also relevant before initiating enforcement measures or even a trial. Legitimate questions are:

- Is the detected substance endogenous or exogenous?
- Is the detection of a substance a result of off-label use?

The issue of endogenous versus exogenous was first recognised in doping control. As an example 19-norandrosterone (NA) is used as a marker to detect misuse of the synthetic anabolic steroid 19-nortestosterone [187] in sports doping. However, small amounts of NA are biosynthesised by pregnant women and further evidence exists for physiological origin of this compound and thus WADA defined NA as endogenous in 2006 [187]. Examples in animals are the endogenous production of the banned anabolic steroid 17 β -nortestosterone in intersex pigs [188,189], the banned thyreostat thiouracil that proved to occur endogenously in animals through a brassicaceae diet [190,191] and semicarbazide, a marker for the banned antibiotic nitrofurazone, that is not exclusively related to nitrofurazone use, but can occur naturally in shrimp [192].

Note that many antibiotics, among which chloramphenicol and penicillin, were first isolated from bacteria and thus do occur naturally as well. ***Research is needed to determine whether these antibiotics can occur in products of animal origin via a natural route.***

Challenge 3

Another challenge in antibiotic residue analysis is related to the emergence of antibiotic resistance. It is recognised only recently that antibiotic usage in

veterinary practice and the presence of low levels of antibiotics in food products and the environment contribute to the emergence of antibiotic resistance [29]. Therefore, *methods capable of detecting off-label use of antibiotics should be developed*. Legitimate questions are:

- Are detection limits sufficient to detect off-label use?
- Are adequate marker metabolites monitored to be able to detect off-label use?
- Are all relevant compounds, including metabolites, detected?

A clear example is the analysis of β -lactams in poultry production. A main difficulty in β -lactam analysis is that some penicillin antibiotics are unstable (mainly ampicillin, amoxicillin, penicillin G and penicillin V) [4] and that some cephalosporins, including ceftiofur, are known to rapidly metabolise after intramuscular administration. To allow detection of off-label use, methods need to be developed that include a broad spectrum of β -lactam antibiotics and that not only detect the administered drug, but also are able to detect metabolites thereof, including protein bound residues.

Thesis outline

This thesis is a result of research carried out within the field of antibiotic residue analysis. In chapter 2 a literature review is presented on the latest trends observed in the sample clean-up procedures in the field of veterinary drug residue analysis using LC-MS/MS. As a consequence of the advances in the available instruments the number of compounds that are analysed in a single run increased rapidly throughout the last decade. As a prerequisite, to be able to simultaneously analyse compounds having different physical and chemical properties, extraction and sample clean-up procedure had to change as well. This has resulted in the development of generic, non-selective sample preparation procedures. The most frequently reported generic sample preparation methods are a solvent extraction only, solid phase extraction and a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) approach.

In chapter 3 the selectivity of analytical LC-MS/MS methods based on the identification points concept is studied. The chance of an incorrect identification depends on parameters related to the LC separation like the polarity of a

compound. and parameters related to the MS detection, like the molecular mass and product ion mass. Based upon these parameters, models were established to determine the grade of selectivity obtained. If needed, additional measures can be taken to increase the selectivity of the method applied.

In chapter 4 a major analytical challenge is discussed: the analysis of the banned antibiotic chloramphenicol (CAP). CAP is a broad-spectrum antibiotic with historical veterinary use in all major food-producing animals. CAP is a suspect carcinogen and can cause bone marrow toxicity and aplastic anemia, and for this reason the drug is banned for use in food-producing animals in the EU and in many other countries. In recent years, findings of CAP residues in food products such as poultry, honey and sheep casings had a major impact on international trade [193]. CAP can occur in the *meta*-configuration and in the *para*-configuration and has two chiral centers, resulting in eight different isomeric configurations. Only for RR-*p*-CAP regulations were established because this is the active isomer. The challenges related to the analysis of CAP are introduced in more detail in section 4.1.

A complicating factor in the analysis of CAP is that it is biosynthesised by the soil organism *Streptomyces venezuelae* and chemically synthesised for commercial use as well [194]. Evidence of the natural occurrence of CAP in plants and soil is presented in section 4.2.

In section 4.3 research is presented in which the selectivity of generally applied LC-MS/MS methods for the confirmatory analysis of CAP in relation to its isomers was determined. Furthermore, a chiral LC-MS/MS system was developed to be able to discriminate the eight CAP isomers. Using this method, it was studied if the comparison of the isomeric patterns of non-compliant samples with the isomeric pattern of CAP formulations yields information on the origin of CAP residues.

In section 4.4 a new method to distinguish the presence of the active configuration of CAP from the other configurations at trace level in urine is presented. In this especially the development of the sample clean-up was challenging to obtain a robust separation of the isomers on the chiral LC column.

The hypotheses was suggested that CAP, present in soil, is taken up by plants and accumulates there. This would explain the positive findings of CAP in plants,

herbs, straw and corn silage. A model plant experiment was set-up to investigate this hypothesis of which the work is presented in section 4.5.

In chapter 5 the analysis of β -lactam antibiotics in tissue is discussed. The β -lactam antibiotics consist of three main groups: penicillins, cephalosporins and carbapenems. Apart from their human medicinal use, penicillins are the most frequently used antibiotics in poultry breeding [25], which is likely to have contributed to the emergence of extended-spectrum- β -lactamase-producing bacteria. Cephalosporins are assigned as critically important antibiotics in human medicine [195] and should be used sparingly. Nevertheless, bacterial resistance against this group has been reported as well. To prevent further evolvment and dissemination of bacterial resistance, effective analytical methods are needed to detect off-label use of β -lactams in animal breeding. The challenges related to β -lactam analysis are introduced in more detail in section 5.1.

A main difficulty in β -lactam analysis is that some penicillins are unstable and that some cephalosporins, including ceftiofur, are known to rapidly metabolise after intramuscular administration. In section 5.2 the work on the degradation study of ceftiofur and cefapirin under several conditions is presented and the impact of the results on the monitoring of these drugs is discussed.

The ceftiofur MRL was defined as the sum of all active metabolites expressed as DFC [9] and thus all metabolites should be included in the methods. In section 5.3 a new approach for ceftiofur analysis is described which is compared to routinely applied approaches. To allow detection of not only the administered drug but also metabolites thereof, including protein bound residues, the selectivity was intentionally compromised.

Based on the new approach a method was developed for the analysis of a broad range of β -lactam antibiotics including penicillins, cephalosporins (including their relevant metabolites) and carbapenems. This work is presented in section 5.4.

Finally, in chapter 6 general conclusions on selectivity, antibiotic residue analysis, and chlroramphenicol and β -lactam analysis specifically are summarised and future perspectives are discussed.

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Figure A1.1. Mechanisms of antimicrobial action and resistance in Gram-negative organisms. This picture represents a Gram-negative bacteria cell. Black boxes represent mechanisms of drug action and white boxes represent mechanisms of resistance. Below each box there are several examples of drugs presenting those types of mechanisms. Five main mechanisms of antimicrobial action are those who (a) act in the cell wall synthesis; (b) act in the protein translation; (c) act in metabolic precursor biosynthesis; (d) act in the molecular genetics processes (replication, transcription); and (e) disrupt membrane function and permeability. Some of the mechanisms of resistance are (1) enzymatic inactivation of the drug by the presence of β -lactamases (1.1); (2) presence of an enhanced efflux pump; (3) porin mutation obstructing the drug entrance; and (4) target modification of the drug, such as the mutation in the penicillin binding proteins (PBPs). Reproduced with permission from [4].

Chapter 2

Selectivity in the sample preparation for the analysis of drug residues in products of animal origin using LC-MS



B.J.A. Berendsen, A.A.M. Stolker, M.W.F. Nielen, Selectivity in the sample preparation for the analysis of drug residues in products of animal origin using liquid chromatography coupled to tandem mass spectrometry, *TrAC - Trend. Anal. Chem.* 43 (2013) 229-239.

Abstract

Sample preparation is a critical process in relation to analysis time, sample through-put and therefore analysis costs. Due to recent advances in liquid chromatography mass spectrometry instrumentation the detection of many compounds within one run became possible and methods for the simultaneous analysis of different compound groups were developed. To be able to simultaneously analyse such compounds having different physical and chemical properties, generic, non-selective sample preparation procedures are applied. The most frequently reported generic sample preparation methods are a solvent extraction only, solid phase extraction and a QuEChERS approach. These multi-analyte methods - sometimes including more than 150 different compounds in one method - are of much interest for analytical laboratories due to the reduction of costs. A clear drawback of generic sample preparation procedures is the occurrence of abundant matrix effects which compromise detection limits, quantitative aspects, method selectivity and maintenance frequency. In contrast to the trend towards non-selective sample preparation, an opposite trend towards more selective sample preparation methods is expected to be able to unambiguously confirm the identity of compounds, e.g. stereoisomers. This review gives an overview of generic sample preparation procedures in the field of the analysis of veterinary drug residues in products of animal origin using liquid chromatography coupled to mass spectrometry as a detection technique and an outlook towards expected future trends.

Introduction

Sample preparation is the process of extracting chemical residues from the matrix, removing interfering substances and, if needed, the subsequent concentration of the extract and the deconjugation or derivatisation of the compounds of interest. The sample preparation in the field of residue analysis of veterinary drugs in products of animal origin is of critical importance. Since the composition of the final extract introduced into the instrumental system, mainly depends on the sample preparation procedure, it highly impacts the final results in terms of detection limits, reproducibility, ruggedness and selectivity.

Furthermore, sample preparation is a critical process in relation to analysis time, sample throughput and therefore analysis costs.

Sample preparation is not a goal by itself but merely a logical consequence of the detection technique applied. Therefore a clear relation between the selectivity of applied sample preparation procedures and the selectivity of detection techniques available is observed. Until the end of the 20th century mainly liquid chromatography (LC) was used coupled to triple quadrupole (QqQ) mass spectrometers that needed relatively long dwell times (> 100 ms) in the multiple reaction monitoring (MRM) acquisition mode in order to obtain a sufficient number of ions for accurate detection of an ion transition [1]. This dwell time in combination with the low resolution of the LC system limited the number of compounds that could be detected within one run. As a result multi- methods that included compounds belonging to a single compound group, were developed [2]. In the last decade, high resolution LC (HRLC) featuring sub-2 μm stationary phase particles, and fast scanning (< 10 ms) triple quadrupole instruments became available facilitating the detection of a larger number of compounds within one run. Meanwhile, with developments in the detection limits of time of flight mass spectrometry (TOF-MS) and the development of orbitrap instruments, highly selective, high resolution MS (HRMS) detection techniques became routinely available [3]. Because HRMS is a full scan technique, in theory, an indefinite number of compounds can be analysed simultaneously. Both HRLC and HRMS resulted in the development of new analytical strategies that included over a hundred compounds belonging to different compound groups, including antibiotics, growth promoters, pesticides and natural toxins. As a consequence of the advances in the available instruments the number of compounds that are analysed in a single run increased rapidly throughout the last decade.

With the increasing number of compounds that are analysed simultaneously, analytical methods, including the sample preparation procedure, have to be applicable to compounds having very different physical and chemical properties. Therefore multi-residue protocols include very generic sample preparation procedures to obtain sufficient recovery for all compounds. However, as a result of applying a generic and inherently non-selective sample preparation procedure, the final extract contains a significant amount of matrix constituents that might interfere in the detection and overall method selectivity can be compromised.

Therefore, one should be aware of the pronounced matrix effects and the limited selectivity of the sample preparation procedure, which might result in higher detection limits, interfering signals and more variation in the quantitative result [2,4-7].

Reviews on sample preparation techniques applied since 2004 have been published previously [2,8-10]. Reviews mainly covered new analytical techniques [8-16], automation [8,9,17], miniaturization [8,18], regulations for confirmatory methods [2,6] or specific matrices [2].

The scope of the current review is limited to the trend observed towards more generic sample preparation procedures in the field of the analysis of veterinary drug residues in products of animal origin using liquid chromatography coupled to mass spectrometry as a detection technique and thus the focus is on multi-compound methods. This includes the use of solvent extraction only, solid phase extraction and QuEChER-based approaches. Furthermore, expected future trends are discussed. The scientific literature was evaluated covering 2008 - mid 2012 based on the SciFinder and Scopus databases using the following keywords and combinations thereof: veterinary drugs, antibiotics, multi compound analysis, residues, sample preparation, liquid chromatography, solid phase extraction, QuEChERS.

Generic sample preparation techniques

As a generic sample preparation procedure solvent extraction only, very generic solid phase extraction (SPE) procedures and modifications on the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) approach are most frequently reported and discussed in the following sections.

Solvent extraction

In the simultaneous analysis of multiple classes of veterinary drugs in products of animal origin, solvent extraction without further purification is frequently reported as the method of choice. Here solvent extraction includes conventional liquid-liquid extraction as well as the liquid extraction of homogenised tissues such as muscle, liver and kidney. A summary of the applications reported since 2008, giving a good overview of the current trends in sample preparation procedures, is presented in table 2.1.

Table 2.1. Summary of applications using solvent extraction without further clean-up for the analysis of multi-class veterinary drugs in products of animal origin.

Compound groups	Matrix	Method	Ref.
A, AVM, B, Q, T, TMP	Meat-based baby food, powdered milk	Soak with water. Extraction with acidic ACN. Filtration (nylon). HRLC-QqQ-MS.	[19]
Q, S, T	Kidney, liver	Extraction with acidic ACN/water (7:2 v/v). Partly evaporation of solvent and dilute with water/ACN (95:5 v/v). Filtration. HRLC-Orbitrap-MS	[7]
AG, C, L, M, P, Q, morantel	Milk	Extraction with ACN. Evaporation of solvent and dissolve in water/ACN (1:1 v/v). Filtration (PVDF). HRLC-QqQ-MS.	[20]
C, P, M, S	Milk	Extraction with ACN. Evaporation of solvent and dissolve in NH ₄ Ac. Filtration. LC-QqQ-MS	[21]
A, AG, M, P, S, T	Honey	Extraction with ACN. Extraction of the pellet with 10 % TCA. Extraction of the pellet with nonafluoropropionic acid in ACN followed by neutralisation. Hydrolysis of the pellet followed by extraction with ACN. Evaporation of the individual extracts and dissolve in water/MeOH (8:2 v/v). LC-QqQ-MS.	[22]
B, COC, M, N, NIZ, Q, S, T, TQ	Honey, egg, milk, meat	Soak the sample with water. Extraction with acidic ACN, MeOH or acetone (ACE). HRLC-QqQ-MS.	[23,24]
A, AG, C, M, NSAID, P, Q, T, S	Muscle	Extraction with ACN/water (86:14 v/v) at 60 °C. Second extraction with water. Dilution of the combined extracts with water. Fat removal with hexane. LC-QqQ-MS.	[25]
AG, COC, L, M, P, Q, S, T	Muscle	Extraction with 2 % TCA/ACN (1:1 v/v). fat removal using hexane. Dilution with acidic water/ACN (1:9 v/v). Filtration (Nylon). HILIC-LC-QqQ-MS	[26]
AG, L, Q, T	Milk	Extraction with 5 % TCA. Filtration (PVDF). LC-QqQ-MS.	[21]
S, TMP, TPM	Shrimp	Extraction with 6 mL 20 % TCA in ultrasonic bath. Evaporation of solvent and dissolve in MeOH/water (1:4 v/v). Filtration (PTFE). LC-TOF-MS.	[27]
M, P, Q, S, T	Muscle	Add EDTA solution. Extraction with MeOH/water (7:3 v/v). Dilution with water. LC-QqQ-MS	[28]
M, P, Q, S, T	Muscle	Extraction with MeOH/water (7:3 v/v), EDTA. Dilution with water. Filtration. HRLC-QqQ-MS.	[29]

C, L, M, NIZ, P, Q, S, T, TMP	Muscle	PLE with water and EDTA. Evaporation of solvent. LC-QqQ-MS. [30]
M, NIZ, P, Q, S	Muscle	PLE with water and EDTA. Evaporation of the solvent. LC-QqQ-MS [31]

A:amphenicols, AG:aminoglycosides, AVM:avermectins, β A: β -agonists, B:benzimidazoles, C:cephalosporins, COC:coccidiostats, L:lincosamides, M:macrolides, NIZ:nitroimidazoles, P:penicillins, Q: quinolones, S:sulfonamides, T:tetracyclines, TMP:trimethoprim, TPM:triphenylmethanes, TQ:tranquilizers.

To obtain optimal results, the extraction solvent has to be selected in such a way that efficient extraction of the target compounds is obtained, whereas the extraction of matrix constituents remains limited in order to prevent excessive matrix effects to occur. Therefore the selection of the solvent not only depends on the target compounds, but also on the matrix. Mol *et al.* [23] compared the use of ACN, MeOH and acetone (ACE) for the extraction of veterinary drugs, pesticides and toxins from honey, milk, eggs and muscle. For milk, MeOH showed the highest extraction recoveries, but the final extracts were turbid, probably due to insufficient protein precipitation [23,32] and the amount of fat present in the final extract [33]. Nevertheless, MeOH proved to be a quick alternative for the extraction of veterinary drugs from muscle [28,29]. In terms of extraction recoveries, ACE was found to be the most suitable extraction solvent across all tested matrices [23]. ACE did not show undesired side-effects like phase separation or turbid extracts but it showed sub-optimal performance in terms of co-extraction of matrix interferences and as a result ion suppression during detection. ACN typically provides high extraction recoveries, minimises co-extraction of lipids [26] and is efficient for denaturation of proteins. Mol *et al.* [23] reported that when using ACN for the extraction of veterinary drug, pesticide and toxins the overall matrix effects decrease from muscle > milk > egg > honey, although for veterinary drugs specifically the matrix effect of honey seems worse and for muscle less. Furthermore, it was reported that MeOH performed worst in term of matrix effects and that although ACE showed the highest recovery overall, it also showed more severe matrix effects compared to ACN. As a result ACN was selected as the solvent for extraction of muscle and ACE for the extraction of milk, egg and honey [23]. For the analysis of veterinary drugs in products of animal origin ACN is the most frequently reported extraction solvent. However, it is reported that ACN does not sufficiently extract polar

analytes [32] as was demonstrated for the extraction of some quinolones from meat-based baby food and powdered milk [19]. Also low recoveries were observed for the ACN extraction of tetracyclines, which was most likely caused by complex formation of the analytes. As a result Kaufmann *et al.* [4] proposed a bipolar extraction combining an extraction with ACN and an extraction using a McIlvain buffer containing complexing agents. A double extraction using ACN significantly improved the extraction recoveries [33] but this enhances matrix effects as well [28].

Another frequently applied extraction solvent is trichloroacetic acid (TCA), which is an efficient solvent for protein removal [26,32] but might cause degradation of pH sensitive compounds such as β -lactams and some macrolides [26,32]. In contrast Chiaoan *et al.* [26] reported that TCA is favorable for releasing protein bound compounds and stabilises basic aminoglycosides. As a result, Gaugain-Juhel *et al.* [21] reported two parallel solvent extraction methods to cover the complete range of antibiotics in the analysis of milk: ACN was used for the extraction of β -lactams, macrolides and sulfonamides, whereas 5 % TCA proved to be better for the extraction of tetracyclines, aminoglycosides, lincosamides and quinolones. TCA (20 %) was reported not to be applicable for the extraction of shrimp, because it results in turbid extracts [27].

Hammel *et al.* [22] studied the effect of subsequent extraction of antibiotics from honey with (1) ACN, (2) 10 % TCA/ACN (1:2 v/v), (3) nonafluoropentanoic acid (NFPA) in ACN and (4) a hydrolysis during 1 hour at 65 °C followed by extraction with ACN. The results are presented in figure 2.1. The amphenicols, macrolides, all sulfonamides except sulfanilamide and the penicillins except ampicillin and amoxicillin were extracted in the first extraction step using ACN with recoveries > 50 %. The extraction using TCA was necessary to effectively extract the tetracyclines, ampicillin and amoxicillin (recoveries > 50 %) and proved to be beneficial for all compounds except the aminoglycosides and sulfanilamide. NFPA was needed to extract sulfanilamide and the aminoglycosides for 90-100 % and to increase the recovery for the tetracyclins by 20-40 %. The subsequent hydrolysis did not significantly increase the extraction recovery of any of the compounds. However, Sheridan *et al.* [34] stated that an acid hydrolysis is needed for the extraction of sulfonamides from honey and found that this is disadvantageous for the analysis of β -lactams and some macrolides, which are instable at low pH [4,26].

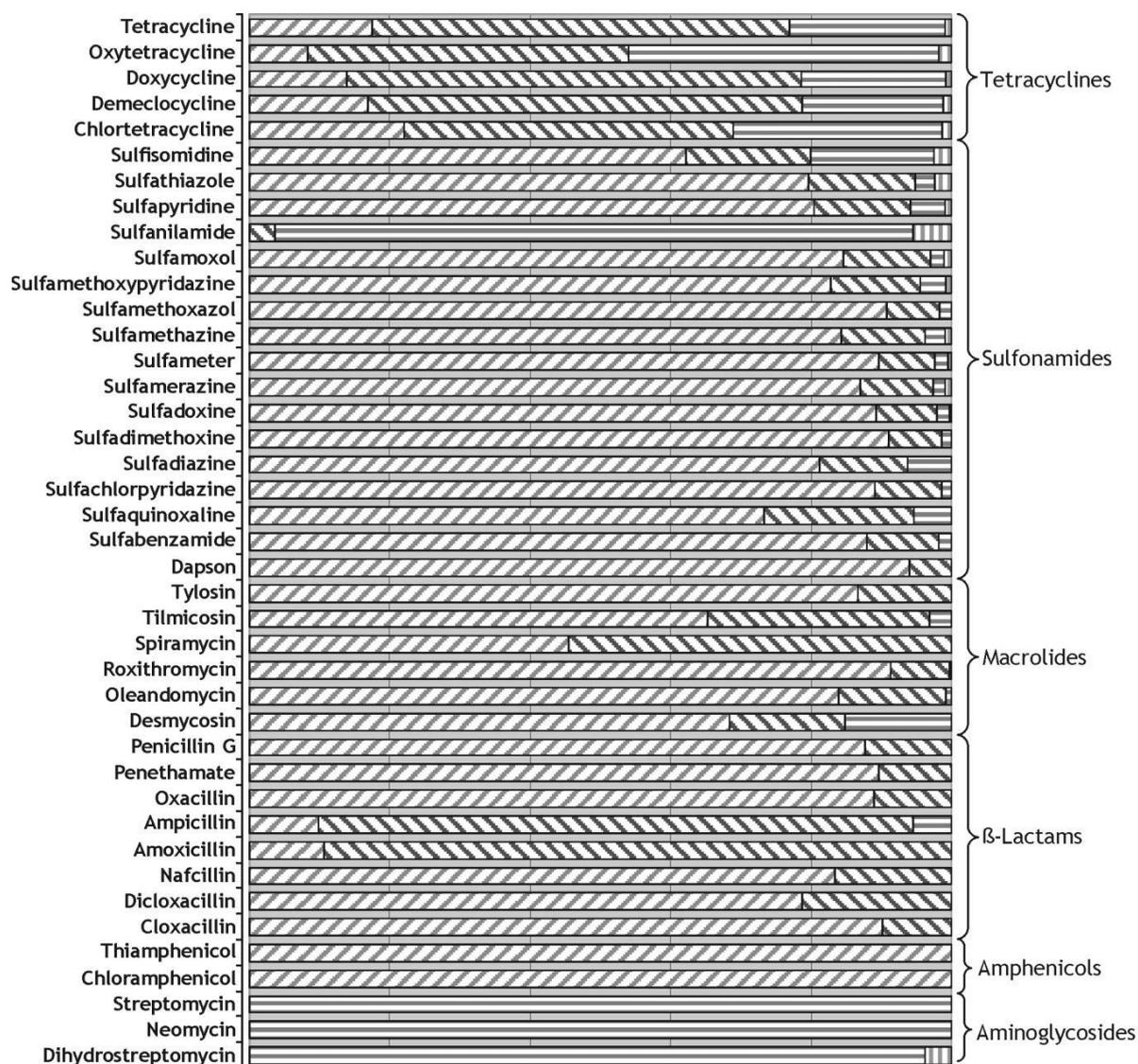


Figure 2.1. Relative recovery (estimation based on an area normalization) of each analyte based on the four liquid-liquid extraction fractions analysed separately. Blank honey sample was spiked at a concentration of $50 \mu\text{g kg}^{-1}$. Reproduced from [22] with permission from the author.

Extraction solutions	
	1mL acetonitrile
	0.5 mL TCA 10% + 1 mL acetonitrile
	40 μL NFPA + 1 mL acetonitrile
	Hydrolysis step + 1 mL acetonitrile

Frequently, the addition of a complexing agent like EDTA (ethylenediaminetetraacetic acid) is used during extraction [4,28-32,35-38]. Complexing agents are reported to be mandatory for the extraction of tetracyclins and some macrolides because these compounds have a strong tendency to form chelates with divalent metallic cations present in food samples [26,31,35,39]. The use of EDTA negatively affects the extraction of benzanthine, which is a marker residue for penicillin use [40].

The use of pressurised liquid extraction (PLE) instead of regular solvent extraction using either water or an organic solvent was reported [30, 31] and proved to be beneficial for the recovery of most veterinary drugs at 70 °C and elevated pressure. For all compounds included, recoveries between 64 and 122 % were obtained. Another advantage is that also when using heated water instead of an organic solvent, acceptable extraction recoveries can be obtained which is environmental friendly and compatible with clean-up procedures like SPE.

Methods that only consist of a solvent extraction procedure usually result in final extracts containing a large amount of matrix interferences and thus matrix effects are pronounced [7,19-23,26,30,41,42]. Ion suppression is a well-known matrix effect, which can result in an increase in the detection limits, negatively influences the quantitation and increases maintenance frequency. Suppression effects are usually related to the retention time of the compounds and are most pronounced for early and late eluting compounds [41,43] due to co-elution of e.g. salts, sugars, fatty acids and proteins. Also ionisation effects occurring in the orbitrap system, and therefore post-ionisation, were observed for low m/z compounds eluting within a particular retention time period when using orbitrap-MS as the detection technique [7].

An approach to reduce matrix effects is the addition of tungsten ions to further remove proteins [32]. Unfortunately tungsten ions covalently bind to organic amines and thus lead to low recovery for some target compounds like sulphonamides. Saturated ammonium sulphate was reported to be as efficient for protein precipitation, but co-precipitation of target compounds was observed. Kaufmann *et al.* [32] reported an optimum concentration of ammonium sulphate to facilitate additional protein precipitation for the extraction of honey and tissue, whereas Peters *et al.* [44] reported no improvement using ammonium sulfate in the extraction of egg, fish and muscle.

Another approach to reduce matrix effects without additional clean-up procedure, is to dilute the final extract to counteract suppression [28,29,32,36]. If matrix effects cannot be eliminated, the use of isotopically labeled internal standards and, to some extent, the use of matrix matched calibrants are useful tools to still obtain reproducible quantitative results.

Besides the occurrence of ion suppression effects, another risk of a limited clean-up is a drop in overall method selectivity due to the appearance of interfering peaks in the chromatogram. Granelli *et al.* [28] observed an interfering peak in one of both traces for tylosin when analysing muscle, ChiaoChan *et al.* [26] reported matrix interferences that eluted close to tylosin and amprolium, and Schürmann *et al.* [45] reported false positive findings when applying a generic sample preparation procedure in combination with MS detection according to 2002/657/EC [46] for the pesticide sebuthylazine.

Solid phase extraction

A procedure that is frequently used as a clean-up technique in the simultaneous analysis of multiple classes of veterinary drugs in products of animal origin is SPE. Three types of SPE can be distinguished (figure 2.2): (1) regular SPE in which the extract is applied onto an SPE cartridge to retain the compounds of interest, followed by a wash step to remove matrix interferences and a subsequent elution of the compounds from the cartridge; (2) SPE applying a highly organic raw extract onto an SPE cartridge and immediately collecting the eluent for further analysis; (3) Dispersive SPE (dSPE) by adding sorbent material to a raw extract, followed by shaking and centrifugation and subsequent isolation of the supernatant. dSPE is usually included in the QuEChERS approach and is discussed in 2.3. A summary of the SPE applications in cartridge format reported since 2008, giving a good overview of the current trends in sample preparation procedures, is presented in table 2.2.

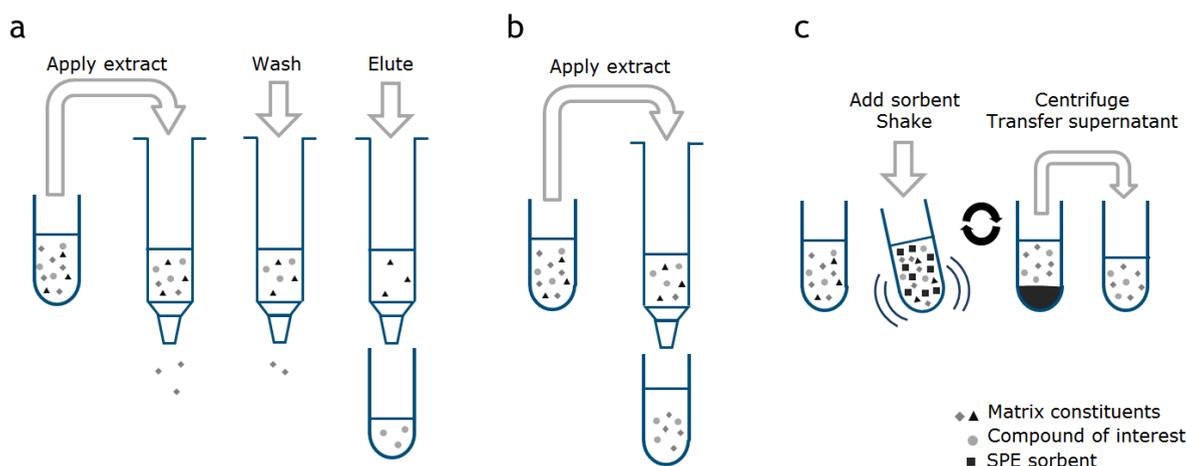


Figure 2.2. Schematic presentation of (a) regular SPE, (b) pass-through SPE and (c) dSPE.

Table 2.2. Summary of applications using SPE for the analysis of multi-class veterinary drugs in products of animal origin.

Compound groups	Matrix	Method	Ref.
S, TMP, TPM	Shrimp	Extraction with metaphosphoric acid in ACN (6:4 v/v). Filtrate (PTFE). Partially evaporate the solvent. SPE Oasis HLB, wash with ACN/water (1:4 v/v), elute with ACN. Partially evaporation of solvent. Filtration (PTFE). LC-TOF-MS.	[27]
B, M, Q, S	Egg	Extraction with 0.1 M Na ₂ EDTA (pH=4). Oasis HLB, wash with n-hexane, elution with ACN, MeOH, alkaline MeOH. Evaporation of solvent and dissolve in acidic MeOH/water (1:1 v/v). Filtration (nylon). HRLC-QqQ-MS.	[36]
B, C, NIZ, P, Q, S, TMP, TQ	Meat	Extraction with water/ACN (1:1 v/v), EDTA, (NH ₄) ₂ SO ₄ . Add (NH ₄) ₂ SO ₄ . Evaporation of organic solvent. Adjust to pH=6.5. SPE Oasis HLB, wash with water, elution with ACN and succinate buffer/ACN (1:1 v/v). Add DMSO. Evaporation of the organic solvent. Dilution with water. HRLC-TOF-MS.	[4]
L, M, Q, T, TMP	Honey	Extraction with McIlvain, Na ₂ EDTA. Filtration. SPE Oasis HLB, wash with water/MeOH (95:5 v/v), elution with MeOH. Evaporation of the solvent. Dissolve in 0.2 % formic acid.	[38]
C, M, P, Q, S, T, bacitracin	Milk	Extraction with ACN. Dilution in 0.1 % formic acid. SPE Oasis HLB, wash with 0.1 % formic acid, elution with ACN/MeOH (7:3 v/v). Add 0.1 % formic acid. Partial evaporation of solvent. Ultrafiltration 30 kD.	[47]
M, Q, S, T	Honey	Extraction with Na ₂ EDTA (pH=4). SPE Oasis HLB	[39]
A, B, COC, I, M, P, NIZ, NSAID, Q, S, T, TMP, TQ	Egg, fish, meat	Extraction with ACN/water (6:4 v/v). Dilution of supernatant in water. SPE Strata-X RP, wash with water. Egg: elution with MeOH/ethyl acetate (EtAc) (1:1 v/v). Fish/meat: elution MeOH/ACN (1:1 v/v). Evaporation of solvent and dissolve in ACN, dilute in acidic water. HRLC-TOF-MS.	[44]
A, AG, I, L, M, S, T, fumagillin	Honey	Extraction with water. Aliquot for streptomycin analysis. Filtration (nylon). SPE Strata-X RP, wash with water and hexane, elute with MeOH, ACN and alkaline MeOH. Partial evaporation of solvent. Dilution with water. LC-QqQ-MS.	[42]
A, B, I, M, NIZ, NSAID, P, Q, S, T, TMP, TQ	Milk	Extraction with ACN. Dilution with water. SPE Strata-X RP, wash with water, elution with MeOH. Evaporation of the solvent. Dissolve in ACN. Dilute in acidic water. HRLC-TOF-MS.	[48]

COC, CS, Q, S, TMP, griseofulvin	Milk	Extraction with ACN. Dilution with water. SPE Strata-X RP, elution with MeOH. Evaporation of the solvent. Dissolve in acidic MeOH.	[33]
B, C, I, NIZ, P, Q, S, TMP, TPM, TQ	Honey, fish	Extraction with water/ACN (35:45 v/v), EDTA, (NH ₄) ₂ SO ₄ . Add (NH ₄) ₂ SO ₄ . Evaporation of organic solvent. Adjust to pH=6.5. SPE Evolute ABN, wash with water, elution with ACN and succinate buffer/ACN (1:1 v/v). Partial evaporation of the solvent. Dissolve in water. HRLC-Oribtrap-MS.	[32]
BA, CS, stimulants, narcotics	Urine	Hydrolysis at pH=4.8 with β-glucuronidase/aryl sulphatase. SPE Oasis MCX, wash with 1 M acetic acid and NaAc buffer/ACE (85:15 v/v), elution with alkaline ethyl acetate. Evaporation of the solvent. Dissolve in water/ACN (5:95 v/v). HRLC-TOF-MS.	[49]
B, BA, BDP, CS, NIZ, Q, S, T, TPM	Meat, milk, egg	Mix with anhydrous Na ₂ SO ₄ . Double extraction with acidic ACN. Extraction EtAc. Evaporate combined extract and dissolve in alkaline MeOH. Pass through SPE Oasis HLB, elute with alkaline MeOH. Evaporation of solvent and dissolve in water/ACN (7:3 v/v). HRLC-QTOF-MS.	[50]
AVM, B, M, Q, T, S	Egg	Extraction with ACN, citric acid (pH=4), 0.1 M Na ₂ EDTA. Pass through Oasis HLB cartridge, Partial evaporation of solvent and dilute with acidic MeOH/water (1:1 v/v). Filtrate (nylon). HRLC-QqQ-MS.	[36]

A:amphenicols, AG:aminoglycosides, AVM:avermectins, βA:β-agonists, B:benzimidazoles, BDP:benzodiazepams, COC:coccidiostats, CS:corticosteroids, I:ionophores, L:lincosamides, M:macrolides, NIZ:nitroimidazoles, NSAID:non-steroidal anti-inflammatory drugs, P:penicillins, Q:quinolones, S:sulfonamides, T:tetracyclines, TMP:trimethoprim, TPM:triphenylmethanes, TQ:tranquilizers.

In general, matrix effects can be significantly reduced by using an SPE procedure [50]. However, in order to use an SPE approach for a broad range of compounds, having different physical and chemical properties, the choice of the interaction mechanism is limited. Peters *et al.* [49] used a mixed mode cation exchange material for the clean-up of hydrolysed urine samples for the analysis of multi-class prohibited small substances. Others use C₁₈, Oasis HLB, Strata-X RP or Evolute ABN which are all based upon hydrophilic-lipophilic interaction. To retain even the polar target compounds, a low organic content of the extract that is applied onto the SPE cartridge is mandatory. Therefore, the extraction solvent should have a high water content or additional procedures have to be applied to change the solvent of the raw extract prior to SPE [44]. Even though the use of an high organic extraction solvent like ACN or ACE is favorable for high solvent extraction recoveries (2.1), aqueous solvents are frequently applied if the extraction is followed by an SPE procedure [36,38,39,42]. In other cases the

organic solvent is evaporated before SPE [4,32,50], which is time-consuming or the organic phase is diluted with an aqueous solvent [33,44,47,48].

In single-compound methods selective wash steps and a selective elution can be used to effectively separate the target compounds from matrix interferences. Because the chemical properties of the target compounds in multi-class methods are usually very diverse, the options for clean-up in SPE are limited. Water, formic acid, water/methanol (95:5 v/v) and hexane [36,42] are used for washing the SPE cartridges to remove highly hydrophilic and highly lipophilic matrix interferences. Elution of the target compounds, which are mostly polar or semi-polar, is carried out by pure organic solvents or mixtures thereof at neutral or alkaline conditions. The analytical separation is usually based on the same separation principle (reversed phase (RP)) and therefore the additional selectivity obtained by SPE is limited. Therefore, co-elution with interfering compounds and thus ion suppression effects remain [33,42] but surprisingly, in some cases lower matrix effects and lower detection limits are reported [48]. An evaluation of the matrix effects of milk samples after SPE is reported by Ortelli *et al.* [43]. It is shown that matrix effects especially occur during the first and last part of the chromatogram and therefore influence the analysis of the most hydrophilic and lipophilic compounds. For milk the matrix effects after SPE are relatively variable throughout different samples [43], complicating quantitative analysis when no isotopically labeled internal standards are available.

RP SPE is somewhat limited towards polar compounds [44,48]. As an example the aminoglycosides, which are highly polar compounds, are usually not included in procedures that use SPE as a clean-up technique. Lopez *et al.* [42] included the aminoglycosides in their multi-class method, but a fraction of the raw extract was isolated before SPE to be able to detect the aminoglycosides. Another example is the low recovery found for tetracyclins in the generic SPE procedure applied by Frenich *et al.* [36]. They also show the limitations of SPE towards highly lipophilic compounds like ivermectin.

Kaufmann *et al.* [32] presented the use of an Evolute ABN cartridge which has a smaller pore size (40 Å) compared to other commercially available polymeric reversed phase materials (60 Å). As a result, interfering high molecular weight compounds are more effectively removed and the protein content of the final extract dropped by a factor two to three compared to the use of Oasis HLB

cartridges. On the other hand the ABN material shows slightly lower retention power which negatively affects its performance towards polar compounds due to the increase of break-through.

Deng *et al.* [50] and Frenich *et al.* [36] developed a procedure in which an organic extract is passed through a reversed phase SPE cartridge. This is effective for removal of lipophilic matrix interferences and is comparable to dSPE.

QuEChERS approach

A third frequently applied clean-up technique in the multi-class analysis of veterinary drugs in products of animal origin is a QuEChERS procedure which consists of the extraction with an organic solvent and phase separation using a high salt content, in some cases followed by dSPE. This procedure is considered to be quick, easy, cheap, efficient, robust and safe and found its origin in pesticide analysis [51]. Since a few years QuEChERS approaches are also applied in the analysis of multi-class veterinary drugs in products of animal origin; a summary, giving a good overview of the current trends in sample preparation procedures, is presented in table 2.3.

Table 2.3. Summary of applications using QuEChERS for the analysis of multi-class veterinary drugs in products of animal origin.

Compound groups	Matrix	Method	Ref.
AVM, B, M, Q, S, T	Egg	Extraction with acidic ACN and 0.1 M Na ₂ EDTA. Shake with anhydrous MgSO ₄ and NaAc. Dilution with acidic MeOH/water (1:1 v/v). Filtration (nylon). HRLC-QqQ-MS.	[36]
B, COC, M, NSAID, NIZ, Q, S, T, TQ	Honey, egg, milk, meat	Soak with water. Extraction with acidic ACN, MgSO ₄ , NaAc. HRLC-QqQ-MS.	[23]
A, AVM, B, Q, T, TMP	Meat-based baby food, powdered milk	Extraction with acidic ACN. Shake with anhydrous MgSO ₄ and NaAc. Filtration (Nylon). HRLC-QqQ-MS.	[19]
B, M, Q, S, T	Milk	Extraction with acidic ACN and EDTA. Shake with anhydrous MgSO ₄ and NaAc. Filtration (nylon). Dilution with acidic MeOH/water (1:1 v/v). HRLC-QqQ-MS.	[35]

AVM, B, M, Q, S, T	Milk	Extraction with acidic ACN and EDTA. Shake with anhydrous MgSO ₄ and NaAc. Filtration (nylon). Dilution with acidic MeOH/water (1:1 v/v). HRLC-QqQ-MS. HRLC-TOF-MS. HRLC-Orbitrap-MS.	[52]
P, S, T	Muscle	Extraction with acidic ACN. Shake with anhydrous MgSO ₄ and NaAc. Evaporation of solvent. Dissolve in MeOH and dilute in water. HRLC-Orbitrap-MS.	[53]
P, S, T	Milk	Extraction with acidic ACN/water (5:6 v/v). Shake with anhydrous MgSO ₄ and NaAc. Evaporation of solvent. Dissolve in MeOH and dilute in water. HRLC-Orbitrap-MS.	[53]
I, DNC, NIZ, Q, S	Tissue	Extraction with ACN, acidic ACN, ACN/water (7:15 v/v) or acidic ACN/water (7:15 v/v). Shake with anhydrous NaSO ₄ or MgSO ₄ . For NIZ additional SPE Oasis MCX. LC-QqQ-MS.	[54]
AVM, B, M, Q, S, T	Milk	Extraction with acidic ACN and Na ₂ EDTA. Shake with anhydrous MgSO ₄ and NaAc. Dilution with acidic MeOH/water (1:1 v/v). HRLC-QqQ-MS.	[37]
S, TMP, TPM	Shrimp	Extraction with acidic ACN. Shake with anhydrous MgSO ₄ and g NaCl. dSPE PSA and anhydrous MgSO ₄ . Evaporation of solvent and dissolve in MeOH/water (1:4 v/v). Filtration (PTFE). LC-TOF-MS.	[27]
P, S, T	Liver	Extraction with acidic ACN. Shake with anhydrous MgSO ₄ and NaAc. pSPE with PSA, C ₁₈ and anhydrous MgSO ₄ . Evaporation of solvent. Dissolve in MeOH and dilute in water. HRLC-Orbitrap-MS.	[53]
AVM, B, F	Milk, liver	Extraction with ACN. Shake with anhydrous MgSO ₄ and NaCl. pSPE with C ₁₈ or PSA and MgSO ₄ . LC-QqQ-MS.	[55]
AVM, B, M, Q, S	Muscle	Add water and extraction with ACN/water (8:2 v/v). Shake with anhydrous MgSO ₄ , sodium citrate dibasic sesquihydrate and sodium citrate dehydrate. dSPE with PSA. Filtration (nylon). Dilution with acidic water/ACN (1:1 v/v). HRLC-QqQ-MS.	[40]
M, NIZ, P, Q, S	Muscle	Extraction with acidic ACN. Shake with anhydrous MgSO ₄ . dSPE with PSA. Evaporation of the solvent. Dissolve in water/ACN (9:1 v/v).	[31]

A:amphenicols, AVM:avermectins, B:benzimidazoles, COC:coccidiostats, DNC:dinitrocarbanilide, F:flukicides, M:macrolides, NSAID:non-steroidal anti-inflammatory drugs, NIZ:nitroimidazoles, P:penicillins, Q:quinolones, S:sulfonamides, T:tetracyclines, TMP:trimethoprim, TPM:triphenylmethanes, TQ:tranquilizers.

The majority of methods based on the QuEChERS approach consist of a solvent extraction with acidic ACN in the presence or absence of EDTA followed by phase separation using anhydrous magnesium sulphate as a drying agent. A few methods include a subsequent dSPE procedure using C₁₈ [55], primary secondary amine (PSA) [27,31,40,55] or a combination of both [53].

The preferred extraction solvent when using a QuEChERS approach is acidic ACN, whereas the preference for acidic solvents is not as clear for solvent extraction methods without further clean-up. It is demonstrated that recoveries in a QuEChERS approach significantly improve for especially quinolones when using acidic ACN [31].

Several drying agents have been compared. Low recoveries for especially quinolones when using silica due to adsorption were reported [31]. No differences in terms of recovery were observed when using anhydrous MgSO₄ or Na₂SO₄ [31,54], but MgSO₄ was selected for its better dispersion characteristics [31]. It is observed that the amount of drying agent is an important parameter. Too low amounts result in incomplete water removal and therefore a less efficient clean-up during dSPE. A too high amount results in lower recoveries for the quinolones [31], probably caused by adsorption to the drying agent.

Also the sorbent used for subsequent dSPE is critical. Carbon is not suited because it results in low recoveries or complete loss for a broad range of compounds [31]. Blasco *et al.* [31] reported that recoveries for PSA and C₁₈ were similar, whereas others reported lower recoveries for especially the flukicides [55], for polar compounds [31,50] and spiramycin [31] when using PSA. PSA resulted in cleaner extracts for muscle [31] and milk [55] and is thus favourable for these matrices. For the analysis of liver, because of its high fat content, the use of C₁₈ is reported to be beneficial [53,55]. The amount of sorbent is not a critical factor, but too high amounts can result in cloudy extracts [31]. In dSPE (as well as in regular SPE) the organic content of the extract has to be critically reviewed: when using C₁₈ sorbent, if the aqueous phase is not completely removed by phase separation and thus an excess of water is present, immediate loss of lipophilic compounds onto the sorbent material will occur.

Blasco *et al.* [31] reported that the QuEChERS approach resulted in more severe matrix effects for bovine muscle compared to PLE. Matrix effects were primarily observed for early eluting compounds. As a result the detection limits for some compounds are higher using QuEChERS compared to PLE. Others observed significant matrix effects for the QuEChERS procedure as well [35,40].

Other generic clean-up procedures

Some other clean-up techniques that have shown to be applicable for multi-class analysis of veterinary drugs in products of animal origin were reported. These include ultrafiltration and ultra-low temperature phase separation. A summary of such methods published since 2008 is presented in table 2.4.

Ultrafiltration is a very quick technique and is applied in the analysis of samples with a high protein content. It was reported that using an ultrafiltration procedure, some compounds show low recoveries when using a 30 kD filter [48]. Low recoveries and high variation for the avermectins were specifically observed when using a 3D cut-off filter, which was contributed to the voluminosity of these compounds [43].

As expected, a cut-off filter of 3 kD clearly showed cleaner extracts compared to a 10 and 30 kD filters [43]. Ortelli *et al.* [43] showed that ultrafiltration removes more lipophilic matrix interferences compared to SPE, but that the removal of hydrophilic interferences is worse. The matrix effects of different milk samples after ultrafiltration are very comparable, facilitating quantitative analysis by using matrix match calibrants.

A method for the analysis of muscle based upon phase partitioning at ultra-low temperatures was reported by Lopes *et al.* [57]. This method consists of a solvent extraction using ACN and phase partitioning by freezing the aqueous fraction by immersing the test tube in liquid nitrogen. This approach is fast and selective, but proved to be problematic for ampicillin, clindamycin and erythromycin. Extracts were reported to be clean, but matrix effects were not specifically reported.

Table 2.4. Summary of applications of other generic sample preparation procedures for the analysis of multi-class veterinary drugs in products of animal origin.

Compound groups	Matrix	Method	Ref.
GCS, designer drugs	Urine	Enzymatical hydrolysis (pH=7). pH adjustment to pH=9-10. Extraction with diethylether and anhydrous Na ₂ SO ₄ . Evaporation of organic phase and dissolve in water/ACN (5:95 v/v). HRLC-TOF-MS.	[56]
C, M, P, S, T	Milk	Extraction with ACN. Acidify. Partial evaporation of solvent. Ultrafiltration 3 kD. LC-QTOF-MS.	[41]
AVM, B, βA, C, CS, L, M, NIZ, NSAID, P, Q, S, T, TMP, TQ, others	Milk	Extraction with acidic ACN. Ultrafiltration 3 kD. Partial evaporation of organic phase. HRLC-TOF-MS.	[43]
B, C, M, P, Q, S, T	Muscle	Extraction with ACN. Immerse tube in liquid nitrogen. Evaporation of the organic phase. Dissolve in formic acid solution. Filtration. LC-QqQ-MS.	[57]

AVM: avermectins, βA: β-agonists, B: benzimidazoles, C: cephalosporins, CS: corticosteroids, GCS: glucocorticosteroids, L: lincosamides, M: macrolides, NIZ: nitroimidazoles, NSAID: non-steroidal anti-inflammatory drugs, P: penicillins, Q: quinolones, S: sulfonamides, T: tetracyclines, TMP: trimethoprim.

Outlook and future trends

The trend towards the use of generic sample preparation procedures is not only observed in the field of veterinary drug residue analysis in products of animal origin, but also in the analysis of pesticides in various matrices [14,58] and pharmaceuticals in environmental samples like water, soil and plant material [59,60]. It is expected that the trend towards non-selective sample preparation continuous parallel to the development of more sensitive full-scan mass spectrometers having even higher resolution and scanning speed. Because generic sample preparation methods are usually straight-forward, also the trend towards further automation of the sample preparation will continue to further increase sample throughput [8,9,17] as well as the trend towards green chemistry [8,18].

Although a continuing trend towards generic sample clean-up procedures is observed, a parallel future trend is expected towards more extensive sample clean-up procedures to obtain a precise quantitation at ultra-low levels and to provide ultra-high selectivity for unambiguous confirmation of the identity.

For all regulated compounds [61] quantitative analysis is of crucial importance in order to fulfil method requirements and to prove compliance or non-compliance versus the MRLs [46]. According to 2005/34/EC [62] for all products entering the EU that are analysed for banned veterinary drugs and thus are within the scope of 97/78/EC [63], the minimum required performance limit (MRPL) as stated in 2002/657/EC [46] should be regarded as a reference point of action. In this perspective, with the increasing number of provisional MRPL's, also the quantitative analysis of banned substances becomes increasingly important. Because in generic methods matrix effects can be pronounced and overall analyte recovery is compromised, the precision of the quantitation is compromised as well [64,65]. A common way to deal with these matrix effects and incomplete analyte recovery is by using appropriate calibration strategies [66,67].

In the case the detection limits are not sufficiently low due to the presence of matrix, the solution is to be found in a more selective (and therefore usually more extensive) sample preparation procedure to remove excessive matrix interferences. A clear example is the use of a multiple stage SPE procedure in the quantitative analysis of antiviral drugs in combination with a column switching liquid chromatographic system to overcome excessive matrix effects [68], the use of molecular imprinted polymers [69] and immunoaffinity based techniques [70].

According to criteria concerning the performance of analytical methods and the interpretation of results methods used for the analysis of samples taken for monitoring residues in animal products have to be validated according to the described procedures [46]. In these procedures selectivity is mentioned as a main characteristic of an analytical method. Selectivity is defined as “the power of discrimination between the analyte and closely related substances like isomers (...)”. To obtain sufficient selectivity to be able to comply with this definition and to discriminate among different stereo-isomers, selective methods are needed, usually involving chiral selectors. Recently, it has been reported that an

extensive clean-up procedure is mandatory for the stereo-isomeric selective analysis of clevudine in plasma [71] and chloramphenicol in urine [72].

Nevertheless, from the experience in the authors' laboratory it is concluded that for the analysis of a broad range of veterinary drugs in products of animal origin, solvent extraction only or in combination with an SPE clean-up is suitable [23,44,48,49,73]. Such methods are considered screening methods that are useful for application in a routine situation where high sample throughput is of major importance. In this, ACN is the preferred extraction solvent but in case tetracyclines are included in the method, EDTA should be added to the extraction solvent to prevent complex formation. Although ACE results in higher recoveries for especially lipophilic compounds, co-extraction of matrix interferences and thus matrix effects increase as well. The use of acidic or alkaline conditions during extraction can improve the procedure for specific compounds, but overall recoveries are compromised. Even though it was found that in some cases QuEChERS approaches are advantageous with regard to sample clean-up, from our experience this techniques limits the scope of the method especially for very hydrophilic compounds.

Conclusions

For the analysis of multi-class compounds, having very different physical and chemical properties, very generic procedures should be applied. The first challenge is the extraction of the target compounds from the complex matrices encountered in food analysis. The selection of the extraction solvent is related to the target compounds in order to obtain high extraction efficiency, but also to the matrix of interest to prevent excessive matrix effects and to obtain a final extract that is compatible with injection into the chromatographic system. Although acetonitrile and TCA are frequently used as extraction solvent in generic methods, no single extraction solvent is available for the extraction of the entire range of veterinary drugs from products of animal origin. Therefore, a combination of extraction methods is needed that have to be carried out subsequently or in parallel or a compromise has to be made in terms of recovery or the number of compounds included in the method. PLE seems more promising but data are scarce.

SPE procedures can be useful for the clean-up in the analysis of a broad range of veterinary drugs, but the scope is limited, especially toward very hydrophilic compounds. The advantage in terms of matrix effects is sometimes limited and SPE procedures can be quite laborious. In some cases the extraction solvent is sub-optimal or additional procedures allowing a solvent change in order to ensure compatibility with the SPE procedure are needed.

QuEChERS is useful for veterinary drug analysis, but its scope is somewhat limited. Matrix effects using the QuEChERS procedure are more severe compared to PLE, but more data on the matrix effects when using QuEChERS approaches in the analysis of veterinary drugs are needed for an adequate comparison with solvent extraction, SPE and PLE.

Besides the continuation of the development of generic non-selective sample preparation methods and the automation of these straight forward procedures, an expected parallel and opposite future trend is towards highly selective sample preparation to produce precise quantitative results at low levels and to be able to comply with regulations regarding confirmation of the identity of a compounds, e.g. when analysing compounds having a chiral centre like chloramphenicol or clenbuterol.

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Chapter 3

The (un)certainty of Selectivity in Liquid Chromatography Coupled to Tandem Mass Spectrometry



B.J.A. Berendsen, A.A.M. Stolker, M.W.F. Nielen, The (un)certainty of selectivity in Liquid Chromatography coupled to tandem mass spectrometry, *J. Am. Soc. Mass Spectr.* 24, 1 (2013) 154-163.

Abstract

In the analysis of food contaminants and residues, sports doping and forensic sciences, quantitative and qualitative aspects are involved in declaring a sample non-compliant (positive). For the quantitative aspect of a method, validation procedures are available on basis of which the measurement uncertainty is determined and the quantitative uncertainty is taken into account in the decision making process. For the qualitative aspect, criteria for confirmation of the identity of a compound using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) are laid down in several regulations. These criteria regard the minimum requirements for the instrumental set-up and criteria for retention time (RT) and relative product ion abundances in LC-MS/MS operated in the multiple reaction monitoring (MRM) mode. A positive confirmation indicates that the result is not against the presumptions made: the compound shows the same characteristics as the selected reference compound, but it does not exclude the possibility of the presence of another compound showing the same characteristics. No validation procedure is described to express this uncertainty of a qualitative confirmation result and only limited criteria were set for the selection of product ions: in most regulations, all product ions are assumed to have similar identification power. A procedure was developed on basis of which the identification power of an LC-MS/MS method, which is related to its selectivity, can be determined. A measure for the (un)certainty of the selectivity is the probability of any compound showing the same precursor ion, product ions and RT as the compound of interest. In this work this is calculated based upon empirical models constructed from three very large compound databases. Based upon the final probability estimation, additional measures to assure unambiguous identification can be taken, like the selection of different or additional product ions. The reported procedure in combination with criteria for relative ion abundances result in a powerful technique to determine the (un)certainty of the selectivity of any LC-MS/MS analysis and thus to decrease the risk of false positive results. Furthermore, the procedure is very useful as a tool to validate method selectivity.

Introduction

In residue analysis of food contaminants, sports doping and forensic sciences two criteria exist for declaring a sample as being non-compliant (positive). The first criterion is quantitative: does the concentration of the contaminant significantly exceed the maximum tolerated limit? The second is a qualitative criterion: is the identity of a contaminant confirmed? There is no universally-accepted definition of confirmation or identification [1,2]. In this thesis, the term ‘confirmation’ presumes knowledge on the identity of the compound present, e.g. from previous analysis. A positive confirmation indicates that the result is not against the presumptions made: the compound shows the same characteristics as the selected reference compound, but it does not exclude the possibility of the presence of another compound showing the same characteristics. In contrast, in ‘identification’ no a priori presumption on the structure of the substance present is made and thus identification requires that all other substances are excluded, so that the reported substance is the only possible candidate [3].

For the quantitative aspect of a method, validation procedures are available, stating criteria for trueness, repeatability and within-laboratory reproducibility [4]. On basis of the validation results the measurement uncertainty is determined, allowing this uncertainty to be applied in the decision making process. For the identification of a compound, the main validation parameter is selectivity which is defined as “the power of discrimination between the analyte and closely related substances...” [4]. Although some regulations have been established for the confirmation of the identity of a compound [4-11], these are all based on comparison of the hypothesised identity with a single reference standard thereby ignoring that another compound on this planet might yield a similar result. No validation procedures are described to express this aspect of the uncertainty of the qualitative result in LC-MS/MS analysis.

Guidelines for confirmatory analysis using mass spectrometry with electron impact (EI) ionisation were reported for the first time in 1978 [12]. Based on the empirical study of product ion spectra databases, using diethyl stilbestrol as a model compound, it was concluded that the minimum number of ions in an EI mass spectrum needed for identification of a compound is three. This observation became known as the “three-ion criterion” [1] and was applied in practice.

Although three ions should be sufficient, the choice of ions used for confirmatory purposes is critical to prevent false positive findings [12-15].

Criteria concerning the performance of analytical methods and the interpretation of results were established in the European Union in 2002 [4]. In residue analysis, mass spectrometry (MS), either in combination with liquid chromatography (LC) or gas chromatography (GC), was assigned as the main technique for identification of banned and regulated substances [4]. In 2001 the “identification point concept” was introduced [16], which was later included in 2002/657/EC [4]. This approach was derived from the “selectivity indices approach” in which different extraction, clean-up, and detection techniques were assigned a certain number of points [17,18], but because in the establishment of 2002/657/EC [4] selectivity was predominantly considered a function of the measuring technique, the selectivity obtained by the extraction and clean-up were not included in the final regulation.

When using GC- or LC-MS a minimum number of identification points is required for ‘unequivocal identification’ [4]. For regulated substances (table 1 of EU 2010/37 [19]) three identification points are considered adequate and for banned substances (table 2 in EU 2010/37 [19]) four identification points are required [4,20]. When full scan single MS is used, a minimum number of four ions must be present in the spectrum having a relative intensity of > 10 %, which is a more stringent criterion compared to the “three-ion criterion” [12]. If low resolution MS/MS is used, four identification points are earned for example when monitoring one precursor ion and two product ions and five identification points are earned when monitoring two precursor ions, each having one product ion. In all cases the relative ion abundance of a set of product ions (ion ratio) must comply with the criteria established in 2002/657/EC (table 3.1) before the sample can be considered non-compliant (positive). Slightly deviating criteria for relative ion abundances have been established by other organizations (table 3.1). Previously, a quantitative assessment of the probability of false positive results in EI-MS was carried out and it was concluded that more stringent criteria are needed to prevent false positive results [21]. This underlines that MS/MS criteria do not seem to originate from statistically supported data, but are a result of experts’ judgment and therefore it cannot be concluded that one criterion is better than the other. Moreover, this will depend on the point of view: more stringent

criteria result in an increased probability of false negative results whereas less strict criteria result in an increased probability of a false positive result [3].

A critical review on the ion ratio criteria was presented by Kaufmann *et al.* [22,23] and false positive findings based upon EU criteria have been reported [22,24,25]. It was concluded that, although the current criteria are a good starting point for confirmation of the identity of a compound, an additional procedure is needed to determine the (un)certainty of this qualitative result.

Table 3.1. Maximum allowed tolerance of relative ion abundance for confirmation of the identity based upon LC-MS/MS according to different guidelines.

Guideline	Ion ratio (%)	Maximum tolerance ion ratio deviation relative to the reference standard
EU [4]	> 50	± 20 % relative
	> 20 - 50	± 25 % relative
	> 10 - 20	± 30 % relative
	≤ 10	± 50 % relative
SANCO [5]	Equal to EU	
SOFT / AAFS [6]	All	± 20 - 30 % relative
AORC [7]	All	± 20 % absolute or ± 40 % relative, whichever is larger
CVM [8]	All	± 10 % relative in case of two transition
		± 20 % relative in case of three transition
IOC [9]	All	± 10 % absolute or ± 25 % relative, whichever is larger
UK Drug testing [10]	All	± 20 % relative
WADA [11]	> 50	± 10 % absolute
	25 - 50	± 20 % relative
	5 to < 25	± 5 % absolute
	< 5	± 50 % relative

SOFT / AAFS = Society of Forensic Toxicologists / American Academy of Forensic Sciences

AORC = Associating of Official Racing Chemists

CVM = Center for Veterinary Medicine

EU = European Union

IOC = International Olympic Committee

WADA = World Anti-Doping Agency

In this chapter a procedure is presented to judge the (un)certainty of the selectivity of a method using LC coupled to triple-quadrupole (QqQ) MS based on the precursor ion mass, the product ions selected and the RT on a specific reversed phase chromatographic system. With this procedure the uniqueness of the monitored LC-MS/MS characteristics can be estimated and on the basis of this result, it can be decided if additional data, e.g. a third product ion or more extensive sample clean-up, are needed for a trustworthy identification of the compound present.

Methods

Databases

Three databases were constructed on basis of which the (un)certainty of the selectivity of an LC-MS/MS identification procedure was determined.

Database A contains molecular mass data and was extracted from the eMolecules database [26], which contains over 5.3 million commercially available compounds. Because residue analysis focusses on small molecules, only the compounds having a molecular weight of 100-1000 were taken into account. Of these, all compounds containing at least one carbon in combination with at least oxygen, nitrogen, sulphur and/or phosphorous were selected because these compounds can most likely be ionised by electrospray ionisation. All salts and metal containing compounds were removed from the dataset, which resulted in a total of over 5.1 million compounds. Of these compounds the mono-isotopic nominal mass was calculated.

Database B contains product ion spectra data and is constructed from the product ion spectra of 3629 compounds obtained from our own research, the Massbank database [27] and literature [28-35]. Of 3629 compounds, the positive ion mode electrospray ionisation (ESI) collision induced dissociation (CID) MS spectra were converted to binary spectra with a cut-off level at 5 % relative intensity. The combination of precursor ion mass and product ion mass, and the corresponding neutral losses, were included in the database, resulting in a total of 18314 entries.

Database C contains theoretical RT data on a specific high resolution LC system and is constructed from the qualitative structure-retention relationship (QSRR) model presented in this chapter. For 451 compounds, the relevant physical and chemical parameters were used to calculate the theoretical RT.

Modeling the retention time

UPLC grade water and methanol were obtained from Biosolve (Valkenswaard, the Netherlands). Ammonium formate was obtained from Merck (Darmstadt, Germany). Reference standards of 126 model compounds consisting of veterinary drugs, growth promoters and pesticides, were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Ultra Scientific (North Kingstown, RI, USA).

The LC system consisted of a vacuum degasser, autosampler, and a Waters (Milford, MA, USA) model Acquity binary pump equipped with a Waters Acquity HHS-T3 analytical column of 2.1×100 mm, $1.7 \mu\text{m}$ placed into a column oven at 30°C . The gradient (solvent A, water containing 2 mM ammonium formate, pH 3.5; solvent B, methanol/water (9:1, v/v) containing 2 mM ammonium formate): 0 - 1.0 min, 0 % B, 1.0 - 11.0 min, linear increase to 100 % B with a final hold of 0.5 min, operating at a flow rate of 0.4 mL min^{-1} . The injection volume was 10 μL .

Detection was carried out using a Bruker Daltonics (Billerica, MA, USA) Micro-QTOF MS in the positive electrospray ionisation (ESI) mode. The operating parameters were: end plate offset, -500 V; capillary voltage, 3.5 kV; nebulizer, 2.0 bar; dry gas, 8.0 L min^{-1} ; dry temperature, 200°C .

Of 126 compounds the RT in the analytical system was determined. Furthermore, of each of these compounds 22 physical and chemical parameters were determined: Bioconcentration factor (BCF) at pH=5.5, K_{oc} at pH=5.5, polarizability and molecular volume were obtained from Chemspider [36]; Log P, Log D at pH=3.0 and Log D at pH=5.5 were obtained from SciFinder [37]; total energy, Connolly accessible area, Connolly molecular area, Connolly solvent excluded volume, molecular mass, ovality, molecular refractivity, partitioning coefficient, Balaban index, molecular topological index, polar surface area, shape attribute, total connectivity, total valance connectivity and Wiener index were calculated from BioChem3D Ultra 12.0 after MM2 energy optimization [38].

Extreme correlations among parameters were studied using GenStat software [39] and high correlations were excluded before further analysis. Next, using Genstat, the optimal parameters were selected to be included in the QSSR model to obtain the best relation between the parameters and the observed RT. Using the selected parameters, multiple linear regression was carried out to establish an optimal linear model.

From the constructed model, the theoretical RT of an additional 325 randomly selected compounds were calculated. All compounds for which a theoretical RT below 0.6 min was calculated, being the dead time of the system and thus impossible in practice, were set at 0.6 min. From this data and the theoretical RT of the 126 model compounds the probability distribution of the theoretical retention time on the selected chromatographic system was constructed.

Proposed procedure

A measure for selectivity is the probability of the occurrence of a compound showing the same LC-MS/MS characteristics as the compound of interest: the probability of an interference, $P(I)$. $P(I)$ is calculated based upon independent probability matching:

$$P(I) = P(M_{pc}) * \max(P(M_{pd1}), P(M_{nl1})) * \max(P(M_{pd2}), P(M_{nl2})) * P(RT), \quad (1)$$

in which $P(M_{pc})$ is the probability of the occurrence of the precursor ion, $P(M_{pd})$ the probability of the occurrence of the product ions of transition 1 and 2, $P(M_{nl})$ the probability of the occurrence of a neural loss of transition 1 and 2, and $P(RT)$ the probability of the occurrence of a RT in the chromatographic system. $\max(P(M_{pd}), P(M_{nl}))$ is the maximum of $P(M_{pd})$ or $P(M_{nl})$ to represent a worst case situation. These parameters are determined based on the databases and empirical models presented in this chapter. Next, $P(I)$ is compared to an a priori established criterion to rank the method selectivity, e.g. as sufficient or insufficient. Based on the result, corrective measures can be taken to obtain additional selectivity like the selection of another or an additional product ion. A flow chart of the proposed procedure is presented in figure 3.1.

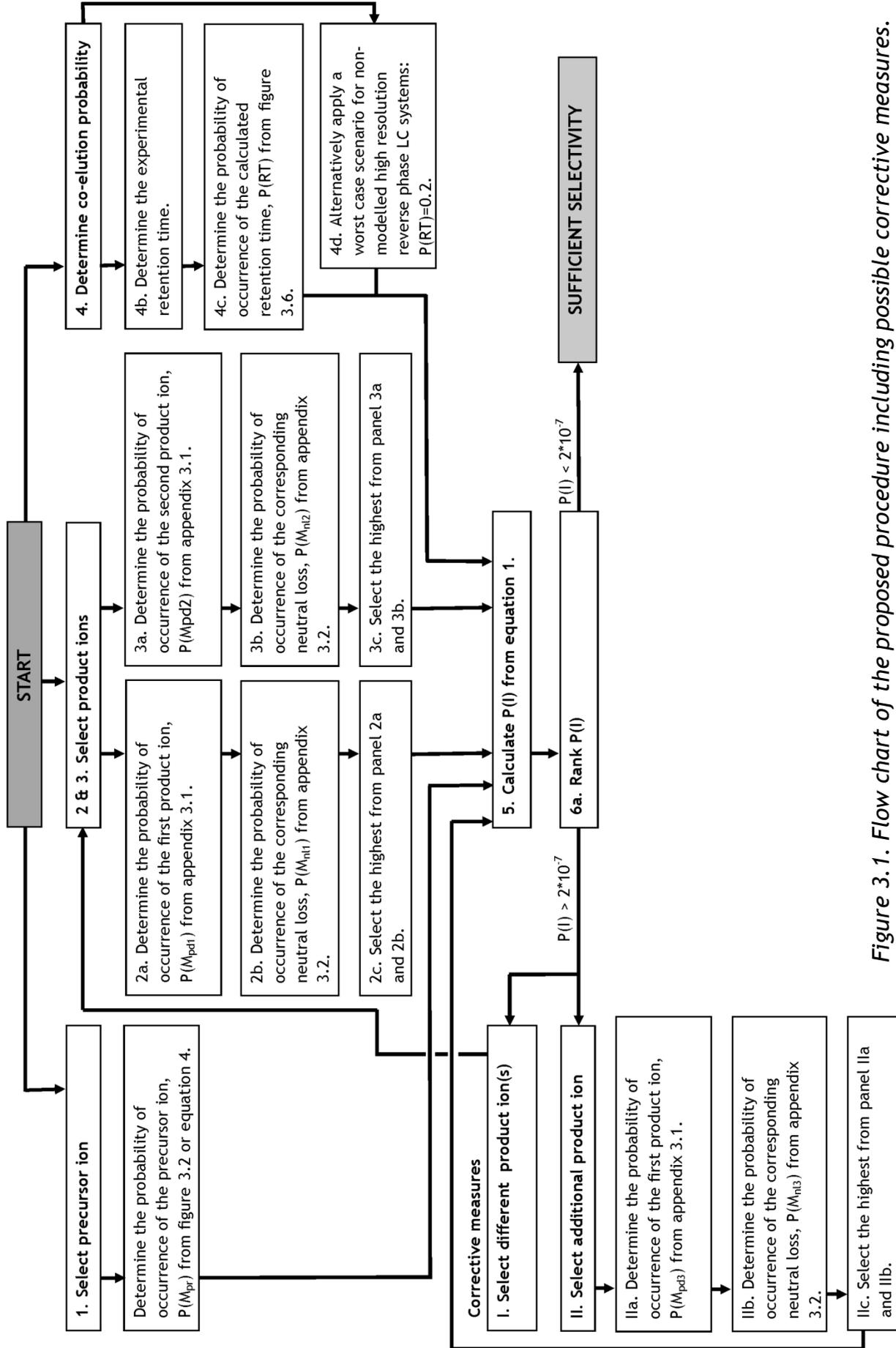


Figure 3.1. Flow chart of the proposed procedure including possible corrective measures.

Results and discussion

An important parameter influencing the (un)certainty of the identification using MS, as was already pointed out by Sphon in 1978 [1,12], is the relative importance of certain transitions. EU legislation indicates that “the selected diagnostic ions should not exclusively originate from the same part of the molecule” [4], but even though clear differences in the selectivity of transitions were reported in literature [40], in most regulations very non-selective transitions like the loss of water or ammonia during CID are considered equally selective as compound specific fragmentations. The UK guidelines for urine drugs testing mention product ions that are considered insufficiently selective, viz. $m/z < 50$, 58, 86, 91 and 105 [10] and SANCO/12495/2011 states that high m/z ions arising from loss of water or from common moieties are of little use [5].

McLafferty *et al.* [41] and Pesyna *et al.* [42,43] regarded the probability of ions to occur in a mass spectrum. From a large collection of GC-EI-MS spectra, the occurrence of ions between m/z 29 - 400 were modeled indicating that ions at m/z 29, 39, 41, 43, 55, 57, 73, 91 and 105 have a high probability of being a high abundant ion in a EI mass spectrum, in contrast to ions at m/z 37, 38, 49 - 53 and 62 - 66. On the obtained data, they carried out probability matching based upon the probability theory which states that if a number of events occur with a certain probability, the probability of all these events to occur simultaneously is the multiplication of all the individual probabilities. This overall probability was considered a measure for the uniqueness of a spectrum and thus for selectivity.

Probability matching of selectivity

Selectivity is a parameter that can be expressed in terms of the uniqueness of a compounds' characteristics, in other words in terms of the probability of the occurrence of another compound possessing the same characteristics, $P(I)$. If $P(I)$ is high, the probability of an incorrect confirmation result is high and thus the method selectivity is considered low, whereas if $P(I)$ is low the system used can be considered as highly selective. The selectivity for identification purposes using low resolution LC-QqQ-MS/MS, and thus the probability of an incorrect identification, is primarily determined by: (1) the combination of the selected precursor and product ions, (2) the RT of the compound and therefore the

probability of co-eluting interfering compounds and (3) the selectivity of the sample preparation method [17,18].

According to EU criteria [4], using low resolution MS/MS, at least two transitions from precursor ion to product ion should be monitored to obtain a sufficient number of identification points. The selectivity of the monitored transitions is directly related to the m/z of the precursor ion(s), M_{pc} , and the product ions, M_{pd} . In theory, the most exact calculation of the probability of the occurrence of a combination of two transitions, $P(MS)$, is given by applying dependent probability calculations. In that case $P(MS)$ is the probability of M_{pd2} , given that the M_{pd1} occurs and that both originate from the selected precursor ion(s). This can only be determined empirically from a very large MS/MS database and the final result will be limited by the total number of spectra included in this database. For instance if database B, containing 3629 spectra, is used and a unique combination of M_{pc} , M_{pd1} and M_{pd2} is found, it can be concluded that $P(MS)$ is $2.8 \cdot 10^{-4}$, or in other words 1 in 3629. This is a severe underestimation of the selectivity of the method and can only be overcome by increasing the total number of spectra in the database. However, if a result of e.g. 1 in 5,000,000 is considered the minimum to conclude that sufficient selectivity is obtained, at least 5,000,000 product ion spectra should be included in the database, which is far off reality for the time being. Therefore independent probability matching is proposed here, in which the individual probabilities of M_{pc} , M_{pd1} and M_{pd2} are multiplied. The selectivity of the precursor ion(s) is given by the probability of the occurrence of compounds showing the same precursor ion mass: $P(M_{pc})$. The selectivity of the product ions is related to the product ion mass (M_{pd}) itself, but also to the resulting neutral loss ($M_{pc}-M_{pd}$). For instance a product ion of m/z 400 has a low probability, but if it originates from a precursor ion of m/z 418, and thus if a neutral loss of water (18 Da) is involved, this transition should be considered non-selective. In the proposed worst case approach, the selectivity of the product ion is determined by selecting the highest of either $P(M_{pd})$ or the probability of the corresponding neutral loss, $P(M_{nl})$. As a result, the probability of the occurrence of a compound having the same MS characteristics as the compound of interest is given by formula 2.

$$P(MS) = P(M_{pc}) * \max(P(M_{pd1}), P(M_{nl1})) * \max(P(M_{pd2}), P(M_{nl2})) \quad (2)$$

These parameters can all be derived from the constructed databases A and B.

Additional selectivity is obtained from the liquid chromatographic system. From the regression model, based on 11 physical and chemical properties, the theoretical RT of any compound, including its uncertainty, can be estimated. Based on the theoretical RT of 451 compounds included in database C, the probability of co-elution with other compounds can be determined. Then the total probability of the occurrence of the selected characteristics, $P(I)$ is calculated by formula 3.

$$P(I) = P(MS) * P(RT) \quad (3)$$

A disadvantage of the application of independent probability statistics is that dependency of the data is assumed not to exist. Obviously, some dependencies will exist, for example singly charged precursor ions always result in product ions at lower m/z . Therefore no exact quantitation of $P(I)$ is obtained in this work, but rather a very good estimate. The result should therefore not be considered as a quantitative measure, but as a ranking tool for selectivity, e.g. insufficient, sufficient, high. The calculated value of $P(I)$ in relation to the ranking of selectivity depends on fitness for purpose [1] and other factors affecting method selectivity like the preceding sample preparation procedure. In this work the applied sample preparation procedure is not taken into account in calculating $P(I)$. A very selective sample preparation procedure, e.g. the use of bio-affinity solid phase extraction will decrease the probability of interfering signals and thus a higher $P(I)$ could be acceptable, whereas for very generic sample preparation techniques, which are increasingly being applied [44], a lower $P(I)$ is needed. Furthermore, if a derivatization procedure is used in the sample preparation procedure, e.g. a hydrolysis using nitrobenzaldehyde as is common for nitrofurans analysis [25], all product ions or neutral losses related to nitrobenzaldehyde should be considered less selective than calculated from the method reported here, since an excessive number of compounds containing a nitrobenzaldehyde moiety will be present in the derivatised solution and thus database B would not be representative for this specific case.

Note that in this approach the relative ion abundances are not taken into account, whereas this would usually result in additional certainty on the identity of the compound. Therefore, the method presented here is on the safe side and gives an overestimation of the true $P(I)$.

Precursor ion probability

The probability distribution of the m/z of the protonated molecules of all entries in database A is presented in figure 3.2. It is observed that, in database A, an m/z of 387 is most common having a probability of 0.0052. Consequently, interfering signals are more likely for compounds in the mass range around m/z 400 compared to the lower and higher mass range. Therefore, the detection of compounds with M_{pr} around m/z 400 is considered less powerful than detection of compounds with an M_{pr} of e.g. 200 or 600. The obtained probability distribution is in good agreement with the distribution reported by Little *et al.* [45] who used ChempSpider [36] as a reference. Kind and Fiehn, who used the Pubchem database [46] as a reference, found the highest probability at a slightly higher mass and reported a longer tail at the high mass range [47]. This might be a result of the presence of complexes and compounds containing metal atoms present in the Pubchem database, which have been removed from database A. At around m/z 500 a non-linear transition is observed in the dataset. This is likely a result of the content of the eMolecules database, rather than a true lower relative occurrence of compounds at this specific mass.

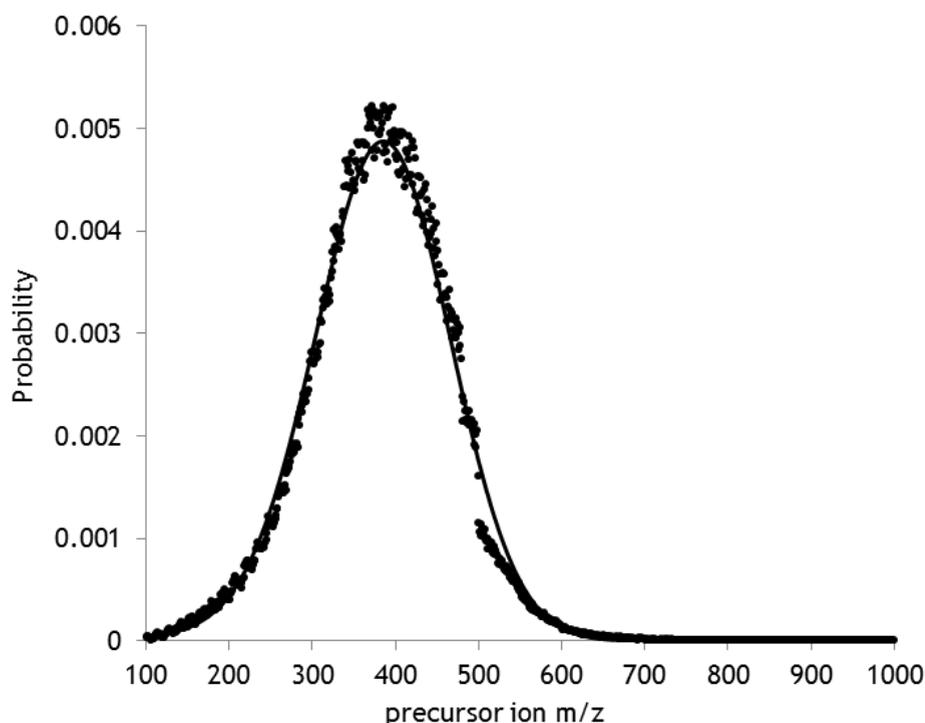


Figure 3.2. Probability distribution of the mass over charge ratio of the protonated molecules included in database A ($n > 5,000,000$). Solid line: fit according to equation 4.

$P(M_{pr})$ can be empirically determined from database A. For application of the method without access to the original database, the probability distribution was modeled assuming binomial distributed data and using a logit link function. By doing so, $P(M_{pr})$ can be calculated by formula 4, in which M_{pr} is the m/z of a precursor ion.

$$P(M_{pr}) = \frac{\exp(-19.8 + 0.067 M_{pr} - 0.000084 M_{pr}^2 + 405 M_{pr}^{-1})}{1 + \exp(-19.8 + 0.067 M_{pr} - 0.000084 M_{pr}^2 + 405 M_{pr}^{-1})} \quad (4)$$

Product ion probability

From database B, the probability distribution of the product ions is presented in figure 3.3 for four categories of precursor ions: m/z 100 - 200, m/z 200 - 300, m/z 300 - 400 and > 400. These groups were defined to obtain at least 500 product ion spectra per group. From the data presented in figure 3.3, for each precursor ion category a continuous model was constructed assuming binomial distributed data using a logit link function, to facilitate calculation of $P(M_{pd})$ (appendix 3.1). Some product ions show an exceptional high or an exceptionally low probability compared to this model (high residual) and for these cases the probabilities are presented individually in appendix 3.1.

In the low mass range ($M_{pr} = 100-200$) the probability of the occurrence of a product ion around m/z 100 is the highest, in the mass range 200-300 this is m/z 140 and at the higher mass range ($M_{pr} > 300$) this is m/z 170. Overall a product ion at m/z 91 has the highest probability and is therefore the least selective, followed by 105, 121, 109, 100 and 72. From these, only the product ions at m/z 91 (tropylium cation) and 105 (benzoyl cation) were previously indicated as being insufficiently selective in LC-MS/MS by the UK guidelines for urine drugs testing [10].

The relation between the product ion probability distribution and the precursor ion mass was briefly studied. A clear decrease of the probability of the product ion at m/z 91 is observed with increasing precursor ion mass. To less extend this decrease is observed for m/z 105. One explanation for this observation is that for large precursor ion masses, sometimes only a limited product ion mass range has been acquired, missing certain low mass product ions. Therefore, the probability of product ions at m/z < 100 originating from large precursor ions can be

somewhat underestimated. However, this effect is not apparent for the continuous distribution of product ions observed for high mass precursor ions and thus it is concluded that this effect is relatively small. The second explanation is that in large molecules, more sites are available to dissipate the dissociation energy and thus the number of fragment stabilization options increase with the molecular mass. As a result, the probability of producing a specific low weight product ions decreases.

Modeling product ion probability for the selected precursor ion mass ranges yielded large differences in models compared to the overall product ion probability model. The differentiation in the selected precursor ion mass ranges was found to be effective in coping with the obvious dependency between product and precursor ion mass probabilities.

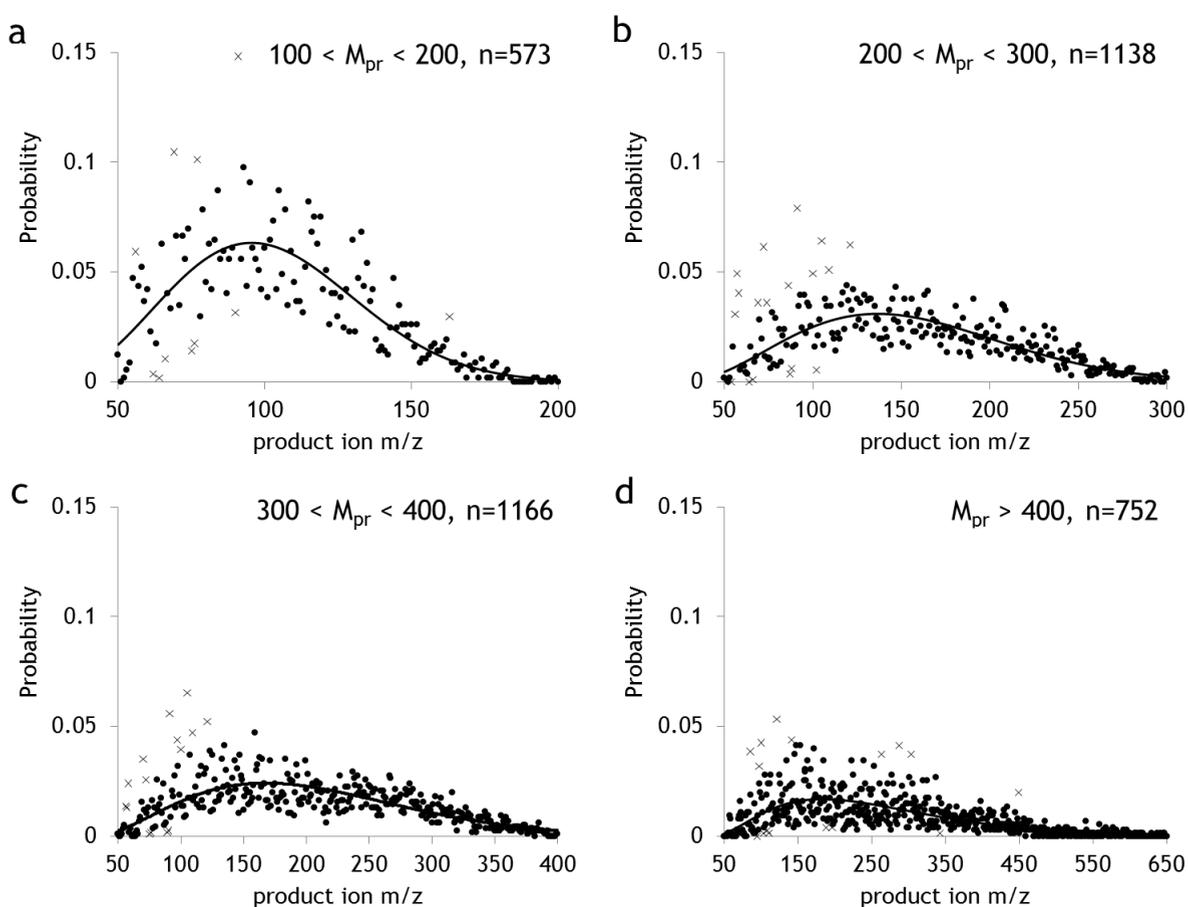


Figure 3.3. Probability distribution of product ion masses for precursor ions of m/z (a) 100 - 200, (b) 200 - 300, (c) 300 - 400 and (d) > 400. Product ions showing a high residual from the constructed model are indicated with a cross. n is the number of spectra per group. Solid line: fit according to the equations as presented in appendix 3.1.

Neutral loss probability

From database B, the neutral losses were calculated ($M_{pr}-M_{pd}$). Although for neutral losses it is less obvious, a clear dependency between the neutral loss and the precursor ion mass was observed. Therefore, also for the neutral losses four product ion mass categories were established. The probability distribution of the neutral losses is presented in figure 3.4.

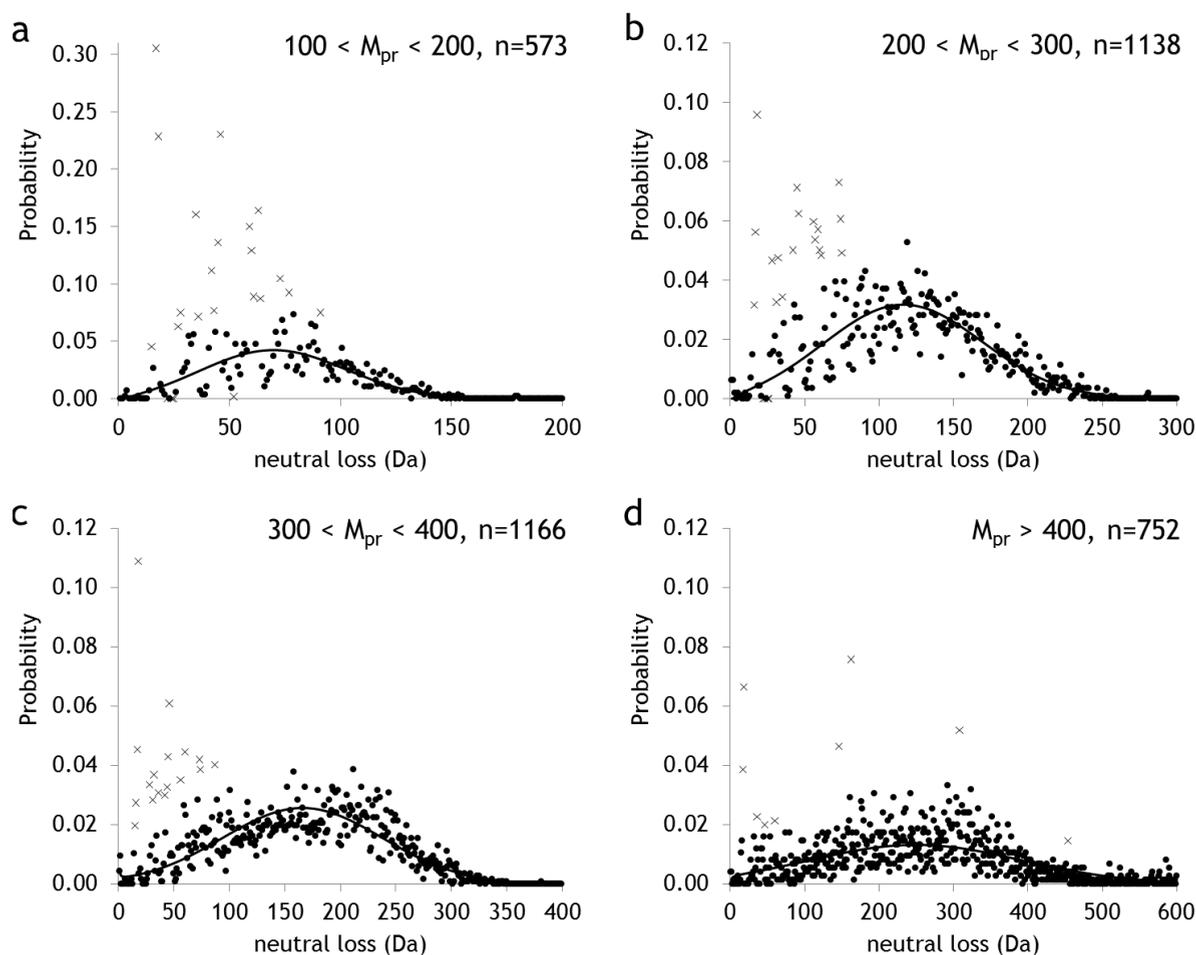


Figure 3.4. Probability distribution of neutral losses for precursor ions of m/z (a) 100 - 200, (b) 200 - 300, (c) 300 - 400 and (d) > 400. Neutral losses showing a high residual from the model are indicated with a cross. n is the number of spectra per group. Note the extended y-axis in (a). Solid line: fit according to the equations as presented in appendix 3.2.

From the data presented in figure 3.4, for each precursor ion category a continuous model was constructed assuming binomial distributed data using a logit link function to facilitate calculation of $P(M_{nl})$ (appendix 3.2). Again, some neutral losses show an exceptional high or an exceptionally low probability

compared to this model (high residual) and for these cases the individual neutral loss probability is presented in appendix 3.2.

Overall a neutral loss of 18 Da has the highest probability and is therefore the least selective (as was reported previously [2]), followed by 46, 17, 45, 60, 73 and 59. From experience, it was expected that the loss of water (18 Da), ammonia (17 Da), formic acid (46 Da) and acetic acid (60 Da), and for larger molecules glycoside (162 Da), have a high probability and are thus non-selective. However, other observations are not considered general knowledge, like the low selectivity of the neutral loss 59 and 73 Da. The neutral losses of 22 - 25 Da have an exceptionally low probability ($P_{nl}=0$) compared to the empirical model. This can be explained because neutral losses at these masses are chemically impossible; no molecular structure can be drawn that is in agreement with these neutral loss masses.

In a previous version of database B the high probability of neutral loss 162, 176, 194 and 308 Da were quite dominant. These neutral losses are all to some extent related to the loss of glycoside, galactoside and glucuronide moieties. Especially the high probability of 176 Da was caused by the inclusion of the data obtained from Wissenbach *et al.* [32], who presented the fragmentation of a large number of antidepressant glucuronides. After removal of these data from database B, the probability of 176 Da dropped from 0.11 to 0.03, demonstrating that this single paper largely biased the dataset. This effect was only limited for the other often occurring neutral losses, e.g. for 162 Da the probability dropped by 10 %. This demonstrates that the construction of the product ion spectra database is crucial for correct interpretation of the selectivity. To further improve this procedure the database should be extended to equally represent compounds from different groups.

The relation between the neutral loss probability distribution and the precursor ion mass was studied. The probability of the smaller neutral losses, e.g. 18, 17 and 46 Da clearly decreases with increasing precursor ion mass. This might be explained by the increasing number of dissociation reactions possible with increasing molecular mass, e.g. multiple water losses. As expected, the probability of multiple dissociation reactions in a high mass molecule increases, together resulting in higher neutral loss values.

Chromatographic co-elution probability

Besides the selectivity provided by the MS detection, the liquid chromatographic system adds additional selectivity. To predict the probability of a co-eluting compound, the RT of an infinite number of unknown, possibly interfering compounds, should be estimated. Because this is impossible, a theoretically predicted RT can be used. A QSRR model can be constructed to predict a compounds' RT based upon its physical and chemical properties [48,49]. This approach has been applied by Zheng *et al.* [50] who obtained an excellent relation between the true and the predicted RT ($r^2 > 0.997$) for nucleic acids. However, they divided the compounds in eight groups that were modeled individually, each group containing compounds with similar properties. Baczek, *et al.* [51] and Ghasemi *et al.* [52] applied QSRR to predict the RT of compounds on a previously characterised chromatographic system, resulting in accurate RT predictions; $r^2 > 0.89$ and 0.9 respectively.

In this work a linear regression model was constructed on the basis of the experimentally determined RT in a reversed phase LC system of 126 compounds and a set of 22 physical and chemical parameters for each of these compounds. From the calculations, 10 parameters proved to result in the best prediction of the RT. The empirically determined model is:

$$RT_t = 4.22 + 0.20 * \log D_{pH3} + 0.61 * \log D_{pH5.5} - 2.1 * 10^{-7} * B - 9.5 * 10^{-4} * T + 0.19 * P - 1.9 * \frac{A_{pol}}{A_{mol}} + 0.12 * Shape - 3.1 * 10^{-3} * E_{tot} + 6.8 * 10^{-3} * W \quad (5)$$

in which B is Balaban index, T is molecular topological index, P is partitioning coefficient, A_{pol} is polar surface area, A_{mol} is Connolly molecular area, E_{tot} is total energy and Wiener is Wiener index. The relation between the experimental RT and the calculated RT from the model is presented in figure 3.5; a coefficient of correlation (r^2) of 0.91 is obtained, which is comparable with previous QSRR models that include different compound classes [51,52]. From this model, the prediction error was calculated, which is the combination of the residual standard error of the model and the standard error of the regression line parameters. Because the residual standard deviation of the model is significantly larger than the standard deviation of the regression line parameters, the prediction error is 0.7 min over the whole RT range. Therefore, using this model

the RT of unknown compounds on the reversed phase system tested, can be predicted on the basis of its physical and chemical properties with a prediction error of ± 1.4 min ($\alpha = 0.05$).

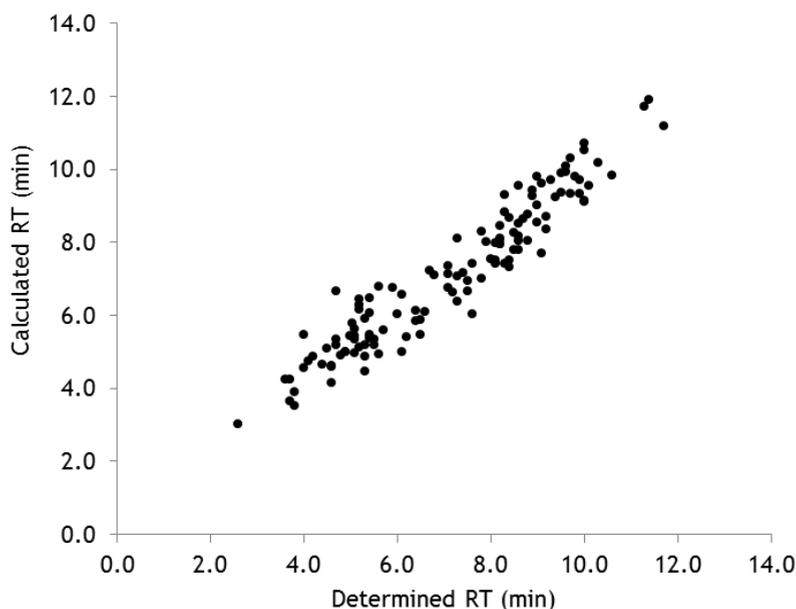


Figure 3.5. Calculated RT from the model as a function of the experimentally determined RT on the reverse phase LC system and using a gradient of acidified water and methanol as the mobile phase.

The probability distribution of the theoretical RT of the 126 compounds used to construct the model plus 325 randomly selected compounds is presented in figure 3.6. The largest probability of co-elution is observed for extremely hydrophilic compounds that do not show retention on the chromatographic system. Next, the highest probability of co-elution is observed for compounds that elute at RT 8.5 min. A high or low RT (as long as compounds undergo some retention) is less probable and therefore more selective. From this, the fraction of compounds eluting within a certain RT window can be calculated and the probability of co-elution of a target compound with other unknown compounds can be estimated. According to EU regulations, the maximum deviation of the RT of a compound compared to the reference is 5 % in case no internal standard is used [4]. Therefore, for a compound of interest that has an experimental RT of x , the best estimate of the probability of interference due to co-elution of unknown compounds is the probability of compounds with a theoretical RT of $x - 5\%$ and $x + 5\%$. The fraction of compounds eluting from the presented system between these theoretical RTs can be determined from database C and figure 3.6.

A prerequisite for obtaining a good estimate for $P(\text{RT})$ is that the used chromatographic system is modeled and that an RT probability distribution is constructed from a significant number of compounds. If not, a worst case approach can be applied. From our data it was found that the highest probability for co-elution of interfering compounds within the established RT interval is for compounds having an RT of 9.5 min. The corresponding $P(\text{RT})$ is 0.11. To challenge the suggested procedure, a second RP chromatographic system using a different analytical column and acetonitrile as the organic modifier was modeled. For this system the relation between the experimental RT and the calculated RT from the model is linear with a coefficient of correlation (r^2) of 0.90. Co-elution showed to be most likely at $\text{RT} = 8.0$ minutes and the corresponding $P(\text{RT})$ is 0.16. From this, it was assumed that for a high resolution RP system using gradient elution, a worst case value of $P(\text{RT}) = 0.2$ can be adopted for calculating $P(I)$ in case the used system is not modeled.

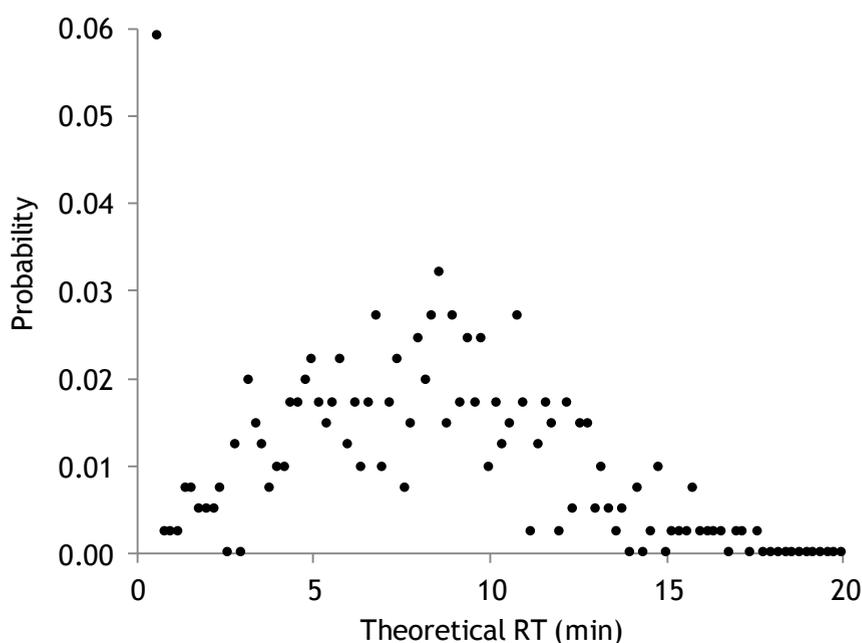


Figure 3.6. probability distribution of the theoretical RT (in categories of 0.2 min) of the compounds in database C ($n=451$).

Application of the procedure

As an illustration of the proposed procedure given in figure 3.1, the calculation of $P(I)$ for sebuthylazine, 3[(2-nitrophenyl)-methylene]-amino-2-oxazolidinone (NPAOZ), oxolinic acid, 17 β -trenbolone and ceftiofur are presented in table 3.2.

The preselected precursor mass and product ion masses for these compounds are included in table 3.2. $P(M_{pr})$ is empirically determined from the probability distribution presented in figure 3.2. $P(M_{pd})$ and $P(M_{nl})$ of both mass transitions are empirically determined respectively from the probability distributions presented in figure 3.3 and appendix 3.1, respectively figure 3.4 and appendix 3.2, at the corresponding precursor mass category. The theoretical RT is calculated from equation 5 and $P(RT)$ is empirically determined from figure 3.6. The results are presented in table 3.2.

Table 3.2. Precursor ion mass, product ion mass and retention time of sebuthylazine, NPAMAZ, oxolinic acid, β -trenbolone, and ceftiofur including calculation of $P(I)$.

	Sebuthylazine [24]	NPAMAZ [25]	Oxolinic acid [40]	β -trenbolone [53]	Ceftiofur [54]
Precursor ion	230	335	262	271	524
$P(M_{pr})$	$7.5 \cdot 10^{-4}$	$4.0 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$	$1.7 \cdot 10^{-3}$	$7.6 \cdot 10^{-4}$
Product ion 1 (m/z)	174	291	244	253	241
$P(M_{pd1})^a$	0.025	0.019	0.010	0.011	0.024 ^a
Neutral loss 1	56	44	18	18	283
$P(nl_1)^a$	0.060 ^{a,b}	0.033 ^{a,b}	0.10 ^{a,b}	0.10 ^{a,b}	0.012
Product ion 2 (m/z)	104	262	216	199	125
$P(M_{pd2})^a$	0.028	0.018	0.013	0.018	0.028 ^a
Neutral loss 2	126	73	46	72	399
$P(M_{nl2})^a$	0.043 ^a	0.042 ^{a,b}	0.062 ^{a,b}	0.035 ^a	0.004
$P(MS)$	$1.9 \cdot 10^{-6}$	$5.4 \cdot 10^{-6}$	$8.7 \cdot 10^{-6}$	$5.6 \cdot 10^{-6}$	$5.1 \cdot 10^{-7}$
Theoretical RT (min)	8.4	0.6	4.8	9.3	10.3
$P(RT)$	0.09	0.06	0.04	0.11	0.08
$P(I)$	$1.7 \cdot 10^{-7}$	$3.0 \cdot 10^{-7}$	$3.7 \cdot 10^{-7}$	$5.9 \cdot 10^{-7}$	$4.2 \cdot 10^{-8}$
1 in...	5,900,000	3,300,000	2,900,000	1,700,000	24,000,000

^a The maximum value of $P(M_{pd})$ versus $P(M_{nl})$

^b Probability with high deviation from the constructed model; empirical value taken from appendix 3.1 (product ions) or 2 (neutral loss)

The proposed procedure was applied for 200 compounds that are routinely analysed using LC-MS/MS at the authors' laboratory, including veterinary drugs, pesticides and natural toxins. The cumulative probability distribution versus $P(I)$ is presented in figure 3.7 for using one and two MS/MS transitions. It is observed that the proposed procedure supports the legal requirement of monitoring at least two product ions [4]: $P(I)$ strongly increases when using two products ions, as expected. As an example, if for ceftiofur (table 3.2) only one product ion is monitored, $P(I)$ is 1.5×10^{-6} and when considering two product ions, $P(I)$ is 4.2×10^{-8} , which is an improvement of method selectivity of approximately 300 times.

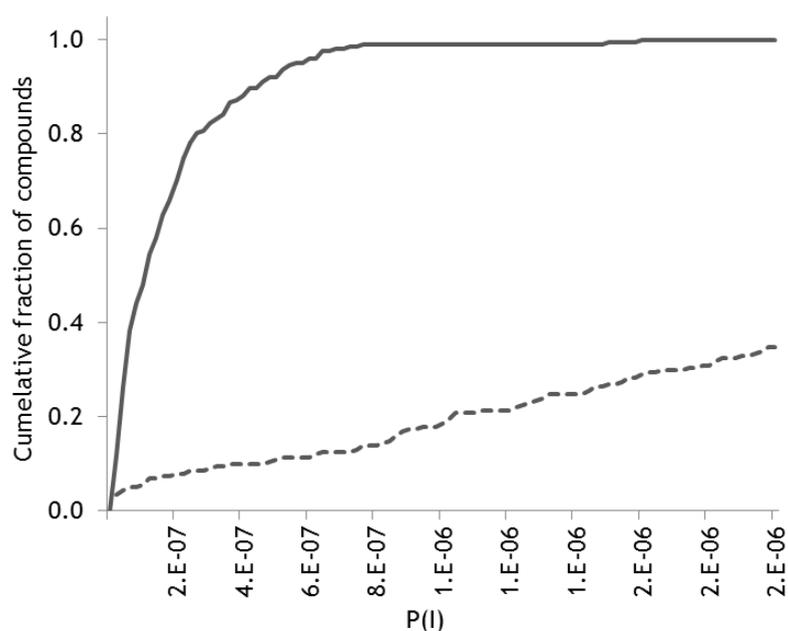


Figure 3.7. Cumulative distribution function of the fraction of the compounds ($n=200$) versus $P(I)$ using (dotted line) one product ion and (solid line) two product ions.

Setting a threshold for $P(I)$

When is a method sufficiently selective? To be able to answer this question a calculated value $P(I)$ should be ranked in terms of method selectivity, e.g. insufficient or sufficient. This can be done using a threshold value for $P(I)$. However, that would suggest the existence of a sharp boundary between 'selective' and 'non-selective', which is not the case. When $P(I)$ is close to any set threshold value, other factors should be considered as well, e.g. the sample preparation procedure and/or results obtained from orthogonal (screening)

analysis which might provide additional information on the compound's identity. In forensic sciences, DNA-matching techniques yield in a probability of 10^{-9} that someone would have the same DNA-profile and thus the probability of an incorrect identification is at most 1 in a billion [55]. However, no threshold value for DNA-matching probability has been established nor for acceptance of a DNA-match in court: DNA evidence by itself is usually not considered sufficient for conviction [56]. Although it is somewhat arbitrary, we feel that a threshold for P(I) can be useful in order to evaluate method selectivity. The threshold should be fixed in such a way that it can be concluded that the method is sufficiently selective (fit for purpose), i.e. (1) the calculated probability of the occurrence of an incorrect identification is low enough to persuade a judge or jury in court and (2) an expert scientist would feel comfortable in defending the probability of an incorrect identification.

According to the authors, the certainty needed for satisfactory confirmation depends on fitness for purpose [1] and thus the threshold value can differ depending on the field of interest, e.g. food safety, sports doping, forensic sciences, and the legal framework involved. To support the establishment of a threshold value for our laboratory, which focusses on residue analysis of registered and banned veterinary drugs, pesticides and natural toxins in food products, sebuthylazine and NPAOZ, compounds for which false positive results were previously reported [24,25] were studied. Furthermore, oxolinic acid and 17 β -trenbolone were studied, being compounds that show difficulties in confirmatory analysis [40,53] and of which the selected neutral losses, water and formic acid for oxolinic acid and water and C₄H₈O for 17 β -trenbolone are considered to be non-selective. The calculated P(I) for NPAOZ, oxolinic acid and 17 β -trenbolone are respectively $3.0 \cdot 10^{-7}$, $3.7 \cdot 10^{-7}$ and $5.9 \cdot 10^{-7}$ (table 3.2). Because of the apparent probability of a false identification for these compounds, in our opinion, the proposed procedure should indicate insufficient method selectivity in these cases and thus we prefer $P(I) = 2 \cdot 10^{-7}$ as a threshold value for evaluation of the method selectivity. This threshold value was challenged on basis of the calculated P(I) of 200 compounds that are routinely analysed at our laboratory by LC-MS/MS (figure 3.7). Of these 200 compounds, 20 are routinely analysed using neutral losses of ammonia, water and/or formic acid. Because these neutral losses are generally considered to be non-selective, the probability of a false positive result should be apparent from the proposed procedure. Of these 20

compounds, calculated $P(I)$ range from $3 \cdot 10^{-8}$ to $1.6 \cdot 10^{-6}$ and only 10 % are below $2 \cdot 10^{-7}$ and would therefore be considered sufficiently selective, which is in agreement with expectations. This supports the choice of $P(I) \leq 2 \cdot 10^{-7}$ in our laboratory as a suitable criterion for sufficient method selectivity. Using this criterion, the analysis of sebutylazine ($P(I) = 1.7 \cdot 10^{-7}$) would be considered just sufficient, even though false positive findings have been reported [24]. It is recalled that $P(I)$ is an estimation of the true probability of interfering compounds, therefore it is suggested to critically review all compounds having a $P(I)$ around the threshold value.

From figure 3.7, it is observed that approximately 70 % of the selected 200 routinely analysed compounds give $P(I) \leq 2 \cdot 10^{-7}$ and thus show sufficient selectivity. For the other 30 % additional measures should be taken in case of confirmatory analysis to prevent false positive results to occur. For example, if for oxolinic acid a product ion of m/z 160 is monitored instead of m/z 244, $P(I)$ would be $1.3 \cdot 10^{-7}$, which can be just sufficient and if three product ions are monitored $P(I)$ would be $1.3 \cdot 10^{-8}$, which is quite safe. These are clear examples of how the calculation of $P(I)$ can assist in selecting product ions and the number of product ions to be monitored during method development to assure adequate method selectivity and thus a high certainty of the confirmation.

Conclusions

In this chapter a procedure is presented that allows the estimation of the (un)certainly of the method selectivity of an LC-MS/MS procedure. Based on empirical models derived from three databases which include data on the precursor ion mass, the product ion mass and a theoretical RT, the probability of the co-occurrence of a compound showing the same characteristics in LC-MS/MS can be estimated. From this, the selectivity of the procedure can be assessed and if found insufficient, corrective measures can be taken like the selection of a different, more selective product ion or the inclusion of an additional third product ion. Acceptance criteria for the presented procedure are suggested and depend on other factors besides LC-MS/MS that possibly influence the method selectivity, like the preceding sample preparation procedure. The proposed procedure is extremely useful to select sufficiently selective multiple reaction

monitoring acquisition parameters during method development. In that way it serves as an additional tool to the established relative ion abundance criteria [4-11] and is a very strong combination for confirmation of the identity of a compound because both relative ion abundances as well as the selectivity of the monitored product ions is taken into account. Furthermore, the procedure reported here can be used as a tool to validate the selectivity of any LC-MS/MS method.

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Appendix 3.1

Empirical model for calculation of product ion probability and extremely deviating values.

Empirical model:	$P(M_{pd}) = \frac{\exp(a + bM_{pd} + cM_{pd}^2 + dM_{pd}^{-1})}{1 + \exp(a + bM_{pd} + cM_{pd}^2 + dM_{pd}^{-1})}$			
	precursor ion m/z range			
	100 - 200	200 - 300	300 - 400	> 400
a	-2.22	-2.03	-2.03	-1.66
b	$4.22 \cdot 10^{-2}$	$8.38 \cdot 10^{-3}$	$1.11 \cdot 10^{-2}$	$5.17 \cdot 10^{-3}$
c	$-3.12 \cdot 10^{-4}$	$-6.62 \cdot 10^{-5}$	$-2.43 \cdot 10^{-5}$	$-5.13 \cdot 10^{-6}$
d	-159	-181	-197	-235

Product ions with a significant higher probability than expected from the empirical model:

Product ion m/z	probability in precursor ion m/z range				Possible product ion
	100 - 200	200 - 300	300 - 400	> 400	
56	0.06	0.03	0.01		$C_4H_8^{+\bullet}$
57		0.05	0.01		$C_4H_9^+$ [33,57]
58		0.04	0.02		$C_3H_8N^+$ [33,57] $C_3H_6O^+$
69	0.10	0.04			
70			0.04		$C_4H_8N^+$ [33] $C_3H_4N_3^+$ [33]
72		0.06	0.03		$C_4H_{10}N^+$ [57]
74		0.04			$C_4H_{12}N^+$ [33]
77	0.10				Phenylum ion [57]
85				0.04	$C_2H_5N_4^+$ [33]
86		0.04			$C_5H_{12}N^+$ [33]
91	0.15	0.08	0.06		Tropylium cation [29,33,57]
97			0.04	0.03	
100		0.05	0.04	0.04	$C_5H_{12}N_2^{+\bullet}$ [33] $C_6H_{14}N^+$ [33]
105		0.06	0.07		Benzoyl cation [57] $C_7H_7N^{+\bullet}$ [33] $C_6H_5N_2^+$ [33]
109		0.05	0.05		$C_7H_5F^+$ [33]
121		0.06	0.05	0.05	Cyclohexatrienecarboxylic acid [33] $C_6H_5N_2O^+$ [33]

141		0.04	$C_{11}H_9^+$ [33]
163	0.03		
263		0.04	$C_{20}H_{23}^+$ [33]
287		0.04	
303		0.04	
449		0.02	

Product ions with a significant lower probability than expected from the empirical model:

Product ion m/z	probability in precursor ion m/z range			
	100 - 200	200 - 300	300 - 400	> 400
54		0		
62	0.003			
64	0.002	0		
66	0.01	0.001		
75	0.01		0.001	
76	0.02		0.002	
87		0.004		
88		0.006		
89			0.002	
90	0.03		0.003	
94				0
97				
102		0.005		
103				0.001
108				0.002
110				0.001
188				0.004
196				0.003

Appendix 3.2

Empirical model for calculation of neutral loss probability and extremely deviating values.

Empirical model:	$P(M_{nl}) = \frac{\exp(a + bM_{nl} + cM_{nl}^2 + dM_{nl}^{-1})}{1 + \exp(a + bM_{nl} + cM_{nl}^2 + dM_{nl}^{-1})}$			
	precursor ion m/z range			
	100 - 200	200 - 300	300 - 400	> 400
a	-5.21	-5.74	-6.37	-5.83
b	$6.47 \cdot 10^{-2}$	$4.14 \cdot 10^{-2}$	$3.30 \cdot 10^{-2}$	$1.23 \cdot 10^{-2}$
c	$-4.75 \cdot 10^{-4}$	$-1.80 \cdot 10^{-4}$	$-9.87 \cdot 10^{-5}$	$-2.47 \cdot 10^{-5}$
d	-7.94	-6.88	1.06	-0.959

Neutral losses with a significant higher probability than expected from the empirical model:

Neutral loss (Da)	probability in precursor ion m/z range				Possible loss
	100 - 200	200 - 300	300 - 400	> 400	
15	0.05		0.02		Methyl [57]
16		0.03	0.03		Methane [57] O, e.g. from sulfones [57] NH ₂
17	0.31	0.06	0.04	0.04	Ammonia
18	0.23	0.10	0.11	0.07	Water [57]
27	0.06				Nitrile
28	0.08	0.05	0.03		CO, e.g. from lactones
31		0.03	0.03		CH ₅ N, e.g. methylamine
32		0.05	0.04		CH ₄ O, e.g. methyl ether
35	0.16	0.03			Water + ammonia
36	0.07		0.03	0.02	2 x water
42	0.11	0.05	0.03		C ₂ H ₂ O, e.g. from acetates
43	0.08				C ₂ H ₅ N, e.g. cyclic amines
44			0.03		CO ₂ , e.g. from lactones
45	0.13	0.07	0.04		C ₃ H ₇ N, trimethylamine CH ₃ NO, formamide
46	0.23	0.06	0.06	0.02	CH ₂ O ₂ , e.g. formate
56		0.06	0.03		C ₄ H ₈ C ₂ H ₂ NO, e.g. isocyanate
57		0.05			C ₄ H ₉ [•] C ₃ H ₅ O, e.g. from cyclopentanol
59	0.15	0.06			C ₃ H ₉ N, e.g. from amines C ₃ H ₅ NO, e.g. from oximes C ₃ H ₇ O, e.g. from polyethers

60	0.13	0.05	0.05	0.02	42 + water 43 + ammonia C ₂ H ₄ O ₂ , e.g. acetate
61	0.09	0.05			43 + water C ₂ H ₅ O ₂ , e.g. from glycols C ₂ H ₅ S, e.g. from aliphatic thioles
63	0.16				45 + water Chloroethyl CH ₃ SO, e.g. from alkyl sulfoxides
64	0.09				46 + water SO ₂ , e.g. sulfoned
73	0.10	0.07	0.04		3 x water + ammonia 56 + ammonia
74		0.06	0.04		56 + water 57 + ammonia C ₂ H ₃ O ₃ , e.g. acetic acid + water C ₃ H ₆ O ₂ , e.g. ethylester C ₄ H ₁₀ O, e.g. tert. Butyl + water C ₃ H ₁₀ N ₂ , e.g. diamino propane
75		0.05			57 + water
77	0.09				
87			0.04		
91	0.08				Tropylium radical (C ₇ H ₇ [•]) [58]
146				0.05	C ₁₁ H ₁₄ C ₅ H ₅ O ₅ , e.g. from glucuronide [35] C ₆ H ₁₀ O ₄ , e.g. from rhamnoside [59]
162				0.08	C ₁₀ H ₁₀ O ₂ C ₆ H ₁₀ O ₅ , e.g. from glycoside [59] or galactoside
308				0.05	162 + 146
454				0.01	162 + 2 x 146

Neutral losses with a significant lower probability than expected from the empirical model:

Neutral loss (Da)	probability in precursor ion m/z range			
	100 - 200	200 - 300	300 - 400	> 400
22	0	0		
23	0	0		
24	0	0		
25	0	0		
52	0.002			

Chapter 4

The Analysis of Chloramphenicol



4.1. General introduction on chloramphenicol analysis

Chloramphenicol

Chloramphenicol (CAP; 2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide) is a broad-spectrum antibiotic used as a human medicine but also as a veterinary drug in all major food-producing animals. The drug is biosynthesised by the soil organism *Streptomyces venezuelae* and several other *actinomycetes* [1], but is produced for commercial use by chemical synthesis [2].

CAP has been evaluated by a number of agencies, including the International Agency for Research on Cancer [3], the European Committee for Veterinary Medicinal Products [2], the United States Food and Drug Administration [4] and more recently in by JECFA (Joint Expert Committee on Food Additives, FAO) at its 62nd meeting [5].

CAP is a suspected carcinogen and due to its linkages to the development of aplastic anemia in humans, the drug is banned for use in food-producing animals in the European Union (EU) and in many other countries, including the United States of America, Canada, Australia, Japan and China. A minimum required performance limit (MRPL) of $0.3 \mu\text{g kg}^{-1}$ was assigned by the European Commission for the analytical methods testing for CAP in products of animal origin [6]. Although CAP is a banned substance, according to 2005/34/EC [7] for all products entering the EU that are analysed for veterinary drugs and thus are within the scope of 97/78/EC [8], the MRPL as stated in 2002/657/EC [9] should be regarded as a reference point of action. In other words, for banned substances, only concentration levels above the MRPL are considered being non-compliant results. However, if the result of an analytical test is below the MRPL “the competent authority has to retain a record of the findings in case of recurrence” [7].

Complications in chloramphenicol analysis

Two major complications exist in the analysis of CAP and the subsequent decision making process. The first is that eight different isomers of CAP exist. A confirmatory method should be able to discriminate among those isomers to assign the correct confirmation in case the drug is detected. The second complication is that CAP is biosynthesised by soil bacteria, which could result in the natural occurrence of the drug.

Discrimination among isomers

CAP occurs in the para-configuration and in the meta-configuration and it contains two chiral centres (carbon 2 and 3, figure 4.1a), thus in total eight different isomeric configurations exist: four (RR, SS, RS, SR) meta-stereoisomers and four para-stereoisomers (figure 4.1). If not explicitly mentioned, the name chloramphenicol generally refers to RR-*p*-CAP, which is also called levomycetin. SS-*p*-CAP is also called dextramycin (DEX) and a racemic mixture of the two isomers is called synthomycin [10]. All para-stereoisomers are biologically active [10-19] but it was reported that only RR-*p*-CAP has antimicrobial properties [10,14]. In contrast, other papers claim < 0.5 % antimicrobial activity for SS-*p*-CAP [15,16] and RS-*p*-CAP [15] compared to RR-*p*-CAP and 1 to 2 % for SR-*p*-CAP [15]. According to literature the structure of the propanediol moiety is critical for the microbial activity whereas the aryl nitro group and the acetamide side chain are not that essential [17]. No information is available on the biological activity *meta*-stereoisomers.

Surprisingly, in the EU regulation [6, 20], no specific configuration of CAP is referred to and thus it is unclear if the regulation applies to all individual isomers or to RR-*p*-CAP only.

In 2002 criteria were established concerning the performance of analytical methods [9]. According to this document samples taken for monitoring of residues in animal products should be analysed using methods that have been validated according to the described procedures [9]. In these performance criteria, selectivity is mentioned as a main characteristic of an analytical method and is defined as “the power of discrimination between the analyte and closely related substances like isomers, metabolites, degradation products, endogenous

substances, matrix constituents, etc.”. Only using highly selective methods, the identity of a compound can be confirmed with high certainty, which is a mandatory in case the analytical result is challenged in court cases. Therefore, methods for the analysis of CAP in animal products should be enantioselective in order to discriminate the antimicrobially active CAP from its isomers.

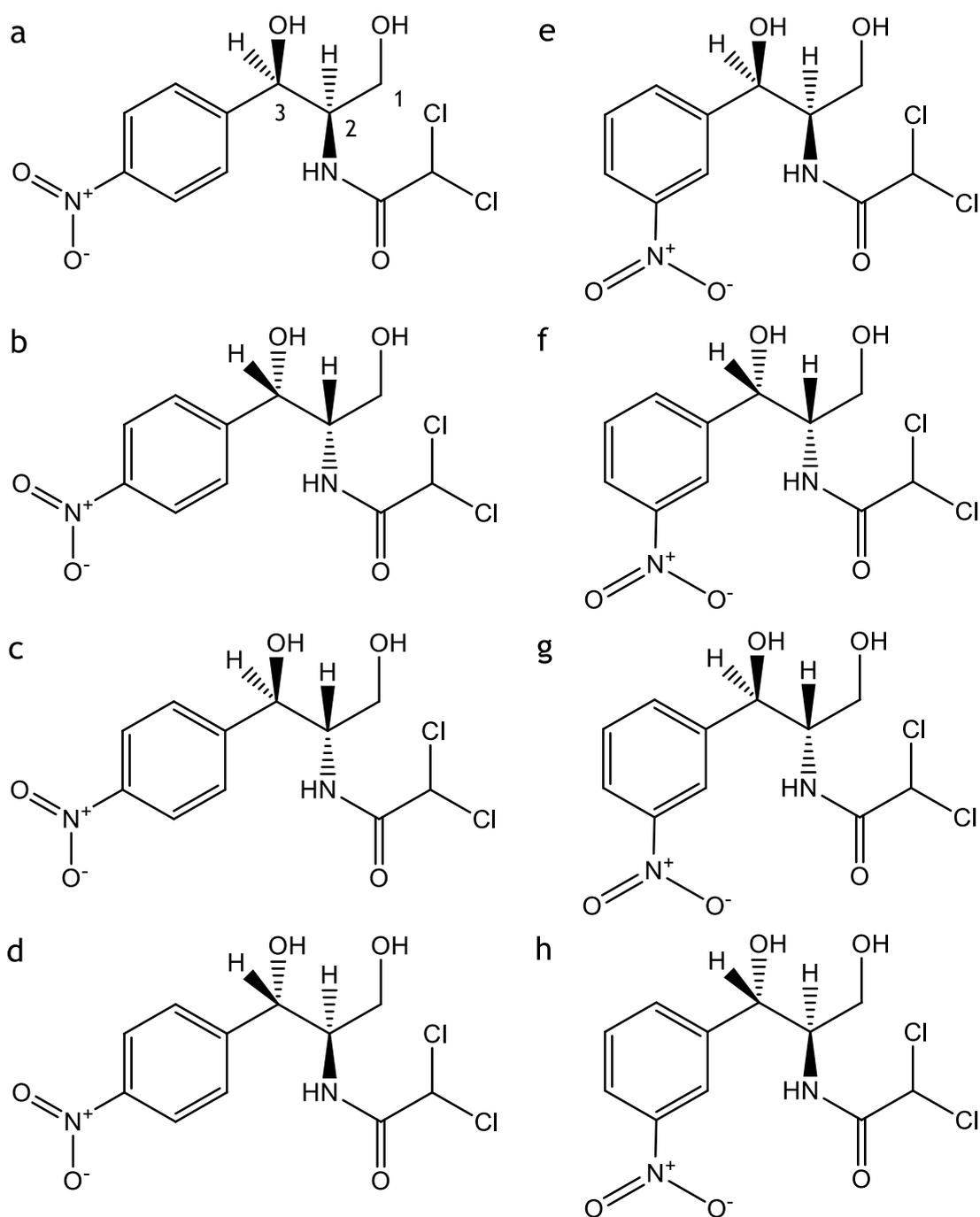


Figure 4.1. Molecular structures of chloramphenicol isomers: (a) *RR-p-CAP*, (b) *SS-p-CAP*, also called *DEX*, (c) *RS-p-CAP*, (d) *SR-p-CAP*, (e) *RR-m-CAP*, (f) *SS-m-CAP*, (g) *RS-m-CAP* and (h) *SR-m-CAP*.

In section 4.3 a high resolution reversed phase LC-MS/MS system was assessed for its ability to separate the eight CAP isomers. In this product ions and the fragmentation pathway in collision induced dissociation was studied in detail. In section 4.4 a newly developed method is presented that is suited for quantitative analysis of eight CAP isomers in urine and which is able to confirm the identity of the target compounds according to EU regulations [9] at part-per-trillion levels, well below the MRPL.

Natural occurrence

In recent years findings of CAP residues in food products such as poultry, honey and sheep casings has had a major impact on international trade [21]. Follow-up investigations in Asian countries related to the non-compliant findings could not identify the origin of CAP residues and no recent history of CAP use was found.

Various hypotheses have been suggested to explain these results. Residues may be caused by the illegal use of the drug in animal production, through contamination of the products by processing workers who were using topical human medicines containing CAP, or by ingestion of naturally occurring CAP from the environment. Due to the fact that recent findings of CAP in several products produced in different countries, such as Thailand and Mongolia, could not be explained by the use of the drug, the hypothesis of naturally occurring CAP warranted scientific investigation.

Several hypotheses for the contamination of food products by possible natural occurring CAP are described by The Joint FAO/WHO Expert Committee on Food Additives [5]. The possibility of contamination due to ingestion of naturally or externally contaminated soil was evaluated. The final conclusion from the evaluation was that the committee could not completely rule out the possibility that foods are occasionally contaminated from environmental sources. However, due to lack of analytical methods to detect the relevant concentrations of CAP in soil, there were no analytical data available to support this suggestion.

Another hypothesis of the natural occurrence of CAP in food product is the production of CAP by *Streptomyces venezuelae* in a typical poultry production environment in poultry litter [21]. The results suggested that residues of CAP on

the farms tested were extremely unlikely to have been caused by natural biosynthesis of the drug in the production environment.

In section 4.2 the monitoring results of plant materials and soil from Mongolia are presented. Several samples of grass and herbs were collected from Mongolian pastures, where the CAP contamination of food products was previously identified. Samples of grass and herbs belonging to the *Atemisia* and *Thalictrum* families were collected. These herbs were selected for collection because it is known that these plants are used as traditional medicines by the local population. To determine if CAP presence could be detected in herbs grown at other locations, samples were purchased from a number of retail outlets in the Netherlands. From this work new hypothesis on the natural occurrence of CAP were suggested.

In section 4.5 the work on the research on the natural occurrence of CAP is presented. The hypothesis is that crops take up and accumulate CAP, that is biosynthesised by soil bacteria, from the soil. If CAP from the soil can accumulate in crops, animals might ingest CAP if these crops are used in animal feed or as stall bedding. As a consequence products of animal origin might be contaminated with CAP residues.

4.2. Evidence of the natural occurrence of the banned antibiotic chloramphenicol in crops and soil

Abstract

Chloramphenicol (CAP), a broad spectrum antibiotic, was detected in several herb and grass samples from different geographic origins. Due to its suspected carcinogenetic properties and linkages with the development of aplastic anemia in humans, CAP is banned for use in food producing animals in the EU and many other countries. However, products of animal origin originating from Asian countries entering the European Market are still found non-compliant (containing CAP) on a regular basis, even when there is no history of chloramphenicol use in these countries. A possible explanation for the continued detection of these residues is the natural occurrence of chloramphenicol in plant material which is used as animal feed, with the consequent transfer of the substance to the animal tissues.

Approximately 110 plant, soil and water samples were analysed using liquid chromatography coupled to tandem mass spectrometry. In 26 samples the presence of CAP was confirmed using the criteria for banned substances defined by the EU. Amongst other, plant materials, samples of the *Artemisia* family retrieved from Mongolia and from Utah, USA, and a therapeutic herb mixture obtained from local stores in the Netherlands proved to contain CAP at levels ranging from 0.1 to 450 $\mu\text{g kg}^{-1}$.

These finding may have a major impact in relation to international trade and safety to the consumer. The results of this study demonstrate non-compliant findings in animal-derived food products may in part be due to the natural occurrence of CAP in plant material. This has implications for the application of current EU, US and other legislation and the interpretation of analytical results with respect to the consideration of CAP as a xenobiotic veterinary drug residue and the regulatory actions taken upon its detection in food.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic with historical veterinary uses in all major food-producing animals. CAP is biosynthesised by the soil organism *Streptomyces venezuelae* and several other *actinomycetes*, but is produced for commercial use by chemical synthesis [2]. CAP is a suspected carcinogen and due to its linkages with the development of aplastic anemia in humans, the drug is banned for use in food-producing animals in the European Union (EU) and in many other countries, including the United States of America (USA), Canada, Australia, Japan and China. A minimum required performance limit (MRPL) of $0.3 \mu\text{g kg}^{-1}$ was assigned by the European Commission for the analytical methods testing for CAP in products of animal origin [6]. In recent years findings of CAP residues in food products such as poultry, honey and sheep casings has had a major impact on international trade [21]. Follow-up investigations in Asian countries that were related to the non-compliant findings could not identify the origin and found no recent history of CAP use.

Various hypotheses have been suggested to explain these results [5]. Residues may be caused by the illegal use of the drug in animal production, through contamination of the products by processing workers who were using topical human medicines containing CAP, or by ingestion of naturally occurring CAP from the environment. Due to the fact that recent findings of CAP in several products produced in different countries, such as Thailand and Mongolia, could not be explained by the use of the drug, the hypothesis of naturally occurring CAP warranted scientific investigation.

A hypothesis, which to our knowledge has never been investigated, is the possibility that grass and herbs (plant materials) absorb and accumulate CAP from the soil. The CAP-containing grass and herbs are used as pasture or harvested as animal feed or forage and consequently products of animal origin are contaminated with residues of CAP. It has been shown that plants are able to absorb veterinary drugs such as tetracyclines from soil [22]. To test this hypothesis several samples of grass and herbs were collected from Mongolia where the contamination of food products with CAP has been identified previously. Samples of grass and of herbs belonging to the *Atemisia* and *Thalictrum* families were collected. To determine if CAP presence could be

detected in herbs grown at other locations samples were purchased from a number of retail outlets in the Netherlands.

For the detection, quantitation and confirmation of CAP different analytical methods are available based on both gas chromatography and liquid chromatography combined with mass spectrometry (GC- or LC-MS) [23-26]. For monitoring purposes, the most frequently used technique is the highly selective, sensitive and relatively quick LC combined with tandem mass spectrometric (MS/MS) detection. This technique is able to detect CAP at the MRPL level of 0.3 $\mu\text{g kg}^{-1}$ in various food products.

In the study reported here plant material and therapeutic herb mixtures were analysed for the presence of CAP using an LC-MS/MS method which was validated in compliance with the EU guidelines in Commission Decision 2002/657/EC [9] and accredited. However, very recently Schürmann *et al.* [27] demonstrated that for a specific matrix/analyte combination a false non-compliant result is obtained by using the EU identification points approach. Therefore, for additional selectivity a few representative samples were reanalysed using a highly selective high resolution LC system (HRLC) monitoring three selected reaction monitoring (SRM) ion transitions.

The major aim of the present study was to determine if CAP can occur naturally in herbs and grass. If confirmed, this observation would help to explain the non-compliant findings of CAP in products of animal origin even when there is no recent history of CAP use. Animals grazing on pasture where such herbaceous plants are prevalent, or being fed with feedstuffs containing those plants, may become contaminated with CAP, with the subsequent detection of CAP in the animal products.

Experimental

Chemicals, reagents and solutions

Methanol (HPLC supra-gradient grade), dichloromethane, ammonia (25 %) and toluene were obtained from Biosolve (Valkenswaard, the Netherlands). CAP (Sigma-Aldrich, St. Louis, MO, USA) and $^{37}\text{Cl}_2$ -CAP (RIVM, Bilthoven, the Netherlands) were used as reference standards. The stock solution of the CAP

reference standard was prepared in methanol at $100 \mu\text{g L}^{-1}$ and were stored at $-18 \text{ }^{\circ}\text{C}$. Dilutions of these stock solution were all prepared in Milli-Q water and stored at $4 \text{ }^{\circ}\text{C}$. The stability of CAP stock solution at $4 \text{ }^{\circ}\text{C}$ is at least 6 months. A solution of ammonia (0.025 %) was prepared by diluting 1 mL ammonia (25 %) in 1 L of Milli-Q water.

Samples

Fifteen plant material samples, among which *Artemisia frigida* and *Thalictrum simplex*, were collected from local fields in the neighborhood of the State Central Veterinary Laboratory, Mongolia (Atar province, Autumn 2007). The first set of five samples was transported in May 2008 to RIKILT. A second set of 10 samples arrived in June of the same year. Six therapeutic herb mixtures, including teas claiming an anti-infectious effect, were obtained from a local store in the Netherlands (June 2009). One *Artemisia frigida* sample originating from Utah, USA was obtained by Internet order from a retail outlet in the UK (June 2009).

In September 2009 herb samples (*Artemisia sieversiana*, *Artemisia frigida* and green grass) were collected from five different provinces in Mongolia (Lun province, Atar province, Hui doloon xudag, Erdene province, Bayandelger province). In each province three different locations were selected and at each location three samples of herbs were collected. Each sample of herb was split into leaves, roots and, if available, stalk. Furthermore, together with each sample of herb two samples of soil were collected (directly below the surface and 20 cm below the surface). Finally, a total of five samples of water were collected. The total number of samples collected was 192.

Equipment

The separation of CAP from the sample components was carried out using liquid chromatography (LC) or by high resolution liquid chromatography (HRLC). The LC-system consists of a vacuum degasser, autosampler and a binary pump (Acquity Waters, Milford, MA) equipped - for LC applications - with a X-Bridge C_{18} analytical column, $3.0 \times 15 \text{ mm}$, $5 \mu\text{m}$ (Waters) placed in a column oven at $30 \text{ }^{\circ}\text{C}$. Isocratic elution was performed with a mobile phase of 0.025 % ammonia / acetonitrile (45:55, v/v) at a flow rate of 0.4 mL min^{-1} and the injection volume was $100 \mu\text{L}$.

For HRLC applications the LC was equipped with an Water Acquity UPLC BEH C₁₈ analytical column of 2.1 x 50 mm, 1.7 µm (Waters) placed in a column oven at 50 °C. The gradient (solvent A, water (100 %); solvent B, methanol (100 %)) was: 0-0.5 min, 10 % B; 0.5-3.5 min, linear increase to 100 % B with a final hold of 0.5 min. Under these conditions CAP eluted after 2.7 min. Injection volume was 100 µL.

Detection was carried out using a Waters Quattro Ultima mass spectrometer with electrospray ionisation (ESI) operating in negative ionisation 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 hr⁻¹; and desolvation gas, 500 L hr⁻¹. CAP was fragmented using collision induced dissociation (CID) and selected reaction monitoring (SRM) transitions at $m/z = 321.0 > 152.1$ and $m/z = 321.0 > 194.0$ were monitored. In the HRLC-MS/MS an additional transition was monitored: $m/z = 321.0 > 257.1$. ³⁷Cl₂-CAP was detected by monitoring the transition $m/z = 324.8 > 152.0$. Data were acquired and processed using MassLynx 4.1 software (Waters).

Sample preparation

Plant material was cut into small pieces and pulverised using a Moulinex blender. Small pieces of plant sample material (1 g) or soil (2 g) were weighed into a 50 mL tube and internal standard ³⁷Cl₂-CAP was added. For the quality control (QC) samples, CAP reference standard solution was also added. Next, 10 mL of Milli-Q water (or more, with a maximum of 25 mL in cases the water was completely absorbed by the sample material) was added to the sample and CAP was extracted from the material by shaking (rotary tumbler, 10 min.) after which it was centrifuged (15 min., 3500 g). An aliquot (3 mL) of the extract was transferred to an Extrelut[®] NT3 (Merck, Darmstadt, Germany) column. After at least 20 min equilibration CAP was extracted from the cartridge using 15 mL dichloromethane which was collected in a 12 mm polypropylene centrifuge tube. The dichloromethane was evaporated to dryness under a stream of nitrogen at 35 °C and the residue was dissolved in 0.5 mL milli-Q water. The final extract was shaken with 1 mL toluene after which the aqueous layer was transferred into an LC-vial.

For quantitation, a calibration line was constructed of the response factor (peak area of CAP / peak area of the IS) versus the CAP concentration using the least squares linear regression method. To take matrix effects into account, blank plant (or soil) samples were fortified with different concentrations of CAP (0 - 50 $\mu\text{g kg}^{-1}$). The collected samples were analysed together with the matrix matched calibration standards (MMS).

Confirmation of the identity of CAP was carried out according to EU criteria [9]. LC-MS/MS monitoring two SRM transitions and comparing the ion ratio of the sample with the reference is suitable to obtain the minimal required number of identification points. Furthermore, the following criteria were to be applied:

- The relative retention time of the compound in the sample has to be the same as the relative retention time of the reference within a margin of 2.5 %.
- The ion ratio of two SRM transition ions of the compound in the sample has to fall within a specified tolerance interval around the reference ion ratio (for example: interval of 20 % if the ion ratio is above 50 %).

Validation

The LC-MS/MS method used for the determination and identification of CAP was validated according to guidelines described for quantitative confirmatory methods in Commission Decision 2002/657/EC [9]. Previous full validation was performed for the matrices urine and shrimps at the concentration levels of 0.2, 0.4 and 0.6 $\mu\text{g kg}^{-1}$. All method characteristics, including linearity, repeatability and reproducibility, fulfilled the EU criteria. The CC α and CC β , established by the analysis of 20 blank samples and 20 fortified samples were, respectively, 0.05 and 0.2 $\mu\text{g kg}^{-1}$ for urine and 0.05 and 0.15 $\mu\text{g kg}^{-1}$ for shrimps. Additional validation experiments were performed for the matrices milk, animal feed and plant material including leaves, stalk, roots and soil. The additional one-day validation for plant material was carried out at levels of 0.3 and 0.5 $\mu\text{g kg}^{-1}$ (n=6 at each level). From these experiments the repeatability was established and compared with the results obtained for the matrices urine and shrimps. In case there are no significant differences the CC α for the additional matrices are established based on the results obtained during the initial validation study.

Sample analysis

The samples of plant material and soil were analysed in series of a maximum of 40 samples. Each series of samples started and ended with the analysis of matrix matched calibration standards. The samples were analysed by the method and the experimental conditions as described above and confirmation of the identity was carried out according to 2002/657/EC [9].

For additional selectivity and confirmation purposes the CAP containing samples (confirmed by using LC) and some blank samples were reanalysed by HRLC. As a reference the average relative retention time and SRM ion-ratios of blank plant material samples spiked with CAP at $2.0 \mu\text{g kg}^{-1}$ were used. Three SRM transitions were monitored and the ion ratios of the sample and the spiked samples were compared. In case the ion ratios fulfilled the EU criteria (ion ratio of sample within the tolerance interval of the spiked samples) the identity of CAP was considered unambiguous.

Results and discussion

Validation

The trueness obtained for the analyses of six samples of plant material (leaves, roots + soil, stalk) at levels of 0.3 and $0.5 \mu\text{g kg}^{-1}$ ($n=6$ at each level) were respectively, 100 % and 104 % and the relative standard deviation under repeatability conditions 9 % and 6 % at these levels. These results did not significantly differ from the results obtained in the initial validation for urine and shrimp. Based on these results it was concluded that the CC α for plant material was $< 0.1 \mu\text{g kg}^{-1}$ (second lowest point of the calibration curve). In other words the method is suitable to detect CAP in plant material at least at concentration levels $\geq 0.1 \mu\text{g kg}^{-1}$.

Sample analysis

In August 2009 the first set of 22 samples including: (a) the samples collected in Mongolia during 2007, (b) the samples obtained from a local store in the Netherland, and (c) the *Artemisia fridiga* obtained from Utah, USA, were

analysed for CAP by using LC-MS/MS. The data produced have been presented in table 4.1. It can be seen that in all the herb samples from Mongolia, CAP was detected at concentrations up to 450 $\mu\text{g kg}^{-1}$. Furthermore, in one herb mixture obtained from a local store in the Netherlands and the sample of *Artemisia fridiga* obtained from a retail outlet, CAP was detected at low $\mu\text{g kg}^{-1}$ levels.

Table 4.1. CAP concentrations detected in herb (mixture)s by LC-MS/MS.

Description	Code	Sample description	Type of plant material	Result ($\mu\text{g kg}^{-1}$)*	
First set Mongolian plants (collected autumn 2007)	S1	<i>Thalictrum simplex</i>	Herb	23	
	S2	<i>Artemisia siversiana</i>	Herb	46	
	„	S3	<i>Artemisia frigida</i>	Herb	175
	„	S4	<i>Thermopsis daurica</i>	Herb	21
	„	S5	<i>Thalictrum simplex</i>	Herb	0.3
Second set Mongolian plants (collected autumn 2007)	S6	<i>Artemisia siversiana</i>	Herb	160	
	S7	<i>Thermopsis daurica</i>	Herb	25	
	„	S8	<i>Artemisia siversiana</i>	Herb	20
	„	S9	<i>Thalictrum simplex</i>	Herb	40
	„	S10	<i>Thalictrum simplex</i>	Herb	450
	„	S11	<i>Thalictrum simplex</i>	Herb	15
	„	S12	<i>Artemisia siversiana</i>	Herb	8
	„	S13	<i>Thalictrum simplex</i>	Herb	50
	„	S14	<i>Artemisia siversiana</i>	Herb	4
	„	S15	<i>Artemisia siversiana</i>	Herb	5
Utah, USA**		<i>Artemisia frigida</i>	Herb	1.3	
Dutch local store		Kamillebloesem	Herb	ND	
„		Bandrek 2 pigeons	Herb tea	ND	
„		Parusahaan Jamu	Herb mixture	4	
„		Ge Xian Weng	Herb tea	ND	
„		Giju	Herb tea	ND	
„		Herb mixture	Herb mixture	ND	
„		Echinacea force	Herb medicine	ND	

* ND = < 0.1 $\mu\text{g kg}^{-1}$

** obtained through a retail outlet in the UK (Internet order)

If the concentrations ($0.3 - 3 \mu\text{g kg}^{-1}$) of CAP detected in products of animal origin during monitoring in the EU (EU Rapid Alert System for Food and Feed, 2008) are compared to these findings then the possibility of the sources of some of this CAP originating from plant materials must be considered a possibility. The data generated also show the accumulation of CAP in plants does not occur at all locations and at all times. As a follow up investigation into the variability of CAP concentrations found in the first survey, in September 2009 some additional samples were collected from Mongolian pastures. In total 192 samples of leaves, roots, stalk of *Artemisia sieversiana*, *Artemisia frigida* were collected as well as samples of green grass, soil and water. From these 192 samples a representative set of 87 samples were analysed for CAP. The results for samples containing CAP $\geq 0.1 \mu\text{g kg}^{-1}$ are presented in table 4.2.

Table 4.2. CAP concentrations detected in Mongolian herb samples collected September 2009.

Province	Location No. / Sample No.	Name	Part of the plant	Result ($\mu\text{g kg}^{-1}$)
Lun	9/36	Green grass	leaves	0.6
	9/37	Green grass	roots	0.3
Atar	14/59	<i>A. frigida</i>	roots	0.3
	1563	Green grass	roots	0.6
	16/66	<i>A. sieversiana</i>	leaves	2.8
	17/72	<i>A. frigida</i>	roots	3.8
Hui doloon xudag	19/80	<i>A. sieversiana</i>	roots	2.0
	21/86	Green grass	leaves	1.2
	24/100	Soil	up*	0.1
	25/103	<i>A. frigida</i>	roots	3.0
Bayandelger	39/168	<i>A. frigida</i>	leaves	0.3
	43/185	Green grass	roots	0.3
	43/186	Soil	up*	0.2

* soil samples were taken directly under the surface (=up) and in the plant hole (=below)

From the results in table 4.2 it can be observed that only a small selection of the samples, 13 out of 87 (= 15 %), contain CAP at detectable concentrations. From these 13 samples only five samples contain CAP above $1 \mu\text{g kg}^{-1}$ with a maximum of $3.8 \mu\text{g kg}^{-1}$. No extreme values as found in the first sample set were found

(table 4.1). Furthermore, no specific relationship was found between the concentration of CAP in soil and herbs, and concentrations found at a specific location. The samples containing CAP appear to be randomly distributed across the population of samples. This finding is further demonstrated in figures 4.2a-c.

Figure 4.2a presents the results of the subset of 87 samples per province. All provinces have CAP containing plants except one (Atar province). Figure 4.2b illustrates that CAP was found in all three species of plants tested but not in every sample. Figure 4.2c points towards the plant roots as having the highest concentration of CAP compared to leaves and soil samples. From these data it was concluded that the herbs growing on the Mongolian pastures do not always contain high concentrations of CAP and also that no single herb family appears to be responsible for the presence of the antimicrobial compound.

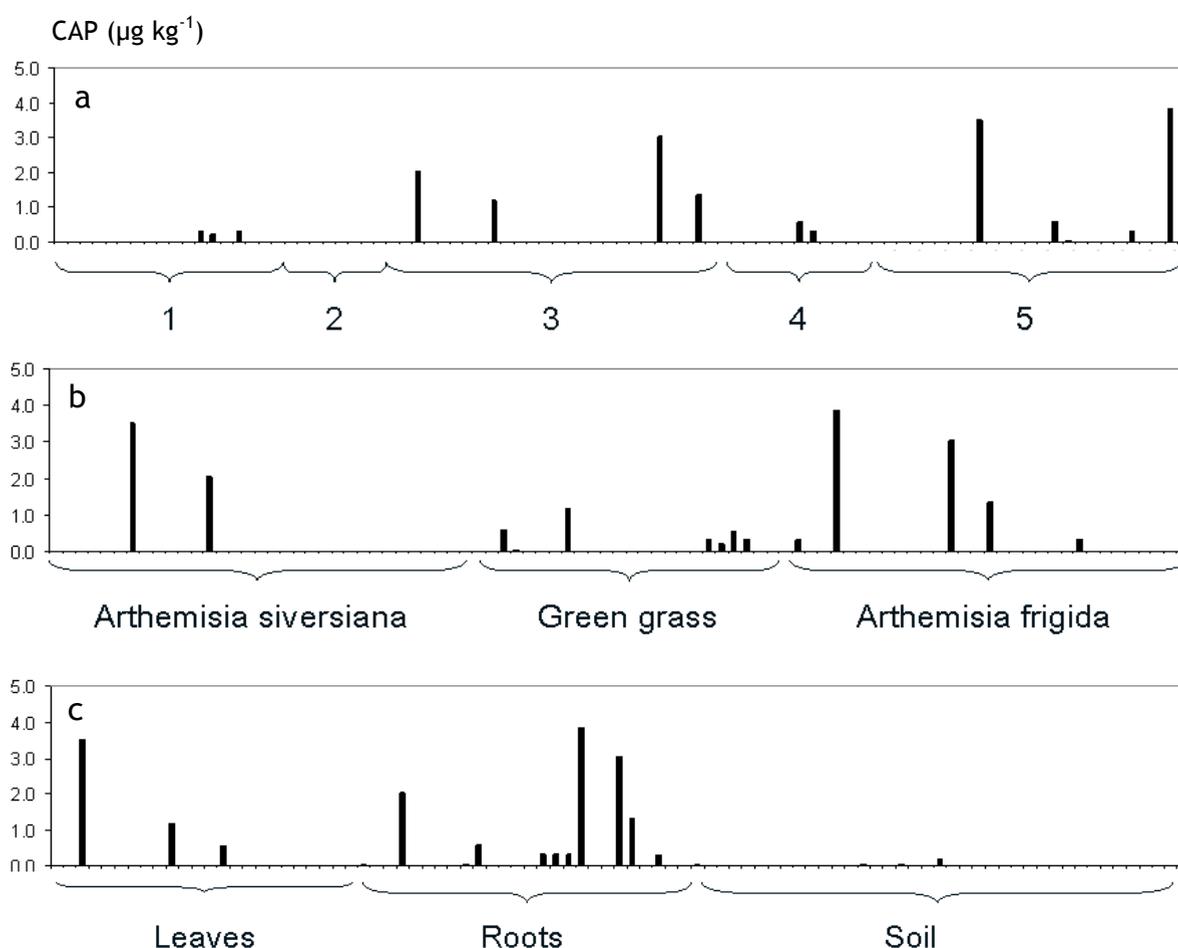


Figure 4.2. Results of the analysis of CAP in $\mu\text{g kg}^{-1}$ for the subset of 87 samples collected in Mongolia, autumn 2009, per (a) province 1) Lun province, 2) Atar province, 3) Hui doloon xudag, 4) Erdene province, 5) Bayandelger province, (b) type of plant and (c) sample material.

It is difficult to draw general conclusions from the observations but it is clear that a relatively large number of root samples contain CAP. It is therefore hypothesised that the CAP originates from the soil and is absorbed through the plant roots, regardless of the plant type. The soil organism *Streptomyces venezuelae*, and some other *actinomycetes*, are known to produce CAP but biosynthesis depends on many factors including the external conditions in the soil. Consequently, it is proposed that the production of CAP may depend on environmental conditions such as the prevailing temperature and the amount of rainfall and consequent moisture content of the soil. The year 2007, for example, was very dry for Mongolia whereas the year 2009 was a very wet year. It is possible (though not proven by our experiments) that the differences in climatic conditions have a strong influence on the biosynthesis of CAP by microorganisms in the soil and its absorption by the plants' root system, and therefore on the concentration of CAP in the plants. Further research is necessary to confirm the biosynthesis of CAP by soil micro-organisms in the vicinity of plants found to contain CAP and to elaborate the various factors influencing CAP biosynthesis and uptake by plants.

Confirmatory analysis

The unambiguous identification of a prohibited compound is of high importance due to the financial consequences of a (false) non-compliant finding, which may include rejection of consignments of contaminated food products by the importing country, increased testing requirements at the expense of the exporter, and possibly prosecution and financial penalties for the producers. Therefore additional precautions are necessary for the identification of CAP in plant materials because of the possible impact the results could have for the international market.

For unambiguous identification of a prohibited (banned) compound the minimum required EU criteria, as previously described, have to be fulfilled. The EU criteria are set up for products of animal origin but these criteria were also used in this study to confirm the identity of CAP in plant material. The results of two representative non-compliant (CAP containing) samples analysed using LC-MS/MS are presented in table 4.3. From the control samples an average ion ratio of 39.5 % and a relative retention time (RRT) of 1.008 were calculated. For confirmatory

Table 4.3. LC-MS/MS results of two samples including identification characteristics.

Description (conc. CAP)	RRT (min)	Rel. deviation of RRT (%)	Response SRM* 321 > 152	Response SRM 321 > 194	Ion ratio (%)	Rel. deviation of ion ratio (%)**
Reference (blank+2 µg kg ⁻¹)	1.008		6048	2389	39.5	
<i>Artemisia frigida</i> (1.3 µg kg ⁻¹)	1.008	0	3736	1445	38.7	-2.0
Herb mixture (4 µg kg ⁻¹)	1.008	0	82603	31611	38.3	-3.0

* Response SRM = peak area

** Max tolerance % according to 2002/657/EC criteria: 25 %

analysis according to EU criteria [9] the maximum allowed relative deviation of the ion ratio is 25 % and thus, in this case the identity of CAP was confirmed if the ion ratio was between 29.5 and 49.1 %. The maximum allowed deviation for the relative retention time is 2.5 %. This approach results in 4 identification points using the approach described in Commission Decision 2002/657/EC [9], which is considered adequate for unambiguous identification within the EU.

The ion ratios obtained for the samples only slightly deviate from the reference ion ratio (maximum relative difference is -2.5 %) and the relative retention time is 1.008 for all samples. From this it was concluded that the identity of CAP is confirmed.

Table 4.4. HRLC-MS/MS results of three samples including identification characteristics.

Description (conc. CAP)	RRT (min)	Rel. dev. RT (%)	Ion ratio 194 / 152 (%)	Rel. dev. ion ratio 194 / 152 (%)*	Ion ratio 257 / 152 (%)	Rel. dev. ion ratio 257 / 152 (%)**
Reference (blank+2 µg kg ⁻¹)	1.004		37.6		70.2	
<i>Artemisia F.</i> (1.3 µg kg ⁻¹)	1.004	0	31.1	-16.4	69.4	-1.1
<i>Artemisia F.</i> (175 µg kg ⁻¹)	1.004	0	36.7	-1.3	66.6	-5.1
Herb mixture (4 µg kg ⁻¹)	1.004	0	35.6	-4.3	68.5	-2.4

* Max tolerance according to 2002/657/EC criteria: 25 %.

** Max tolerance according to 2002/657/EC criteria: 20 %.

For additional selectivity and thus to obtain additional prove for the identity of CAP, the sample extracts were re-injected into an HRLC-MS/MS system to obtain a higher chromatographic resolution. Furthermore three transitions were monitored resulting in a total of 5.5 identification points demonstrating the high selectivity of this method. The results of representative non-compliant samples are presented in table 4.4. Chromatograms of a blank herb mixture sample, a blank herb mixture sample fortified with $2 \mu\text{g kg}^{-1}$ CAP, a non-compliant herb mixture sample ($4 \mu\text{g kg}^{-1}$) and the same herb mixture sample with the addition of $2 \mu\text{g kg}^{-1}$ are presented in figure 4.3.

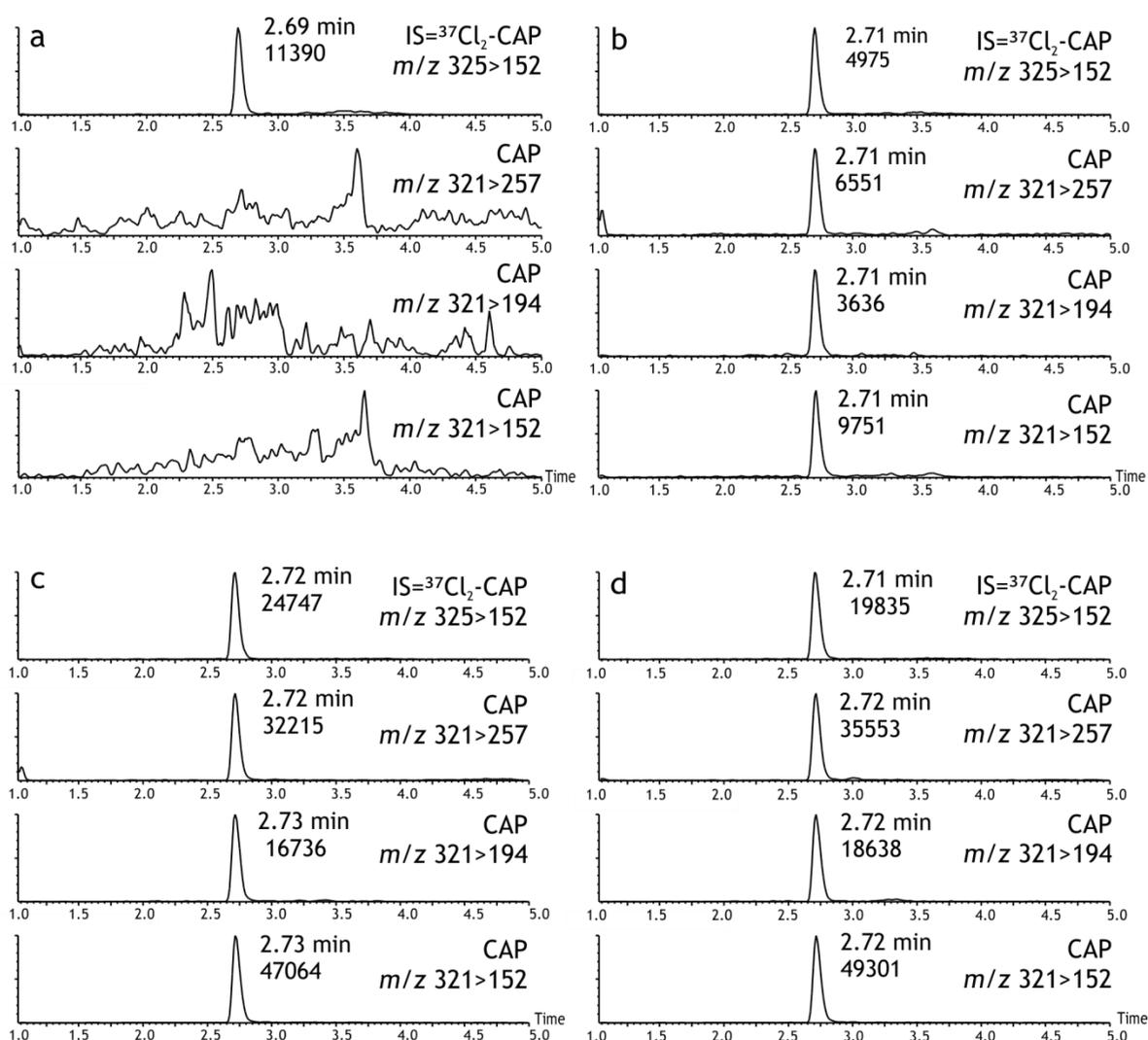


Figure 4.3. HRLC-MS/MS chromatograms showing three SRM ion transitions for CAP and one for the internal standard of (a) a blank herb sample, (b) a blank herb sample with addition of $2 \mu\text{g kg}^{-1}$ CAP, (c) a herb mixture form a local shop and (d) the same herb sample with addition of $2.0 \mu\text{g kg}^{-1}$ CAP.

From the control sample an average ion ratio of 37.6 % was calculated for the product ions $m/z=194$ versus 152 and 70.2 % for $m/z=257$ versus 152. Furthermore a relative retention time (RRT) of 1.004 was calculated. For confirmative analysis according to EU criteria [9] the maximum allowed relative deviation of the ion ratio of $m/z = 194$ versus 152 is 25 % and thus the presence of CAP was confirmed in these samples if this ion ratio was between 28.2 and 47.0 %. For the ion ratio of $m/z = 257$ versus 152 this was 20 % and thus the identity of CAP was confirmed if this ion ratio was between 56.2 and 84.2 %. The ion ratios obtained for the samples were all within these limits and the relative retention time was 1.004 for all samples. From this it was concluded that the identity of CAP was confirmed with high certainty. The LC results did not deviate from the HRLC results. In other words all samples containing CAP based on LC results were (re)confirmed by using the HRLC.

Conclusions

The LC-MS/MS analysis of plant materials from different origins demonstrate that it is possible that plant materials can contain CAP. The concentrations of CAP varied from non-detectable up to $450 \mu\text{g kg}^{-1}$. In cases of positive quantitative CAP results (concentrations above the $CC\alpha$ of $0.1 \mu\text{g kg}^{-1}$) the identity of CAP was unambiguously confirmed according to EU criteria.

From the test results it was concluded that plants belonging to different families can contain CAP. For example, CAP was detected in plants of the families *Artemisa* and *Thalictrum* but it was also detected in grass. It is known that the soil organism *Streptomyces venezuelae* and related organisms can biosynthesise CAP. Therefore it is suggested, based on the results obtained, that CAP is produced in the soil and that the plants absorb CAP through their root systems. Further research is required to confirm this hypothesis and to elaborate the environmental parameters affecting CAP occurrence in plants. This research is presented in section 4.5.

To the best of our knowledge, this is the first time that findings of CAP in plant materials have been reported. These findings make it a much more realistic prospect that products of animal origin can contain residues of CAP that are not due to (illegal) use of the drug, but rather due the natural occurrence of CAP.

The results also have significant implications for the application of legislation with respect to the detection of CAP in food products and may imply, if not a change in the legislation, at least a change in the interpretation of analytical results and in follow-up actions and penalties to producers after the detection of CAP.

4.3. Discrimination of eight chloramphenicol isomer by liquid chromatography tandem mass spectrometry in order to investigate the natural occurrence of chloramphenicol

Abstract

This section describes the discrimination of eight different isomers of chloramphenicol (CAP), an antibiotic banned for use in food producing animals, by reversed phase and chiral liquid chromatography in combination with tandem mass spectrometric detection. In section 4.2, the presence of CAP was confirmed in some grass and herb samples collected on Mongolian pastures up to concentrations of $450 \mu\text{g kg}^{-1}$. It was not possible to establish the cause of CAP residues which has initiated research on the natural occurrence of this drug. CAP occurs in the *para*-configuration and in the *meta*-configuration and contains two chiral centers thus eight different isomeric configurations exist, namely four (RR, SS, RS, SR) *meta*-stereoisomers and four *para*-stereoisomers. It is known that only RR-*p*-CAP has antimicrobial properties. To find out if the CAP detected in the plant material samples is the active configuration, a high resolution reversed phase LC-MS/MS system was tested for its ability to separate the different isomers. This system proved to be able to discriminate among some isomers, but not between RR-*p*-CAP and SS-*p*-CAP, also called dextramycin. Despite a detailed elucidation of the product ions and the fragmentation patterns of all isomers, MS/MS did not add sufficient specificity for full discrimination of the isomers. Therefore a chiral liquid chromatographic separation with MS/MS detection that is able to distinguish all isomers was developed and finally the isomeric ratio of non-compliant plant material samples and some CAP formulations was determined using this system. This showed that Mongolian grass and herb samples only contain the biological active isomer of CAP as do the obtained formulations. Therefore the CAP present in the plant material might origin from the production by soil organisms or from a manufactured source.

Introduction

Chloramphenicol (CAP; 2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide) is a broad-spectrum antibiotic with historical veterinary use in all major food-producing animals. The drug is biosynthesised by the soil organism *Streptomyces venezuelae* and several other *actinomycetes* [28] and is chemically synthesised for commercial use [2]. CAP occurs in the *meta*-configuration and in the *para*-configuration and it contains two chiral centers (carbon 2 and 3, figure 4.1a) thus in total eight different isomeric configurations exist: four (RR, SS, RS, SR) *meta*-stereoisomers and four *para*-stereoisomers (figure 4.1). If not explicitly mentioned, the name chloramphenicol generally refers to RR-*p*-CAP, which is also called levomycetin. SS-*p*-CAP is also called dexamycin (DEX) and a racemic mixture of the two isomers is called synthomycin [10].

All *para*-stereoisomers are biologically active [10-19] but it was reported that only RR-*p*-CAP has antimicrobial properties [10,14]. In contrast, other papers claim < 0.5 % antimicrobial activity for SS-*p*-CAP [15,16] and RS-*p*-CAP [15] compared to RR-*p*-CAP and 1 to 2 % for SR-*p*-CAP [15]. According to literature the structure of the propanediol moiety is critical for the microbial activity whereas the aryl nitro group and the acetamide side chain are not essential [17]. No information is available on the *meta*-stereoisomers.

Chloramphenicol is banned for use in all food producing animals and a minimum required performance limit (MRPL) of 0.3 $\mu\text{g kg}^{-1}$ has been established [6]. Surprisingly, in this document, no specific configuration of CAP is referred to and thus it is unclear if these regulations apply to all individual isomers.

During the last decade, findings of CAP residues in food products such as poultry, honey, and sheep casings have had a major impact on international trade [21]. Follow-up investigations in Asian countries that were related to the non-compliant findings could not identify the origin of CAP residues and no recent history of CAP use was found. Because CAP was also detected in unexpected samples, like wine [29] and plant materials [30] it was suggested that products are contaminated due to the natural occurrence of CAP. More scientific data are needed to be able to support or reject this hypothesis including research on the uptake of CAP by plants, the detection of CAP producing soil organisms and the

presence of isomeric impurities in plant samples and commercially available CAP formulations. The latter is the focus of this section.

Nowadays for the detection, quantitation, and confirmation of CAP mainly reversed phase (RP) LC-MS/MS is used. These RP-LC methods make use of either a C₈ [31,32] or a C₁₈ [23,24,26,30,33] stationary phase combined with isocratic [24,31,32] or gradient [23,26,30,33] elution. Superior resolution is obtained using an analytical column containing sub 2 µm particles [23,30] in combination with gradient elution. These methods have not been tested for other isomers than RR-*p*-CAP and no data are available on the MS/MS detection of CAP isomers.

There are only a small number of papers available on the use of LC to separate CAP isomers. The separation of four *para*-CAP isomers by RP-LC preceded by a derivatization using homochiral isothiocyanate [34] and by addition of cyclodextrin to the mobile phase [35] was reported. However both approaches did not result in full baseline separation of the stereoisomers and are unfavorable because derivatization and complex formation tend to be less robust than direct analysis methods. Chiral LC using 4-(3,5-dinitro benzamido)tetrahydrophenanthrene covalently bound to silica as the stationary phase was applied to obtain a baseline separation for RR-*p*- and SS-*p*-CAP [36] and also for the separation of RR-*p*- and SS-*p*-florfenicol, a compound chemically related to CAP [37]. In literature, no methods are reported combining a chiral LC system with MS detection.

This is the first time results are reported on the LC-MS/MS confirmatory analysis of CAP isomers. The confirmation of the identity of a compound is carried out according to EU regulations on basis of the identification points principle [9]. When using LC-MS/MS the minimum required number of identification points is obtained by selecting one precursor ion and two product ions in Selected Reaction Monitoring (SRM). For a positive identification of a compound in an unknown sample the relative abundance of the two product ions (the ion ratio) should fall within established limits of the ion ratio of the expected compound. Thus, to be able to discriminate among the CAP isomers by MS/MS only, for each isomer a unique combination of two product ions or two product ions with a unique relative abundance are needed.

In the research presented in this section it is tested if a high resolution RP-LC system is capable of separating all CAP isomers and it is the first time MS/MS product ion spectra of CAP isomers are presented and discussed. Furthermore, a chiral liquid chromatographic system was developed to achieve separation of the relevant isomers. The developed method was used to determine the isomeric ratio of CAP isomers in plant material samples obtained from Mongolian pastures that were found positive in previous analyses [30]. Furthermore, CAP formulations were analysed for isomeric impurities. If the isomeric ratio of CAP in plant materials and/or formulations is specific, this could result in tracing the origin of the contamination.

Experimental

Reagents and equipment

HPLC grade water, methanol and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands). Ethanol absolute was obtained from Merck (Darmstadt, Germany). Milli-Q water was prepared using a Milli-Q system at a resistivity of at least $18.2 \text{ M}\Omega \text{ cm}^{-1}$ (Millipore, Billerica, MA, USA). The reference standard of 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, a mixture of RS-*m*-CAP and SR-*m*-CAP and a mixture of RS-*p*-CAP- d_5 and SR-*p*-CAP- d_5 were obtained from Witega (Berlin, Germany). The deuterium atoms were substituted at the phenyl ring and at carbon 3. Non deuterated RS-*p*-CAP and SR-*p*-CAP were not commercially available. Stock solutions were prepared in methanol at 100 mg L^{-1} and all dilutions were prepared fresh daily in Milli-Q water.

The high resolution RP-LC system consists of a vacuum degasser, autosampler, and a binary pump (Acquity Waters, Milford, MA, USA) equipped with a Waters Acquity UPLC BEH C_{18} analytical column of $2.1 \times 100 \text{ mm}$, $1.7 \mu\text{m}$ placed in a column oven at $50 \text{ }^\circ\text{C}$. The gradient (solvent A, water (100 %); solvent B, methanol (100 %)) was: 0 - 0.5 min, 10 % B, 0.5 - 4.0 min, linear increase to 100 % B with a final hold of 0.5 min, operating at a flow rate of 0.4 mL min^{-1} . The injection volume was $35 \mu\text{l}$.

The chiral LC system consisted of the same equipment but combined with a Chromtech Chiral AGP (α 1-acid glycoprotein) analytical column of 2.0 \times 150 mm, 5 μ m (Chromtech inc, Apple Valley, MN, USA) placed in a column oven at 20 $^{\circ}$ C. The gradient (solvent A, water (100 %); solvent B, methanol/acetonitrile (1:1 v/v)) was: 0 - 4.0 min, 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 μ L and of extracted plant material 5 μ L, the latter to extend the column lifetime .

Detection was carried out using a Waters Quattro Ultima mass spectrometer in the negative electrospray ionisation (ESI) mode. The operating parameters were: capillary voltage, 2.7 kV; cone voltage, 25 V; source temperature, 120 $^{\circ}$ C; desolvation temperature, 300 $^{\circ}$ 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-*d*₅ 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), a mixture of *d*₅-RS-*p*-CAP and *d*₅-SR-*p*-CAP, a mixture of RR-*m*-CAP and SS-*m*-CAP and a mixture of RS-*m*-CAP and SR-*m*-CAP. Using this instrumentation a resolution of at least 10000 is 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.

Samples

CAP formulations seized from farms in the Netherlands were analysed for CAP isomers. Plant samples were collected at different Mongolian pastures at different points in time. The people collecting the samples did not use hand creams (possibly contaminated with CAP) and were made aware of the possibility

of contaminating samples due to the use of CAP contaminated gloves to minimise the chance of unintended contamination during this stage. These samples were monitored for the presence of CAP [30] and five highly contaminated plant material samples were selected for analysis of the individual isomers (table 4.5). Extraction and sample preparation were carried out as described before [30] and of the resulting extract 5 μL was injected into the chiral LC-MS/MS system.

Two CAP containing formulations were seized from farms in the Netherlands in 2005. One formulation is a commercially available powder, the other is an unidentified liquid. Of the first formulation a solution of 1 $\mu\text{g kg}^{-1}$ was prepared in water and the second was diluted one million times in water. Of both solutions 20 μL was injected into the chiral LC-MS/MS system.

MS/MS specificity experiments

The discriminating power of the triple quadrupole MS was studied by analysing the product ions formed and comparing their ion ratios for the different CAP isomers. Solutions of RR-*p*-CAP, SS-*p*-CAP (DEX), a mixture of d₅-RS-*p*-CAP and d₅-SR-*p*-CAP, a mixture of RR-*m*-CAP and SS-*m*-CAP and a mixture of RS-*m*-CAP and SR-*m*-CAP were continuously infused in the MS to determine the product ion spectrum at different collision energies (0 through 35 eV). For a closer look, the abundances of the product ions of RR-*p*-CAP and SS-*p*-CAP at each collision energy were compared and the product ions showing the largest relative differences between the two were selected. These product ions were programmed in an SRM experiment and standard solutions of 10 $\mu\text{g L}^{-1}$ CAP and DEX were injected into the LC-MS/MS system alternately (n=8). The ion ratios for both stereoisomers were calculated for all combinations of transitions, resulting in 21 ion ratios. The corresponding ion ratios for RR-*p*-CAP and SS-*p*-CAP were compared using a Students t-test ($\alpha=0.05$) to determine if these stereoisomers can be discriminated based on their fragmentation pattern and relative product ion intensities.

Table 4.5. Mongolian plant materials containing CAP selected for analysis of the isomers.

Sample name	Code [30]	Level of CAP ($\mu\text{g kg}^{-1}$) [30]
<i>Thalictrum simplex</i>	S1	23
<i>Artemisia siversiana</i>	S2	46
<i>Artemisia frigida</i>	S3	175
<i>Artemisia siversiana</i>	S6	160
<i>Thermopsis daurica</i>	S7	25
<i>Thalictrum simplex</i>	S9	40

Results & Discussion

Structure elucidation of product ions

Tentative molecular structures of product ions were suggested based on the presence of chlorine isotopes, the presence of deuterium atoms in RS-*p*- and SR-*p*-CAP- d_5 and the elemental composition derived from the determined exact mass. The chlorine isotope experiment indicated that the product ion $m/z=257$ contains one chlorine atom and that it is the only product ion in which any chlorine is present. The presence of the deuterium atoms in the RS-*p*- and SR-*p*-CAP- d_5 showed that no fragmentation occurs in the penyl ring nor at carbon 3. The exact masses of the major product ions determined using the LC-Q-TOF system are presented in table 4.6. From this information elemental compositions were suggested (table 4.6) for those ions of which the theoretical exact mass is within a 10 ppm error of the experimental assessed value, except for the composition suggested for product ion $m/z=152$ (12 and 35 ppm error for RR-*p*- and SS-*p*-CAP respectively). For this product ion the most likely elemental composition, suggested by the software was selected which is very likely based on the presence of all five deuterium atoms. Tentative product ion molecular structures in combination with the fragmentation pathways as supported by MS^n experiments are presented in figure 4.4. Tentative molecular structures for the product ions $m/z=257$ and 152 were reported by Mottier *et al.* [33] based upon the use of $^{35}\text{Cl}^{37}\text{Cl}$ -CAP and CAP- d_5 . These are in agreement with our findings.

Table 4.6. Exact masses of relevant product ions.

Exact mass (m/z) of product ions				Suggested elemental composition of product ion	Theoretical exact mass of suggested formula
RR- <i>p</i> -CAP	SS- <i>p</i> -CAP	RR- <i>m</i> -CAP / SS- <i>m</i> -CAP	RS- <i>m</i> -CAP / SR- <i>m</i> -CAP		
257.0351	257.0333	257.0329	257.0339	C ₁₀ ClH ₁₀ N ₂ O ₄	257.0329
		249.0512	249.0513	C ₁₁ H ₉ N ₂ O ₅	249.0511
		237.0511	237.0512	C ₁₀ H ₉ N ₂ O ₅	237.0511
			221.0567	C ₁₀ H ₉ N ₂ O ₄	221.0562
		207.0406	207.0440	C ₉ H ₇ N ₂ O ₄	207.0406
194.0469	194.0456	194.0452		C ₉ H ₈ NO ₄	194.0453
		179.0456	179.0458	C ₉ H ₉ NO ₃	179.0457
176.0362	176.0356	176.0350		C ₉ H ₆ NO ₃	176.0348
152.0367	152.0402			C ₇ H ₆ NO ₃	152.0348

The differences in fragmentation pattern might be explained by the three-dimensional configuration of the CAP isomers. The three-dimensional configuration of CAP in solution was extensively studied by X-ray [38], nuclear magnetic resonance (NMR) [39,40] and infra-red spectroscopy [40,41]. It is reported that RR-*p*-CAP only occurs in one configuration (figure 4.5a) and that the formation of intra-molecular hydrogen bonding between the hydroxyl and the methylhydroxyl group occurs [38,39,41]. Furthermore a stabilization of the presented configuration was contributed to the dipolar interaction between the carbonyl oxygen and the nitroaromatic ring [40,41]. RS- and SR-*p*-CAP also form hydrogen bonds and are mirror images. In that research the interaction of the nitrophenyl moiety and the carbonyl oxygen was not discussed. No information is available on the meta-CAP isomers.

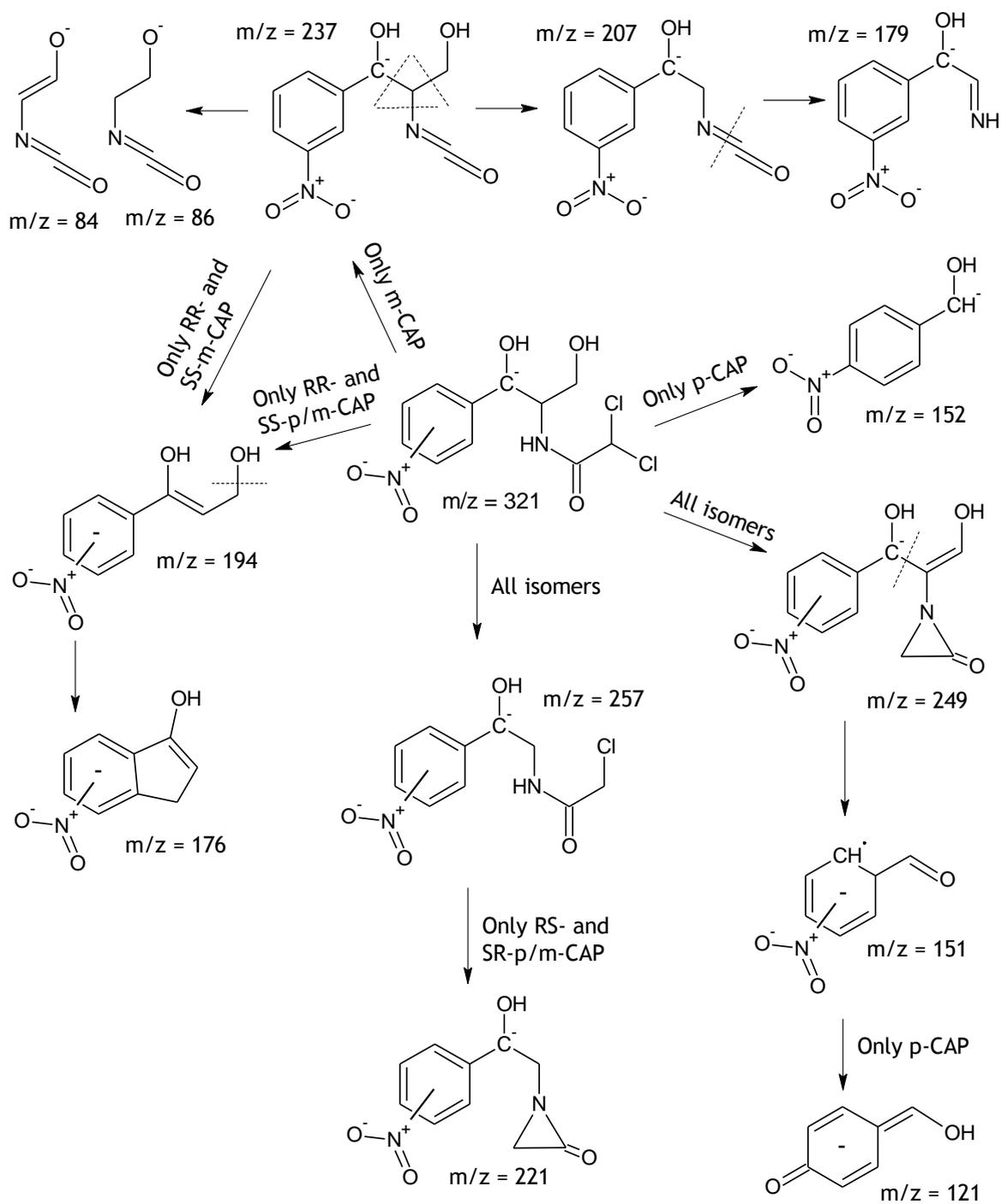


Figure 4.4. Proposed fragmentation pathways for the CAP isomers.

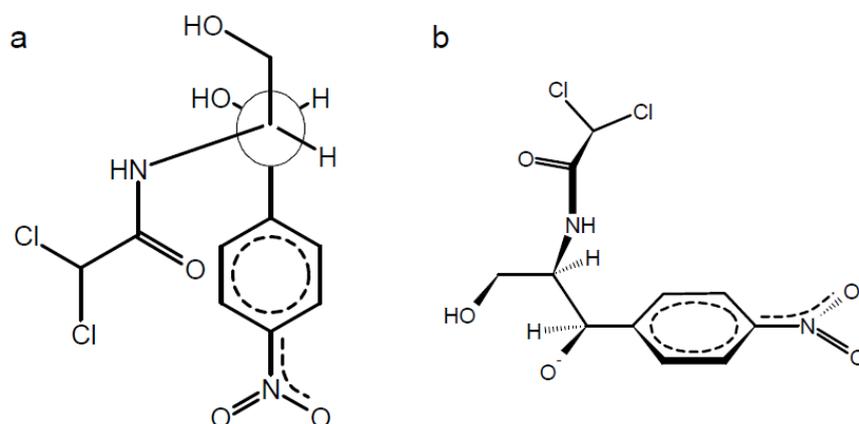


Figure 4.5. Configuration of (a) RR-p-CAP in solvent [41] and (b) the [M-H]⁻ ion of RR-p-CAP in the gas phase.

It should be noted that all reported research so far exclusively focused on the three-dimensional characterization of CAP in solvent whereas this study focused on the fragmentation of the [M-H]⁻ ions of the chloramphenicol isomers in the gas phase under vacuum. Therefore, 3D configurations of all isomers were determined using BioChem3D 12.0 Ultra software (CambridgeSoft, Cambridge, MA, USA). The lowest energetic state of the [M-H]⁻ ion of RR-p-CAP in the gas phase was the benzylic anion having a configuration as presented in figure 4.5b. All isomers show comparable three-dimensional configurations indicating that all isomers form intra-molecular hydrogen bridges between the hydroxyl and hydroxymethyl group. Interaction between the carboxyl and the phenyl group is unlikely. For the RR- and SS-isomers it is observed that the amide nitrogen is on the same plane and within a 3Å distance of the hydroxyl and hydroxymethyl group indicating that it could be part of an intra-molecular hydrogen bridging system, which might influence the fragmentation pathways of these isomers. The presence of the product ion $m/z=152$ for the *para*-CAP isomers and the absence of this product ion for the *meta*-CAP isomers is most likely caused by mesomeric stabilization of the benzylic anion which only occurs when the nitro group is in the *para* position.

Discrimination of CAP isomers by tandem mass spectrometry

Product ion mass spectra of all CAP isomers were recorded at different collision energies. The product ions observed for RR-*p*-CAP are in agreement with previous observations [26,32,33]. From the fragmentation spectra of all isomers obtained during continuous infusion at a collision energy ranging from 0 through 35 eV, product ions that are at least 5 % of the base peak intensity are graphically presented in figure 4.6.

The first important observation from the product ion spectra shown in figure 4.6 is that no discrimination can be made between RR- and SS-*p*-CAP. The configuration of para-CAP isomers is the mirror image of SS-*p*-CAP [39] and thus the same fragmentation pattern is expected. RS- and SR-*p*-CAP are also mirror images of each other [39] as is the case for RR- and SS-*m*-CAP and RS- and SR-*m*-CAP. Although these isomers were only fragmented as a mixture, from this it is expected that the pairs of enantiomers cannot be distinguished by mass spectrometric detection only.

The second important observation from the product ion spectra shown in figure 4.6 is that the para- and meta-isomers dissociate in a different way. Especially a high abundant product ion $m/z=152$ and product ion $m/z=121$ are specific for para-CAP stereoisomers whereas the product ions at $m/z=237$, 207, 179, 86 and 84 which all origin from a fragmentation pathway via the product ion $m/z=237$, are specific for the meta-CAP isomers. From the product ion mass spectra it is concluded that para- and meta-isomers can easily be distinguished by MS/MS detection only.

A third important observation is that different product ion spectra are obtained for the RR- and SS-isomers compared to the RS- and SR-isomers. The product ions $m/z=194$ and 176, originating from a single fragmentation pathway, are solely observed for the mixture of RR- and SS-CAP of both the para- and meta-isomers. The product ion $m/z=221$ (observed as $m/z=226$ for the mixture of RS- and SR-*p*-CAP- d_5) is only observed for RS- and SR-CAP of both the para- and meta-isomers. From this it is concluded that RR- and SS-CAP can be distinguished from RS- and SR-CAP for both the para- and meta-isomers by MS/MS detection only.

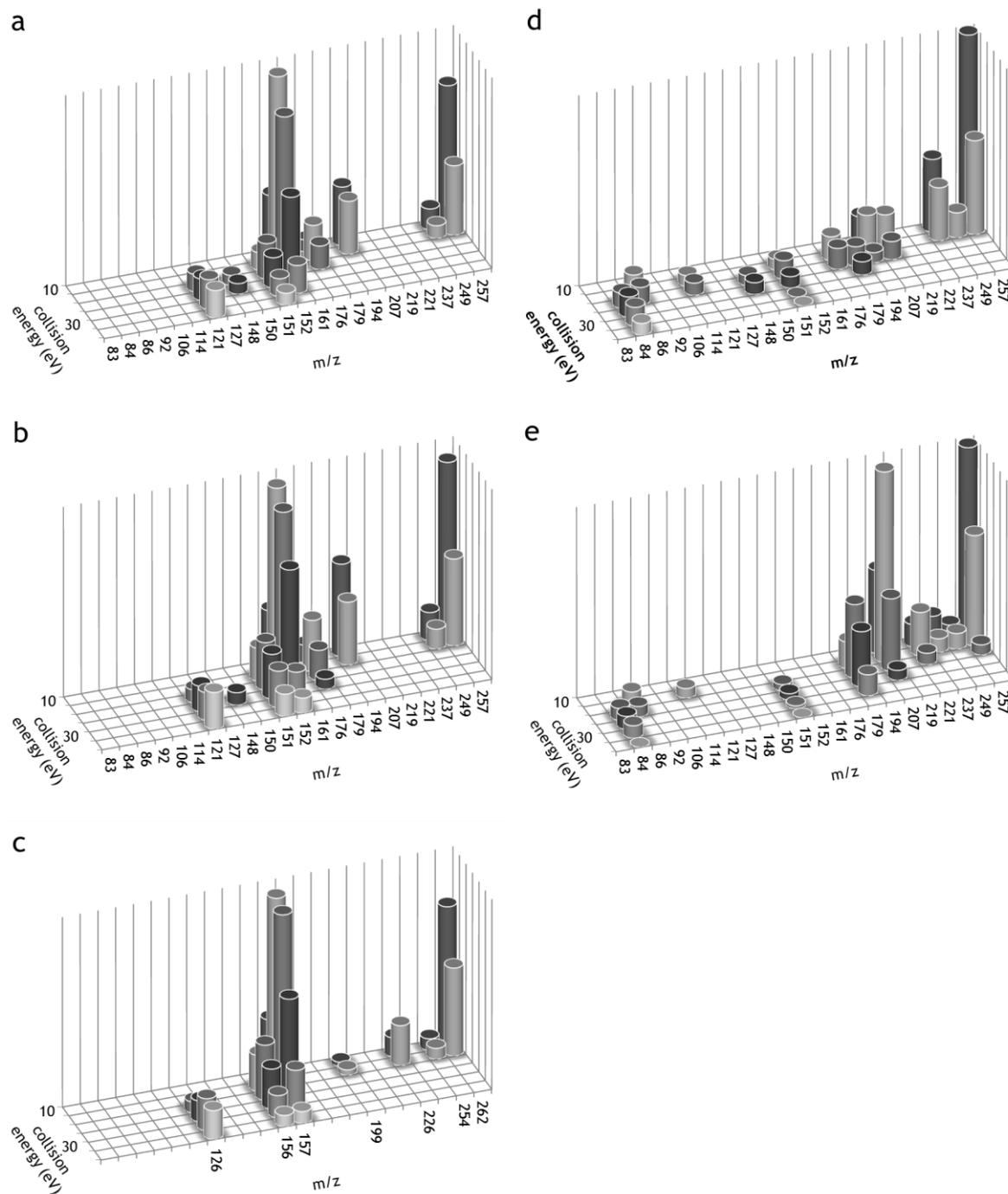


Figure 4.6. Intensity of different product ions recorded at different collision energies for the CAP isomers: (a) RR-p-CAP, (b) SS-p-CAP (c) a mixture of SR- and RS-p-CAP-d₅, (d) a mixture of RR- and SS-meta-CAP and (e) a mixture of SR- and RS-m-CAP.

From these observations it is concluded that the pure form of all isomers, except for the mirror images, can be distinguished by MS/MS detection. The combination of product ions $m/z=194$ and 152 are specific for RR- and SS-*p*-CAP, $m/z=221$ and 152 for RS- and SR-*p*-CAP, $m/z=237$ and 194 for RR- and SS-*m*-CAP and $m/z=207$ and 221 for RS- and SR-*m*-CAP. Of course this will not be the case if several isomers are present together in a mixture.

From the product ion mass spectra RR-*p*-CAP and SS-*p*-CAP (DEX) seem to have a comparable fragmentation pattern. However, using continuous infusion the ionisation conditions (flow and eluent composition) differ from the ionisation conditions during LC-MS/MS. To test the possibility to distinguish RR-*p*-CAP from SS-*p*-CAP based upon the ion ratio concept in relation to Commission Decision 2002/657/EC [9], the product ions of RR-*p*-CAP and SS-*p*-CAP showing a relatively high intensity difference in the continuous infusion experiment were selected being: $m/z=121.0$ (20 eV), $m/z=151.0$ (15 and 30 eV), $m/z=152.0$ (15 and 25 eV), $m/z=176.0$ (25 eV), $m/z=194.0$ (10 eV) and $m/z=257.1$ (10 eV). These product ions were monitored for injections ($n=8$) of RR-*p*-CAP and SS-*p*-CAP into the LC-MS/MS system and all resulting ion ratios were calculated ($n=21$). The difference of the ion ratios of RR-*p*-CAP and SS-*p*-CAP were calculated and compared using a Students t-test ($\alpha=0.05$). No significant ion ratio deviations between RR-*p*-CAP and SS-*p*-CAP were found. From this it is concluded that the pure RR- and SS-*p*-CAP cannot be distinguished using MS/MS detection alone.

Reversed phase-LC-MS/MS

Because the applied RP-LC method makes use of an analytical column containing $1.7 \mu\text{m}$ particles in combination with gradient elution, it is considered a high resolution LC-system. From the LC-methods reported in literature [23,24,26,30-33] it is most likely that this method results in the highest resolution and as a consequence in the best isomeric separation. Chromatograms of a mixture of all *para*-stereoisomers, a mixture of all *meta*-stereoisomers and a mixture of all isomers, injected on the high resolution RP-LC-MS/MS system are presented in figure 4.7. Only two peaks are observed in the chromatogram of the mixture of all isomers: the RS- and SR-isomers of both *para*- and *meta*-CAP elute at a retention time (RT) of 2.60 min and are separated from the RR- and SS-isomers which elute at 2.71 min. As stated RR- and SS-isomers are expected to form an

intra-molecular hydrogen bridging system whereas the RS- and SR-isomers do this to less extent. Therefore the polar groups in the RS- and SR-isomers are able to interact more with the polar mobile phase and therefore they elute from the RP column first. The RR- and SS- as well as the RS- and SR-configurations of both *para*- and *meta*-CAP co-elute using this system. It is expected that the RR- and SS- as well as the RS- and SR-isomers cannot be separated using RP-LC because these isomeric pairs are mirror images to which RP-LC is non-selective. Further optimization was carried out attempting to separate the *para*- from the *meta*-isomers by changing the steepness of the gradient and lowering the injection volume (5 instead of 35 μL). These experiments did not result in a visible separation of the *para*- and *meta*-isomers.

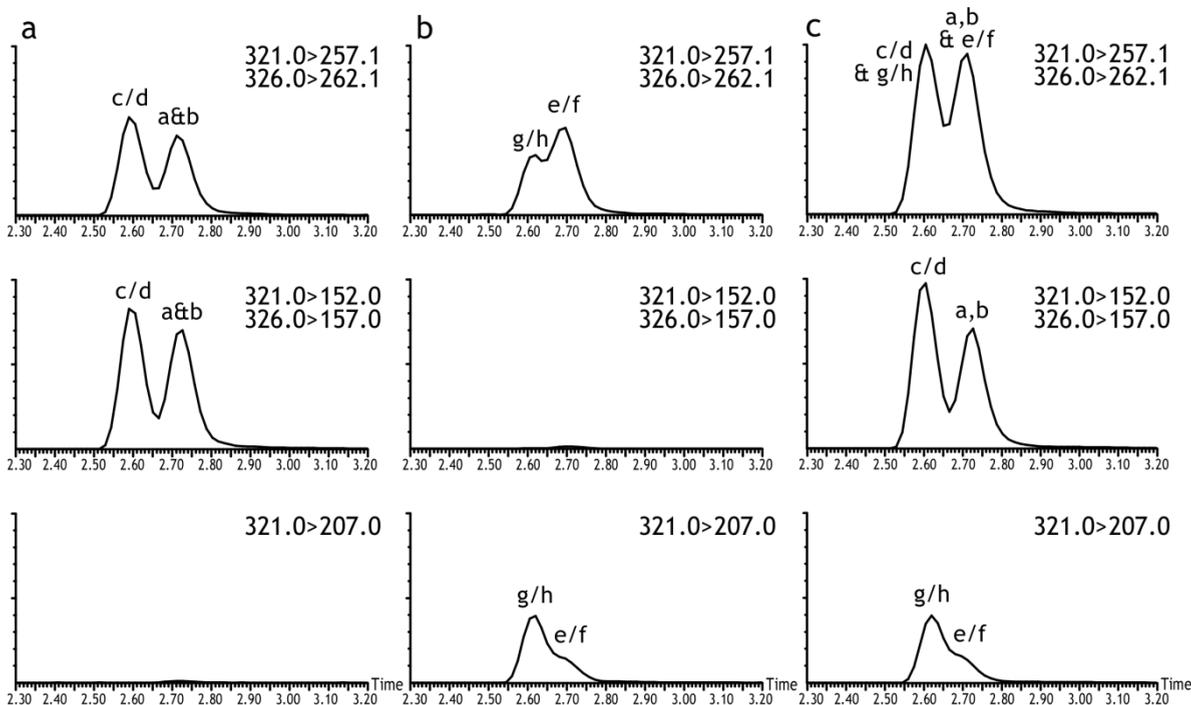


Figure 4.7. High resolution RP-LC chromatograms showing three MS transitions of (a) a mixture of all *para*-stereoisomers, (b) a mixture of all *meta*-stereoisomers and (c) a mixture of all isomers. For the *para*-isomers the transitions of the non-deuterated and deuterated stereoisomers are combined in one chromatogram simulating the use of non deuterated reference standard for all isomers. a = RR-*p*-CAP, b = SS-*p*-CAP, c = RS-*p*-CAP- d_5 , d = SR-*p*-CAP- d_5 , e = RR-*m*-CAP, f = SS-*m*-CAP, g = RS-*m*-CAP and h = SR-*m*-CAP.

Although no chromatographic separation is observed for the *para*- and *meta*-configuration, a mass spectrometric separation based on monitoring different product ions is obtained (cf. MS/MS experiments). From the results it is concluded that high resolution RP-LC-MS/MS method is not able to separate RR- and SS-stereoisomers nor RS- and SR-stereoisomers.

Chiral LC-MS/MS

Several mobile phases were tested to obtain a satisfactory separation of all isomers. The use of methanol, ethanol, acetonitrile and mixtures of methanol and acetonitrile as the mobile phase resulted in a somewhat different selectivity. However, for all mobile phases isocratic elution resulted in co-elution of two *para*- or two *meta*-isomers and in most cases in very broad peaks (> 1 min) for the late eluting isomers. A two-step isocratic elution was introduced running at 9 % methanol/acetonitrile (1:1, v/v) in water for 4 min to obtain satisfactory separation of the *para*-isomers followed by a linear increase of the percentage of organic modifier to 12 % during 0.1 minute with a final hold of 4.9 minutes to speed up the elution of the retained isomers. This system resulted in the chromatographic separation of all eight isomers within eight minutes (figure 4.8).

MS/MS detection provided additional resolution to the system because the *para*-isomers can easily be separated from the *meta*-isomers by monitoring different product ions. The *meta*-isomers are almost baseline separated but the *para*-isomers are not: SS-*p*-CAP (DEX) with RT=5.57 min shows some overlap with RS- or RS-*p*-CAP eluting at RT=5.02 min. However, the two peaks are clearly distinguishable based upon RT. Most important is that RR-*p*-CAP, the active configuration, eluting at RT=3.90 is baseline separated from all other isomers. It is concluded that all eight isomers can be distinguished using the chiral LC-MS/MS system presented here.

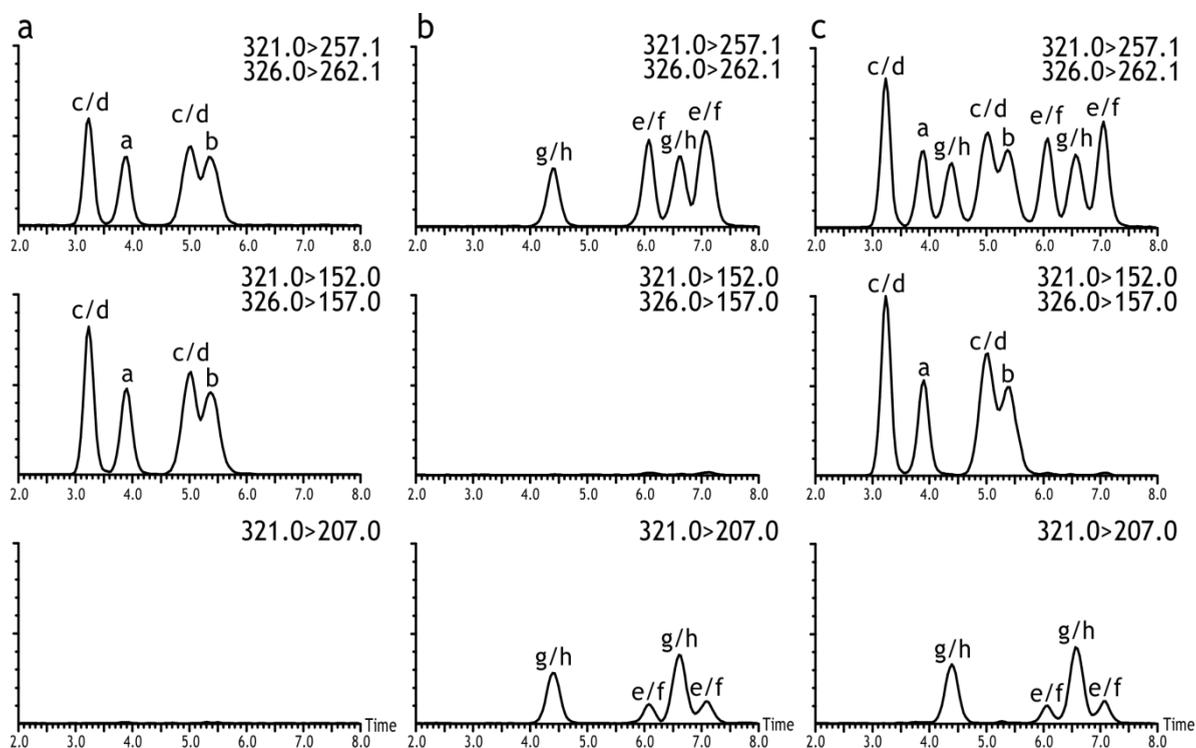


Figure 4.8. Chiral LC chromatograms showing three MS transitions of (a) a mixture of four *para*-stereoisomers (b) a mixture of four *meta*-stereoisomers and (c) a mixture of all isomers. The configuration of the isomers is indicated. A = *RR-p*-CAP, b = *SS-p*-CAP, c = *RS-p*-CAP-*d*₅, d = *SR-p*-CAP-*d*₅, e = *RR-m*-CAP, f = *SS-m*-CAP, g = *RS-m*-CAP and h = *SR-m*-CAP.

Sample analysis

Using the developed chiral LC-MS/MS system the isomeric ratio of different kind of samples was determined. If isomeric impurities are observed, the isomeric ratio of the CAP isomers in plant materials and CAP formulations can be compared to trace the origin of the contamination. The limit of quantitation of the individual isomers in plant material using the chiral LC-MS/MS system is relatively high: 2 - 5 $\mu\text{g kg}^{-1}$ instead of 0.5 $\mu\text{g kg}^{-1}$ for the high resolution RP-LC-MS/MS system. Therefore, isomeric impurities can only be detected for highly contaminated samples.

The chromatograms of a blank plant sample spiked at 100 $\mu\text{g kg}^{-1}$ with all CAP isomers and of plant sample S3 are presented in figure 4.9a and b. From the chromatograms it is clear that the plant material sample contains *RR-p*-CAP, the active configuration. Especially in sample S3 small signals are observed in the MS

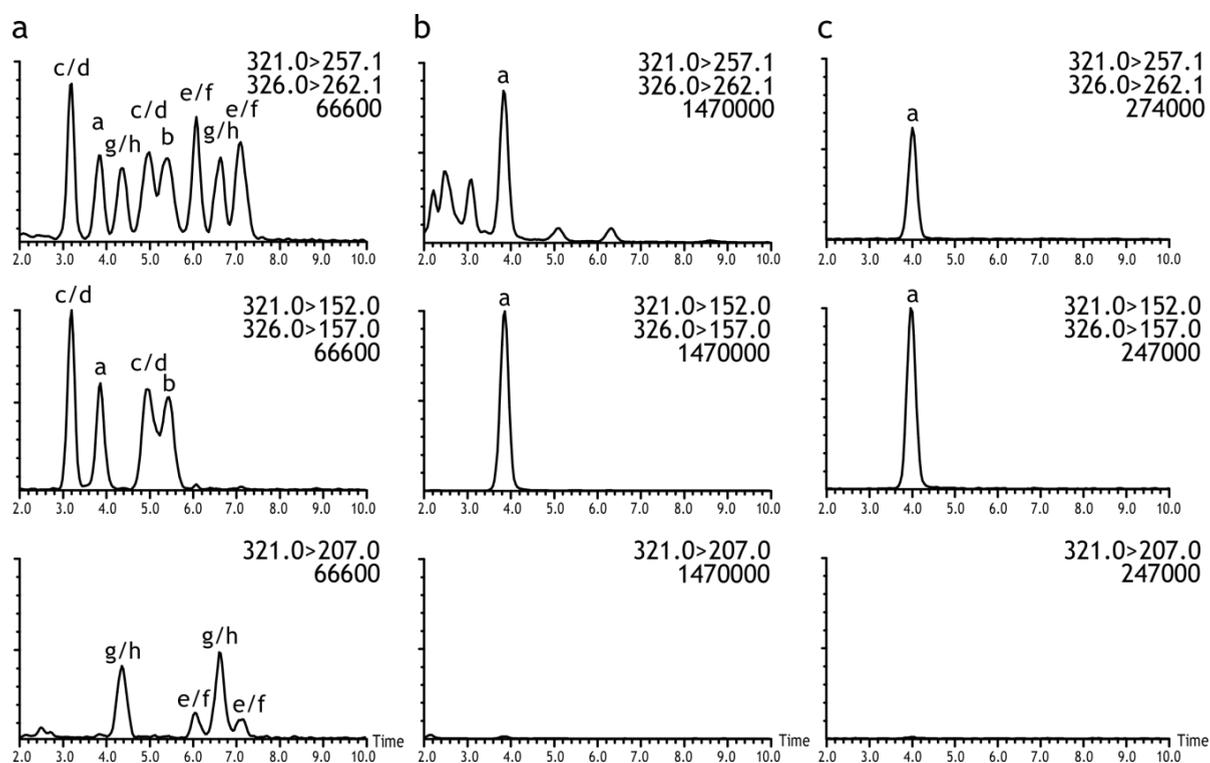


Figure 4.9. Chiral LC chromatograms showing three MS transitions of (a) a blank plant material sample spiked at $100 \mu\text{g kg}^{-1}$ with a mixture of all isomers (b) sample S3 and (c) formulation 1. A = RR-*p*-CAP, b = SS-*p*-CAP, c = RS-*p*-CAP- d_5 , d = SR-*p*-CAP- d_5 , e = RR-*m*-CAP, f = SS-*m*-CAP, g = RS-*m*-CAP and h = SR-*m*-CAP.

transition $m/z = 321.0 > 257.1$ at RT = 3.09, 5.09 and 6.32 min. The RT of these peaks does not correspond to the RT of any of the isomers and furthermore no peaks are observed for the other, more specific transitions. It is concluded that these signals do not originate from CAP isomers and that only the active configuration of CAP was detected in the plant material. This accounts for all tested plant materials.

The analysis of the two seized formulations show very high peaks for RR-*p*-CAP; the chromatogram of the first formulation is presented in figure 4.9c. No isomeric impurities were detected in these formulations. Only RR-*p*-CAP was detected in the tested plant materials and formulations and therefore the origin of the contamination cannot be traced: the CAP present in the plant material can originate from the production by soil organisms or from a manufactured source (illegal use).

Conclusions

In this section it is demonstrated that high resolution RP-LC is suitable to discriminate RR- and SS-stereoisomers from RS- and SR-stereoisomers. Additionally MS/MS detection was found to discriminate *meta*- from *para*-substituted isomers. However, RP-LC methods are unable to discriminate between the mirror images (e.g. the antimicrobial active RR-*p*-CAP and SS-*p*-CAP), neither by RP-LC separation nor by MS/MS detection. Because most LC methods described in literature are based upon RP-LC it is concluded that generally applied methods for the confirmatory analysis of CAP are not specific enough and are thus not suited for the control of food products. Alternative methods, like chiral LC have to be used to specifically detect the administration of the antimicrobial active isomer of CAP.

A chiral LC-MS/MS system was developed which proved to be able to baseline separate RR-*p*-CAP from the other isomers. This system was applied to analyse CAP-containing plant material samples obtained from Mongolian pastures and some CAP formulations. This showed that Mongolian grass and herb samples as well as the formulations only contain the antimicrobial active isomer of CAP. Therefore the CAP present in the plant material can originate from a manufactured source indicating illegal use or from the production by soil organisms (natural occurrence). Additional research on the uptake and accumulation of CAP isomers by plants and herbs, and research on the detection of the presence of CAP producing soil organisms is needed to further elucidate this matter.

Acknowledgements

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4.4. Quantitative trace analysis of eight chloramphenicol isomers in urine by chiral liquid chromatography coupled to tandem mass spectrometry

Abstract

Chloramphenicol is a broad-spectrum antibiotic with, apart from its human medicinal use, veterinary abuse in all major food-producing animals. Chloramphenicol occurs in four stereoisomers (all para-nitro substituted) and furthermore four meta-nitro analogs of chloramphenicol exist. Here, these are referred to as eight chloramphenicol isomers. According to EU regulations an analytical method should be able to discriminate the analyte from interfering substances that might be present in the sample, including isomers. For the first time a quantitative method for the analysis of trace levels of eight chloramphenicol isomers in urine by chiral liquid chromatography in combination with tandem mass spectrometric detection is reported. The separation of the isomers on the analytical column, the clean-up of urine and the selectivity of the monitored product ions turned out to be critical parameters. To obtain reproducible retention isocratic elution on a chiral AGP column was applied. For urine samples matrix compounds present in the final extract caused decreased retention of the isomers on the chiral stationary phase and a lack of chromatographic resolution. Therefore an extended clean-up procedure that combines solid phase extraction and liquid-liquid extraction had to be developed. The final method was fully validated and showed satisfactory performance for all isomers with decision limits ($CC\alpha$) ranging from 0.005 to 0.03 $\mu\text{g L}^{-1}$ and within-laboratory reproducibility of all isomers below 20 % at the minimum required performance limit level of 0.3 $\mu\text{g L}^{-1}$.

Introduction

Chloramphenicol (CAP; 2,2-dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide) is a broad-spectrum antibiotic used as a human medicine but also for veterinary use in all major food-producing animals. However, chloramphenicol is banned for use in all food producing animals and a minimum required performance limit (MRPL) of $0.3 \mu\text{g kg}^{-1}$ has been established [6].

CAP is a para-nitro substituted compound that contains two chiral centers (carbon 2 and 3, figure 4.1a) thus four stereoisomers of CAP exist. Furthermore, four meta-nitro substituted analogs exist resulting in a total of eight different isomeric configurations (figure 4.1), which are all referred to as CAP isomers in this thesis. If not explicitly mentioned, the name chloramphenicol generally refers to RR-*p*-CAP, which is also called levomycetin. SS-*p*-CAP is also called dexamycin (DEX) and a racemic mixture of the two isomers is called synthomycin [10]. All para-stereoisomers are biologically active [10-19] but it was reported that only RR-*p*-CAP has antimicrobial properties [10,14]. In contrast, other papers claim < 0.5 % antimicrobial activity for SS-*p*-CAP [15,16] and RS-*p*-CAP [15] compared to RR-*p*-CAP and 1 to 2 % for SR-*p*-CAP [15]. According to literature the structure of the propanediol moiety is critical for the microbial activity whereas the aryl nitro group and the acetamide side chain are not that essential [17]. No information is available on the meta-analogs.

Criteria concerning the performance of analytical methods and the interpretation of results were established in 2002 [9]. According to this document samples taken for monitoring of residues in animal products should be analysed using methods that have been validated according to the described procedures [9]. In these procedures selectivity is mentioned as a main characteristic of an analytical method. Selectivity is defined as “the power of discrimination between the analyte and closely related substances like isomers (...)”. Only using an enantioselective method, the identity of CAP can be confirmed without any doubt, which is a necessity in case the analytical result is challenged in court cases.

For the quantitation and confirmation of CAP mainly reversed phase (RP) LC-MS/MS has been used. RP-LC methods make use of either a C₈ [31,32] or a C₁₈ [23,24,26,30,33] stationary phase combined with isocratic [24,31,32] or gradient [23,26,30,33] elution. Superior resolution is obtained using an analytical column containing sub 2 µm particles [23,30] in combination with gradient elution. However, recently the lack of selectivity for enantiomers of these methods was demonstrated [42] (section 4.3).

The analysis of isomeric drugs using chiral stationary phase LC-MS/MS is an emerging field. Several methods for the enantioselective analysis of drugs in fungi culture medium [43], waste water [44], saliva [45], plasma [46-51], urine [47,48] and hair [48] have been reported during the last decade making use of a great variety of analytical columns: immobilised α1-acid glycoprotein (AGP) [45,46,49,51], chiral cellobiohydrolase (CBH) [44], chiral phenylcarbamate β-cyclodextrin (CD-Ph) [48], (R)-1-naphthylglycine and 3,5-dinitrobenzoic acid amide linkage [47], teicoplanin [50] or amylose-based analytical columns [43].

Only a few papers are on the use of LC to separate CAP isomers. The separation of four *para*-CAP isomers in solvent by RP-LC using pre-column derivatization using homochiral isothiocyanate [34] and the addition of cyclodextrin to the mobile phase [35] was reported. Both approaches did not result in baseline separation of the stereoisomers and are unfavorable because derivatization and complex formation is usually less robust than direct analysis [52]. Chiral LC using 4-(3,5-dinitro benzamido)tetrahydrophenanthrene covalently bound to silica as the stationary phase was applied to obtain a baseline separation for *RR-p*- and *SS-p*-CAP in formulations [36] and also for the separation of *RR-p*- and *SS-p*-florfenicol, a compound chemically related to CAP, in formulations [37]. Previously a method was reported combining chiral LC using an AGP column material in combination with a two phase isocratic system, with mass spectrometry that is able to discriminate among all eight isomers, but that method lacked sensitivity for regulatory control of animal products at the MRPL level of 0.3 µg L⁻¹ [42] (section 4.3).

Although chiral separations have found their use in clinical and pharmaceutical research [52], to our knowledge no enantioselective LC-MS/MS methods have been reported having their application at sup-ppb levels as required for veterinary drug residue analysis in products of animal origin. The presented

method is suited for quantitative analysis of eight CAP isomers in urine and is able to discriminate *RR-p*-CAP from the other isomers. It is therefore the first method reported that is suited to unequivocally confirm the identity of the individual CAP isomers according to EU regulations [9] at part-per-trillion levels, well below the MRPL.

Experimental

Reagents and equipment

HPLC grade water, methanol (MeOH), acetonitrile (ACN) and ethyl acetate were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium formate, acetic acid, formic acid, 25 % ammonia, sodium hydroxide and β -glucuronidase/arylsulfatase from helix pomatia were obtained from Merck (Darmstadt, Germany). Milli-Q water was prepared using a Milli-Q system at a resistivity of at least $18.2 \text{ M}\Omega \text{ cm}^{-1}$ (Millipore, Billerica, MA, USA). The reference standard of *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, a mixture of *RS-m*-CAP and *SR-m*-CAP and a mixture of *RS-p*-CAP- d_5 and *SR-p*-CAP- d_5 and a mixture of *RR-p*-CAP- d_5 and *SS-p*-CAP- d_5 were obtained from Witega (Berlin, Germany). The deuterium atoms were substituted at the phenyl ring and at carbon 3 (figure 4.1, page 116). Non deuterated *RS*- and *SR-p*-CAP were not commercially available and thus were the deuterated compounds used as a representative reference. *RR*- and *SS-p*-CAP- d_5 were used as the internal standards. Stock solutions were prepared in MeOH at 100 mg L^{-1} and all dilutions were prepared fresh daily in Milli-Q water.

Sample preparation

5 mL of a urine sample was transferred into a polypropylene centrifuge tube. The pH was adjusted to 4.8 using dilutions of acetic acid and sodium hydroxide. 50 μL β -glucuronidase/arylsulfatase were added and the sample was incubated for 16 hours at 37°C or for 2 hours at 55°C . A Phenomenex (Torrance, CA, USA) Strata-X 200 mg / 6 mL solid phase extraction (SPE) cartridge was conditioned with 5 mL MeOH and 5 mL water. The hydrolysed urine sample was applied onto the cartridge and washed with 6 mL 40 % MeOH in water containing 1 % acetic acid

followed by 6 mL 40 % MeOH containing 1 % ammonia (25 %). The cartridges were dried by applying vacuum for 3 minutes. The chloramphenicol isomers were eluted from the cartridge using 3 mL MeOH/water (80:20 v/v) followed by evaporation of the MeOH under a gentle nitrogen stream at 45 °C. 2 mL of ethyl acetate was added to the remaining aqueous extract which was shaken for 5 minutes using a rotary tumbler. After centrifugation (3500 g, 5 min) the ethyl acetate layer was isolated and evaporated under a gentle nitrogen stream at 40 °C until dry. The residue was redissolved in 500 µL water and transferred into an LC-MS/MS sample vial.

LC-MS/MS analysis

The LC system consisted of a vacuum degasser, autosampler, and a Waters (Milford, MA, USA) model Acquity binary pump equipped with a Chromtech (Apple Valley, MN, USA) Chiral AGP (α 1-acid glycoprotein) analytical column of 2.0 × 150 mm, 5 µm placed in a column oven at 30 °C. Isocratic elution was performed using a mobile phase consisting of 2 % ACN in 10 mM ammonium formate buffer adjusted to pH=4.0 with formic acid at a flow rate of 0.4 mL min⁻¹. The injection volume was 10 µL.

Detection was carried out using a Waters model Xevo TQS mass spectrometer in the negative electrospray ionisation (ESI) mode. The operating parameters were: capillary voltage, -0.5 kV; cone voltage, 20 V; source offset, 50 V; source temperature, 150 °C; desolvation temperature, 550 °C; cone gas flow, 150 L h⁻¹; and desolvation gas, 750 L h⁻¹. The CAP isomers were fragmented using collision induced dissociation and the selected reaction monitoring (SRM) transitions are given in table 4.7. As reported before [42], each product-ion is specific for a selected number of CAP isomers. For instance, product ion $m/z=257$ is observed for all isomers, $m/z=152$ only for para-isomers, $m/z=207$ only for meta-isomers and $m/z=194$ only for *RR/SS*-isomers. Therefore, the monitored product ions were carefully selected to obtain adequate isomeric selectivity in combination with high sensitivity. An overview of the fragmentation pattern for each of the isomers including elemental compositions of the product ions was reported before [42].

Because only racemic mixtures of some isomers are available some chromatographically separated peaks could not be specifically assigned to one

isomer. Therefore, they are referred to as 1st and 2nd peak. Data were acquired and processed using MassLynx 4.1 software (Waters). *RR-p-CAP-d₅* was used as the internal standard for *RR-p-CAP* and *SS-p-CAP-d₅* was used as the internal standard for all other isomers.

Method validation

A full validation was carried out according to 2002/657/EC [9]. The following parameters were determined: linearity, trueness, repeatability, within-laboratory reproducibility, decision limit (CC α), detection capability (CC β), selectivity, robustness and stability. The validation was carried out on three different occasions, by two different technicians and including 21 different bovine urine samples.

Table 4.7. SRM transitions of the CAP isomers.

Code (fig. 4.1)	Compound	Precursor Ion (m/z)	Product ion (m/z) ^a	Collision Energy (eV)
a	RR- <i>p</i> -CAP	321.0	152.0	17
			194.0	10
b	SS- <i>p</i> -CAP	321.0	152.0	17
			121.0	28
c	RS/SR- <i>p</i> -CAP-d ₅ 1 st	326.0	157.0	17
			262.0	12
d	RS/SR- <i>p</i> -CAP-d ₅ 2 nd	326.0	157.0	17
			262.0	12
e	RR/SS- <i>m</i> -CAP 1 st	321.0	207.0	14
			86.0	15
f	RR/SS- <i>m</i> -CAP 2 nd	321.0	257.0	12
			207.0	14
g	RS/SR- <i>m</i> -CAP 1 st	321.0	257.0	12
			207.0	14
h	RS/SR- <i>m</i> -CAP 2 nd	321.0	257.0	12
			207.0	14
j	RR- <i>p</i> -CAP-d ₅	326.0	199.0	10
k	SS- <i>p</i> -CAP-d ₅	326.0	199.0	10

^a The most abundant product ion is mentioned first.

Linearity

On three different days a matrix matched calibration line was prepared including the following calibration levels: 0, 0.1, 0.2, 0.3, 0.4 and 0.5 $\mu\text{g L}^{-1}$, (six data points including zero) by adding solutions of the CAP isomers to aliquots of a blank urine sample. Calibration lines were constructed by plotting the peak areas, normalised using the peak areas of the internal standards, versus the added concentration and carrying out least squares linear regression. The linearity was considered acceptable if the coefficient of correlation was at least 0.990.

Trueness, repeatability and within-lab reproducibility

On each of the three days seven different blank urine samples were selected and analysed as such and spiked at 0.15, 0.3 and 0.45 $\mu\text{g L}^{-1}$. For each sample the level of the CAP isomers was calculated using the calibration line constructed on the same day. The trueness was calculated by dividing the average calculated level by the nominal concentration. According to 2002/657/EC the trueness at the selected validation levels should be between 50 and 120 %.

The repeatability and within-lab reproducibility were calculated using single factor analysis of variance (ANOVA). According to 2002/657/EC [9] the relative standard deviation under within-lab reproducibility conditions (RSD_{RL}) and the relative standard deviation under repeatability conditions (RSD_{r}) are considered acceptable if they are below the value calculated from the Horwitz equation [53] respectively two thirds of this value, i.e. below 53.6 % respectively 35.8 % at a level of 0.3 $\mu\text{g L}^{-1}$. Thompson [54] however, demonstrated that the Horwitz equation is not applicable to the lower concentration range ($< 120 \mu\text{g kg}^{-1}$) and suggested a complementary model. Based on this model the RSD_{RL} and RSD_{r} are acceptable if they are below 22.0 % respectively 14.7 %. The latter, more stringent criteria were adopted for this validation study. It is stated that because different urine samples were used within one day, the repeatability is not the pure repeatability as stated in 2002/657/EC [9]. However, using different urine samples a better understanding of the between sample variation is obtained and the validation better reflects a routine analysis situation.

Decision limit and detection capability

According to 2005/34/EC [7] for all products entering the EU that are analysed for veterinary drugs and thus are within the scope of 97/78/EC [8], the MRPL as stated in 2002/657/EC [9] should be regarded as a reference point of action. In other words, for banned substances, only concentration levels above the MRPL are considered being non-compliant results. However, if the result of an analytical test is below the MRPL “the competent authority has to retain a record of the findings in case of recurrence” [7]. Therefore, two values for the decision limit ($CC\alpha$) are needed: one based on the MRPL being the reference point of action [7] to assign samples that contain CAP with a level exceeding the MRPL (α -error is 5 %) and one based on zero tolerance in accordance with 2002/657/EC [9] to assign samples that contain CAP and thus deviate from the blank population (α -error is 1 %). The latter value of $CC\alpha$ indicates the lowest level at which detection and confirmation of CAP is possible.

The $CC\alpha$ and $CC\beta$ based on the MRPL being the reference point of action were calculated according to the procedure described in 2002/657/EC [9] for MRL substances based upon spiked samples at the MRPL level of $0.3 \mu\text{g L}^{-1}$ ($n=21$). Because $CC\alpha$ and $CC\beta$ are calculated from the within-laboratory reproducibility, no specific criteria are set for these parameters.

The $CC\alpha$ and $CC\beta$ based on zero tolerance were calculated based on the band width of the signal in blank samples and the height of samples spiked at $0.15 \mu\text{g L}^{-1}$ at the time window in which the analyte is expected based upon in total 21 individual blank and spiked samples analysed on three different occasions, according to calculations suggested by Antignac *et al.* [55]. According to guidelines on the implementation of 2002/657/EC [56] values for $CC\beta$ should be below or equal to the MRPL and thus the criterion $CC\beta \leq 0.3 \mu\text{g L}^{-1}$ was adopted for this validation.

Selectivity

The selectivity of the method was studied based on a practical and a theoretical study. First, the 21 analysed blank samples were checked for interferences at the retention times corresponding to the CAP isomers. Second, databases were searched for compounds having a molecular mass equal to CAP that could

possibly be present in urine samples. A standard solution of $10 \mu\text{g L}^{-1}$ and a blank urine sample spiked with $100 \mu\text{g L}^{-1}$ of α -zeranol, indicated as a possibly interfering compound, were analysed using the developed method. The resulting chromatograms were checked for interferences at the retention times corresponding to the CAP isomers. Furthermore a blank urine sample spiked with both $0.3 \mu\text{g L}^{-1}$ CAP isomers and $100 \mu\text{g L}^{-1}$ α -zeranol was analysed to check for false negative CAP results due to the presence of interfering compounds.

Robustness

The robustness of the method became clear during the optimisation of the sample preparation, chromatographic conditions and MS parameters. Additionally some minor changes to the method were tested in duplicate being: (1) evaporation of the eluent until only 200 μL of water remained after which ethyl acetate was added, (2) evaporation of the eluent until some methanol was still present after which ethyl acetate was added and (3) evaporation of the ethyl acetate fraction until dryness plus an additional 10 minutes. The trueness and repeatability of these tests were compared to the characteristics of the regular method.

Stability

The stability of chloramphenicol isomers was tested for $100 \mu\text{g mL}^{-1}$ stock solutions and for urine extracts. Individual methanolic solutions of $100 \mu\text{g mL}^{-1}$ of RR-*p*-CAP and SS-*p*-CAP, and mixtures of RS- and SR-*p*-CAP- d_5 , RR- and SS-*m*-CAP, and RS- and SR-*m*-CAP were placed at 4°C and room temperature. After 5 days, these solutions were diluted to $100 \mu\text{g L}^{-1}$ with water and analysed in duplicate.

The stability of urine extracts was determined by storing urine extracts at -20°C after analysis. After 7 days, the calibrants and the samples spiked at $0.3 \mu\text{g L}^{-1}$ ($n = 7$) were defrosted and injected in the LC-MS/MS system again. The linearity and the average and standard deviation of the spiked samples were compared to the results of the freshly injected extracts.

Application to routine samples

Subsequent to the full validation, the developed method was compared to the routine method that was previously used for the control of CAP residues in animal products [30]. In short, that method consisted of a hydrolysis of the urine sample followed by a clean-up procedure based on sorbent-assisted liquid-liquid extraction using Extrelut NT-3 cartridges (Merck) in combination with dichloromethane elution. After evaporation of the solvent, the residue was dissolved in 0.5 mL of water and injected onto the RP LC-MS/MS system.

Two freeze dried bovine urine samples containing CAP glucuronide with an assigned value of 0.20 and 0.36 $\mu\text{g L}^{-1}$ respectively, expressed as free CAP originating from a Dutch proficiency study (2008) were selected. The results obtained by the chiral LC-MS/MS method were compared to the results previously found using the RP LC-MS/MS method.

Results and discussion

General considerations

For the separation of the CAP isomers, the use of an AGP column proved to be suitable. In contrast to a high resolution RP-LC system, an AGP column is limited in terms of back pressure and organic modifier, and equilibration of the column is a relatively slow process. Therefore, the use of isocratic elution at low or moderate flow rate is mandatory to obtain a robust and reproducible method. As a result, peak widths of approximately 0.5 min are obtained using this column, whereas sub-2 μm particle RP-LC generally results in peak widths of about 8 seconds [23]. This, in combination with the presence of only 2 % organic modifier in the mobile phase, which adversely affects the evaporation of the mobile phase in the ion source, results in higher detection limits of the chiral stationary phase LC-MS/MS system. For the analysis of banned substances, detection limits should be as low as reasonably possible and confirmation of the identity of the analyte should at least be possible at MRPL level. Therefore, the loss of sensitivity using the chiral LC system had to be compensated.

Another adverse effect of the chiral LC system was the irreproducibility of the CAP isomers' retention times in urine extracts. If the urine samples were not thoroughly cleaned, a decrease in the retention time of the CAP isomers was observed every subsequent injection up to 50 % for the last eluting compound, resulting in unsatisfactory and irreproducible chromatographic resolution.

To obtain a robust and reproducible chromatographic separation for urine samples and to improve the detection limits, an optimised reversed phase SPE procedure combined with a liquid-liquid extraction using ethyl acetate was developed to clean and concentrate the urine samples prior to LC-MS/MS analysis. Using this sample clean-up a ten-fold concentration of purified urine samples was obtained and when injecting 10 μL in the LC-MS/MS system only slight effects of the remaining urine matrix were observed.

Sample preparation

To optimise the SPE washing and elution procedure 5 mL of a 10 $\mu\text{g L}^{-1}$ standard solution containing all CAP isomers was applied onto conditioned Strata-X RP 200 mg / 6 mL cartridges. The cartridges were washed in duplicate with two times 6 mL water-methanol mixtures ranging from 0 to 100 % MeOH. The retained CAP isomers were eluted from the cartridge using 1 mL MeOH. The solvent was evaporated, the residue reconstituted in 500 μL water and injected into the LC-MS/MS system. The signal of RS/SR-CAP- d_5 1st peak and RR-*p*-CAP are presented in figure 4.10a. It is observed that only from 50 % MeOH, 78 % of RS/SR-*p*-CAP- d_5 eluted from the SPE cartridge whereas RR-*p*-CAP was still fully retained. Because the RS/SR-isomers elute slightly before the RR/SS-isomers using a reversed phase separation mechanism [42] this is according to expectations. It is concluded that the SPE cartridges can be washed twice with 6 mL 40 % MeOH. Because CAP remains in the neutral state it was decided to wash the cartridge with 6 mL acidic 40 % MeOH in water followed by alkaline 40 % MeOH in water to remove additional interferences. For complete (> 95 %) elution of the CAP isomers 6 mL of 70 % MeOH is needed. To further optimise the elution the same procedure was repeated using 3 mL wash volumes containing 60 - 100 % MeOH. The results are presented in figure 4.10b. When using 3 mL of 80 % MeOH, more than 97 % of each of the isomers eluted from the SPE cartridge. This condition was considered favorable for eluting the CAP isomers over 6 mL of 70 % MeOH because less MeOH had to be evaporated.

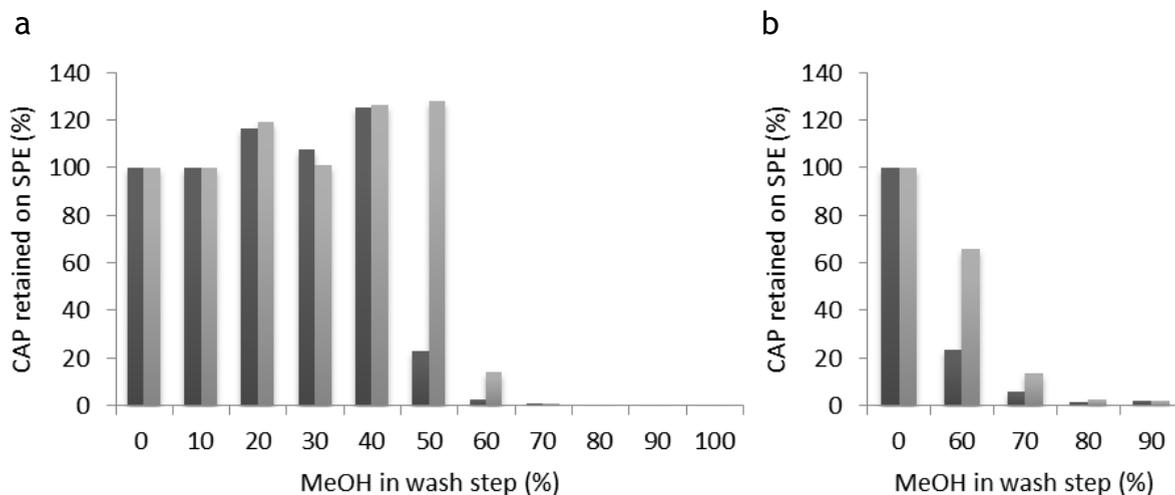


Figure 4.10. Amount of (■) *RS/SR-p-CAP-d₅* and (▒) *RR-p-CAP* still retained on the Strata-X RP SPE cartridge after washing with (a) 6 mL and (b) 3 mL of a mixture of MeOH and water.

LC-MS/MS analysis

The retention mechanism of an AGP column is based upon hydrophobic interaction with amino acids and hydrogen bonding with amides, carbonyl and imido groups [57,58]. The AGP column was selected for optimisation of the separation of CAP isomers because its selectivity towards analytes that contain a ring structure near a chiral center in combination with an alcohol, amide or carboxylic acid group [57].

Aiming for baseline separation of the eight CAP isomers ACN, MeOH, 2-propanol (PrOH), acetone (ACE), tetrahydrofuran (THF) and combinations of these solvents were tested as the organic modifiers. Furthermore the pH and the organic modifier concentration (0 to 5 %) were optimised. The pH did not significantly influence the retention of the isomers but the concentration and the nature of the organic modifier showed to be an important parameter for optimising the enantioselectivity of the LC system. Chromatograms obtained using different organic modifiers are given in figure 4.11.

From the upper two panels in figure 4.11 it is apparent that the retention time decreases with increasing organic modifier concentration, which is comparable to RP separations. This was expected, because in both chromatographic systems hydrophobic interactions are part of the retention mechanism. The effects of the nature of the organic modifier on the isomeric selectivity are not so easily

explained. The elution order of some isomers changes when using a different organic modifier (figure 4.11). For instance, if ACN, MeOH or PrOH is used RS/SR-*m*-CAP 1st elutes before RS/SR-*p*-CAP-*d*₅ 2nd, whereas the elution order changes if ACE or THF is used. Furthermore, SS-*p*-CAP and RR/SS-*m*-CAP 1st co-elute if ACN, MeOH or THF are used, whereas a slight separation is obtained using PrOH and baseline separation of the two isomers is obtained using ACE.

Although the nature of the organic modifier in the mobile phase affected the enantioselectivity, in all cases, at least two CAP isomers co-eluted. The use of 2 % ACN in ammonium formate buffer at pH = 4.0 showed optimal chromatographic resolution. With this system, still SS-*p*-CAP and RR/SS-*m*-CAP 1st peak co-eluted.

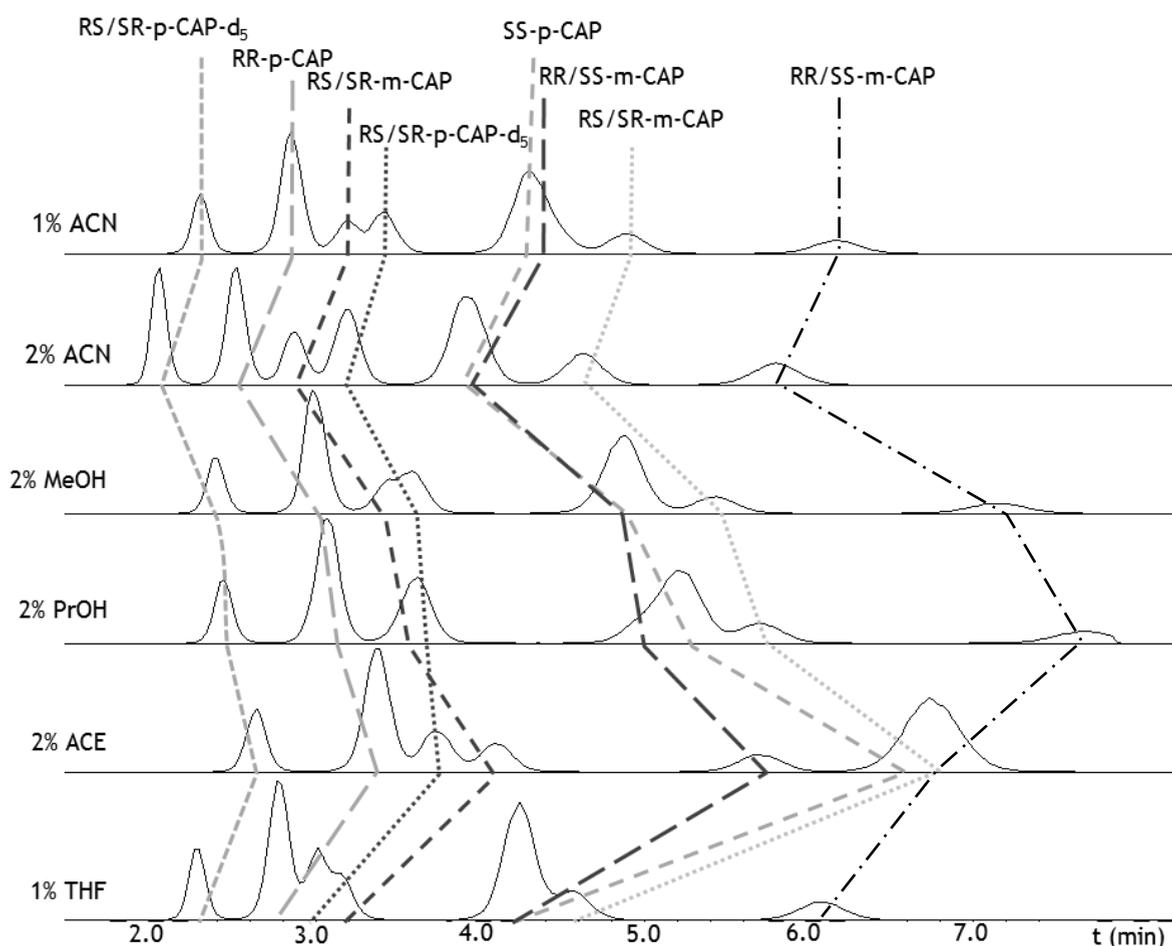


Figure 4.11. Total ion current chromatograms of $40 \mu\text{g L}^{-1}$ mixtures of eight CAP isomers. The chromatographic peaks of each isomer are connected by lines. Crossing lines indicate changed isomeric selectivity.

Because the MS fragmentation pathways of the CAP isomers depend on the substitution of the nitro-group [42], it was possible to select specific product ions for SS-*p*-CAP (321 > 121) and RR/SS-*m*-CAP (321 > 86) allowing discrimination of the co-eluting isomers. Examples of the SRM chromatograms of a blank urine sample and a blank urine sample spiked at 0.3 $\mu\text{g L}^{-1}$ are presented in figure 4.12.

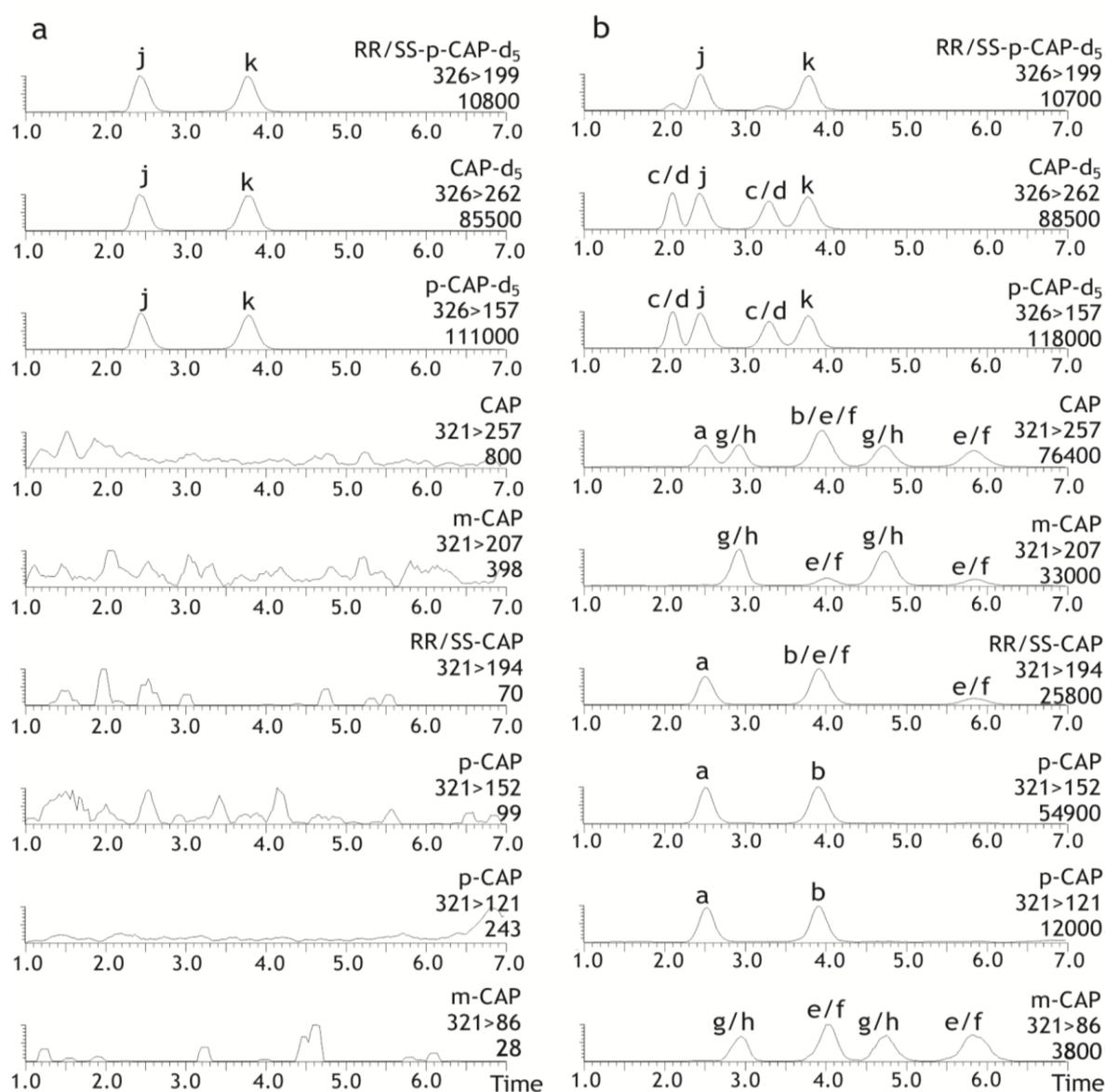


Figure 4.12. SRM chromatograms of (a) a blank urine sample and (b) a urine sample with addition of 0.3 $\mu\text{g L}^{-1}$ of all eight CAP isomers. a = RR-*p*-CAP, b = SS-*p*-CAP, c = RS-*p*-CAP- d_5 , d = SR-*p*-CAP- d_5 , e = RR-*m*-CAP, f = SS-*m*-CAP, g = RS-*m*-CAP, h = SR-*m*-CAP. Internal standards: j = RR-*p*-CAP- d_5 and k = SS-*p*-CAP- d_5 .

Validation

An overview of the validation results is presented in table 4.8.

Table 4.8. Summary of the validation study.

Compound	Validation						
	level ($\mu\text{g L}^{-1}$)	Trueness (%)	RSD _F (%)	RSD _{RL} (%)	CC α^a ($\mu\text{g L}^{-1}$)	CC β^a ($\mu\text{g L}^{-1}$)	Linearity (r^2)
RR- <i>p</i> -CAP	0.15	99	9.1	9.1	<i>0.005</i>	<i>0.13</i>	> 0.995
	0.3	104	10.2	7.4	0.36	0.42	
	0.45	100	11.6	7.4			
SS- <i>p</i> -CAP	0.15	104	8.9	9.0	<i>0.04</i>	<i>0.16</i>	> 0.997
	0.3	104	6.6	7.1	0.34	0.37	
	0.45	105	7.7	8.1			
RS/SR- <i>p</i> -CAP-d ₅ 1 st	0.15	96	9.5	10.1	<i>0.02</i>	<i>0.15</i>	> 0.991
	0.3	99	10.9	13.1	0.36	0.43	
	0.45	99	10.1	15.1			
RS/SR- <i>p</i> -CAP-d ₅ 2 nd	0.15	95	7.7	10.2	<i>0.02</i>	<i>0.14</i>	> 0.992
	0.3	99	11.5	14.3	0.37	0.44	
	0.45	102	10.0	13.4			
RR/SS- <i>m</i> -CAP 1 st	0.15	101	9.7	9.9	<i>0.01</i>	<i>0.14</i>	> 0.990
	0.3	102	6.3	6.4	0.33	0.36	
	0.45	103	7.7	10.9			
RR/SS- <i>m</i> -CAP 2 nd	0.15	98	12.5	13.9	<i>0.04</i>	<i>0.15</i>	> 0.995
	0.3	100	14.5	14.9	0.37	0.45	
	0.45	101	12.2	12.9			
RS/SR- <i>m</i> -CAP 1 st	0.15	97	10.2	20.6	<i>0.005</i>	<i>0.14</i>	> 0.994
	0.3	97	13.4	19.9	0.39	0.49	
	0.45	98	14.1	20.1			
RS/SR- <i>m</i> -CAP 2 nd	0.15	90	9.3	9.8	<i>0.01</i>	<i>0.13</i>	> 0.991
	0.3	91	13.0	13.6	0.36	0.42	
	0.45	93	14.1	14.2			

^a CC α and CC β based on zero tolerance are indicated in italics.

The linearity in the range from 0 to 0.5 $\mu\text{g L}^{-1}$ is above the criterion of 0.990 for all compounds and is therefore adequate for quantitative trace analysis. The trueness is between 90 and 105 % for all isomers, which is amply within the established criterion of 50 to 120 %. The determined RSD_r and RSD_{RL} are below 14.7 % respectively 22 % for all isomers and thus comply with the criteria as well. Based on these outcomes it is concluded that the presented method is suitable for quantitation of the amount of CAP isomers present in a bovine urine sample.

The calculated decision limits ($\text{CC}\alpha$) based on zero tolerance vary from 0.005 $\mu\text{g L}^{-1}$ to 0.04 $\mu\text{g L}^{-1}$ indicating that part-per-trillion levels of CAP isomers are still detectable. The calculated values were verified based upon the signal of the CAP isomers in the chromatograms of the lowest calibration level (0.1 $\mu\text{g L}^{-1}$) and were found to be realistic. The calculated detection capabilities ($\text{CC}\beta$) are between 0.13 and 0.16 $\mu\text{g L}^{-1}$ which is approximately at the lowest validation level and therefore the values could easily be verified using the available data from the blank urine samples spiked at 0.15 $\mu\text{g L}^{-1}$. In 100 % of the cases ($n=21$), based upon the ion ratio, CAP was found non-compliant according to EU criteria [9] indicating that the calculated levels of $\text{CC}\beta$ are slightly overestimated since the criterion is 95 % only. Because this is an initial in-house validation, only limited data are available. Over time the band width of the noise of blank samples and the peak height of samples spiked at 0.1 $\mu\text{g L}^{-1}$ will be monitored to obtain a more exact estimation of $\text{CC}\beta$. However, $\text{CC}\beta$ for zero tolerance substances is below the MRPL of 0.3 $\mu\text{g L}^{-1}$ in all cases and therefore it is concluded that the method is suitable for detection of chloramphenicol at relevant levels.

No interferences were observed in the chromatograms of the blank samples. From the theoretical study α -zeranol, a non-steroidal estrogenic agonist that is banned within the EU [59], but approved for the use as a growth promoter in cattle in the United States and in Canada [60], was found to have the same molecular mass as CAP and might theoretically interfere in the described method. A standard solution of 10 $\mu\text{g L}^{-1}$ and a blank urine sample spiked with 100 $\mu\text{g L}^{-1}$ of α -zeranol were prepared and injected into the chiral LC-MS/MS system. No interfering signals were observed in the chromatogram indicating that the procedure is sufficiently selective for CAP isomers. Furthermore, the presence of a severe amount of α -zeranol in the urine sample does not affect the

detection and confirmation of the CAP isomers and will therefore not result in false negative results for CAP.

For a few urine samples a slight deformation of the chromatographic peaks was observed which was in all cases directly related to the color of the extract indicating that occasionally some matrix components can have some influence on the chromatographic performance. In these cases the peak top can shift to a slightly lower retention time causing a difference in the compound's retention time compared to the compound in the matrix-matched reference standards and therefore extra attention should be given to the confirmatory aspect e.g. by carrying out multi-level standard addition. Another solution is to further dilute the final urine extract in water, but this negatively affects the detection limit and therefore the success of this approach depends on the CAP level in the specific sample.

From the method development, the sample clean-up was the most critical step in the analysis method. Because the percentage of MeOH in the SPE wash and elution procedure are optimised to remove as many interferences as possible, a slight difference in the MeOH content can result in breakthrough or incomplete elution of the CAP isomers. Therefore, the SPE procedure should be carried out accurately for optimum performance.

Three slight deviations to the procedure that might occur in practice, were tested: (1) evaporation of the eluent until only 200 μL of water remained after which ethyl acetate was added, (2) evaporation of the eluent until some methanol was still present after which ethyl acetate was added and (3) evaporation of the ethyl acetate fraction until dryness plus an additional 10 minutes. The duplicates analysed incorporating these deviations in the method showed good trueness and acceptable duplicates, indicating that the tested processes are robust.

When storing methanolic $100 \mu\text{g mL}^{-1}$ standard solutions of the isomers (RR-*p*-CAP and SS-*p*-CAP individually and the others as a racemic mixture) at 4 °C and room temperature, no difference in the peak intensities was observed. Furthermore, neither conversions to other isomeric forms nor any changes in the relative intensities of the isomers in the racemic mixtures were observed. It is concluded that solutions of CAP isomers are stable at room temperature for at least 5 days.

The results of the stability of urine extracts stored at $-20\text{ }^{\circ}\text{C}$ for 7 days are presented in table 4.9. Also after 7 days of storage the calibration lines remain sufficiently linear having a coefficient correlation above 0.990. Furthermore, no significant difference was observed between the average and the RSD_r of the freshly analysed extracts and the stored extracts. It is concluded that urine extracts obtained with the described method are stable for at least 7 days when stored at $-20\text{ }^{\circ}\text{C}$.

Table 4.9. Results of the urine sample spiked at $0.3\text{ }\mu\text{g L}^{-1}$ analysed directly after preparation compared to the results of the same extracts analysed after 7 days storage at $-20\text{ }^{\circ}\text{C}$ ($n=7$).

Compound	Directly			After 7 days		
	Linearity (r^2)	Average ($\mu\text{g L}^{-1}$)	RSD_r (%)	Linearity (r^2)	Average ($\mu\text{g L}^{-1}$)	RSD_r (%)
RR- <i>p</i> -CAP	0.995	0.29	18.0	0.999	0.30	12.0
SS- <i>p</i> -CAP	0.998	0.31	9.9	0.999	0.32	6.8
RS/SR- <i>p</i> -CAP- d_5 1 st	0.992	0.32	12.7	0.995	0.30	13.6
RS/SR- <i>p</i> -CAP- d_5 2 nd	0.992	0.30	10.6	0.994	0.29	12.5
RR/SS- <i>m</i> -CAP 1 st	0.994	0.30	7.6	0.997	0.30	7.1
RR/SS- <i>m</i> -CAP 2 nd	0.998	0.32	15.1	0.999	0.31	10.0
RS/SR- <i>m</i> -CAP 1 st	0.994	0.26	17.5	0.991	0.25	20.3
RS/SR- <i>m</i> -CAP 2 nd	0.993	0.27	10.4	0.995	0.28	12.6

Application to routine samples

Both proficiency test samples that were found non-compliant in previous analyses using the RP LC-MS/MS method were also found non-compliant by the developed chiral stationary phase LC-MS/MS method. The quantitative results of both methods are in excellent agreement being 0.22 and $0.36\text{ }\mu\text{L}^{-1}$ using the RP LC-MS/MS method and 0.19 and $0.32\text{ }\mu\text{g L}^{-1}$ using the chiral LC-MS/MS method. If the results obtained using the chiral method would have been submitted for the proficiency test, z-scores of -0.2 and -0.8 would have been obtained. Note that in a proficiency test, quantitative results are considered satisfactory if the z-score is between -2 and +2 [61], from which it is concluded that the quantitative aspect of the developed method is adequate. From the chiral LC-MS/MS method it is clear that both samples contain the RR-*p*-CAP isomer, the microbial active

isomer of CAP. As an example reconstructed SRM chromatograms of the proficiency test sample having an assigned value of $0.36 \mu\text{g L}^{-1}$ obtained using both systems are presented in figure 4.13. In figure 4.13a no discrimination among RR-*p*-CAP, SS-*p*-CAP, RR-*m*-CAP and SS-*m*-CAP (denoted a, b, e and f respectively) could be obtained, whereas from figure 4.13b it can be unambiguously stated that the sample contains, apart from the internal standards (j and k), only the bioactive CAP isomer (denoted a).

Therefore, a quantitative trace analysis method using chiral stationary phase LC in combination with MS/MS detection, which is able to discriminate eight CAP isomers at trace level in bovine urine, was successfully developed and fully validated according to 2002/657/EC [9] at part-per-trillion levels. Especially the sample clean-up procedure proved to be a critical factor for obtaining reproducible chromatographic resolution. The validation showed good trueness, repeatability and within-lab reproducibility and the selectivity, robustness and stability proved to be sufficient to apply the presented method in routine analyses. As a result, the microbially active RR-*p*-CAP can be discriminated from its inactive isomers and thus the presented method provides the ultimate selectivity in trace evidence, which is expected to survive any legal disputes related to CAP abuse.

Conclusions

A quantitative trace analysis method using chiral stationary phase LC in combination with MS/MS detection, which is able to discriminate eight CAP isomers at trace level in bovine urine, was successfully developed and fully validated according to 2002/657/EC [9] at part-per-trillion levels. Especially the sample clean-up procedure proved to be a critical factor for obtaining reproducible chromatographic resolution. The validation showed good trueness, repeatability and within-lab reproducibility and the selectivity, robustness and stability proved to be sufficient to apply the presented method in routine analyses. As a result, the microbially active RR-*p*-CAP can be discriminated from its inactive isomers and thus the presented method provides the ultimate selectivity in trace evidence, which is expected to survive any legal disputes related to CAP abuse.

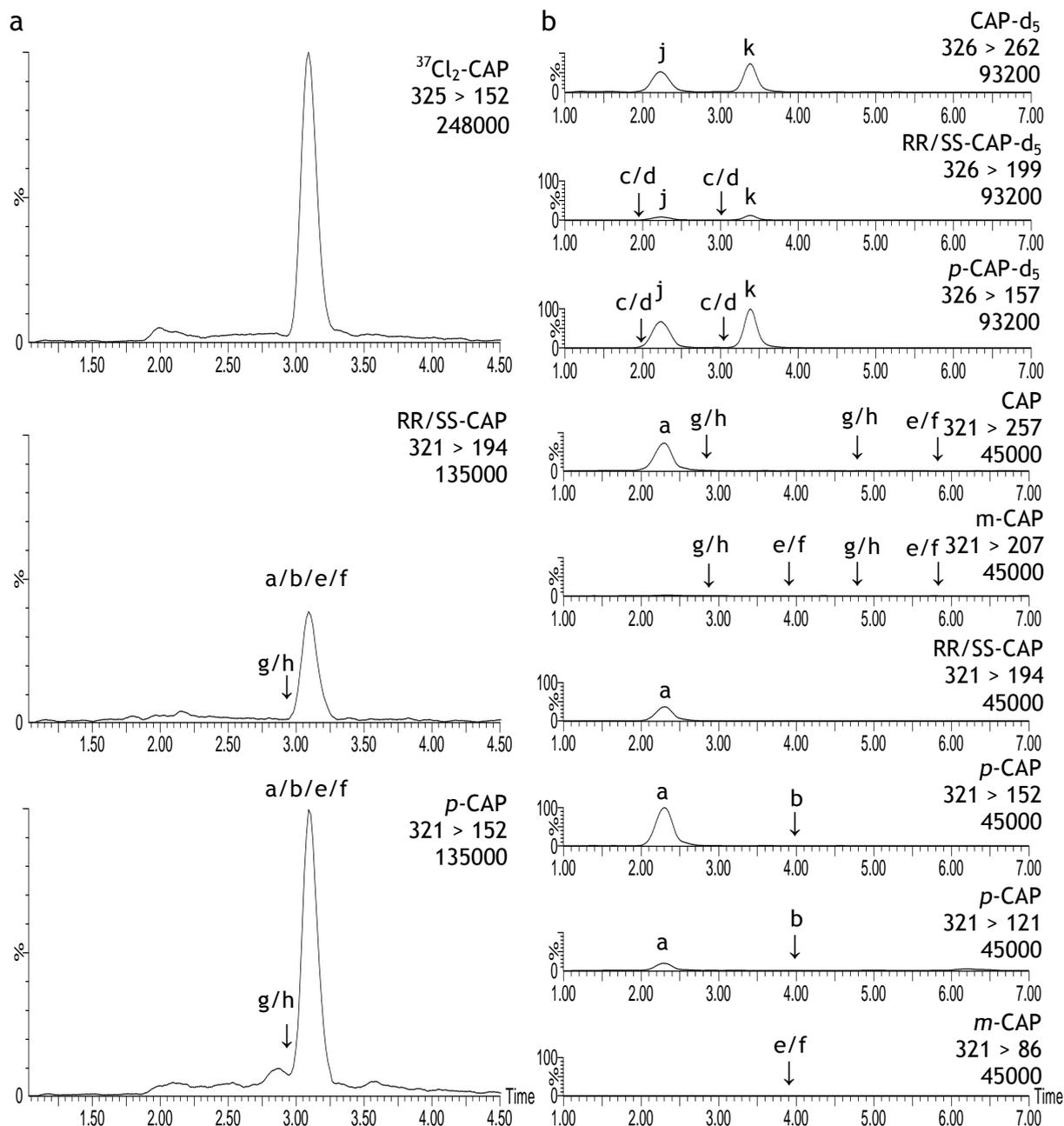


Figure 4.13. SRM chromatograms of a urine sample containing $0.36 \mu\text{g L}^{-1}$ CAP obtained using (a) the RP LC-MS/MS method using $^{37}\text{Cl}_2$ -CAP as the internal standard and (b) using the developed chiral LC-MS/MS method. a = RR-p-CAP, b = SS-p-CAP, c = RS-p-CAP- d_5 , d = SR-p-CAP- d_5 , e = RR-m-CAP, f = SS-m-CAP, g = RS-m-CAP, h = SR-m-CAP. Internal standards: j = RR-p-CAP- d_5 and k = SS-p-CAP- d_5 .

Acknowledgements

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4.5. The occurrence of chloramphenicol in crops through the natural production by bacteria in soil

Abstract

Due to the unexpected findings of the banned antibiotic chloramphenicol in products of animal origin, feed and straw, the hypothesis was studied that the drug is naturally present in soil, through production by soil bacteria, and subsequently can be taken up by crops. First, the stability of chloramphenicol in soil was studied. The fate of chloramphenicol highly depends on soil type and showed a half-life of approximately one day in non-sterile topsoil. It was found to be more stable in sub-soil and sterile soils. Second, the production of chloramphenicol in soil was studied and it was confirmed that *Streptomyces venezuelae* can produce chloramphenicol at appreciable amounts in non-sterile soil. Third, a transfer study was carried out using wheat and maize grown on three different soils, that were weekly exposed to aqueous chloramphenicol solutions at two different levels. Chloramphenicol was taken up by crops as determined by chiral liquid chromatography coupled to tandem mass spectrometric analysis and the levels in crop were found to be bioavailability related. It was concluded that chloramphenicol residues can occur naturally in crops as a result of the production of chloramphenicol by soil bacteria in their natural environment and subsequent uptake by crops.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic that has been used in all major food-producing animals. Traditionally, CAP is produced for commercial use by chemical synthesis [5] but it is biosynthesised by the soil organism *Streptomyces venezuelae* and several other *actinomycetes* [1] as well. The drug has been evaluated by a number of organizations [2-4], most recently in 2005 by the Joint Expert Committee on Food Additives at its 62nd meeting [5]. CAP is a

suspected carcinogen and due to its linkage with the development of aplastic anemia in humans [5], the drug is banned for use in food-producing animals in the European Union (EU) [62] and in many other countries, including the USA, Canada, Australia, Japan and China. A minimum required performance limit (MRPL) of $0.3 \mu\text{g kg}^{-1}$ was set by the European Commission for the analytical methods to be used in testing for CAP in products of animal origin [6].

In recent years, findings of CAP residues in food products had a major impact on international trade [21]. In 2012 non-compliant findings of CAP in the European Union were related to casings, meat products and feed [63]. In 2010, the detection of chloramphenicol in plants and soil of mainly Mongolian origin [30] was reported and a first monitoring of CAP in European straw (n=21) resulted in 57 % positive samples with concentrations mainly below $1 \mu\text{g kg}^{-1}$ but the maximum level was as high as $11 \mu\text{g kg}^{-1}$ [64]. A more extensive follow-up study (n=104) carried out in our laboratories, showed 37 positives (36 %), of which 7 above $0.3 \mu\text{g kg}^{-1}$ and a single result above $1 \mu\text{g kg}^{-1}$: $6.8 \mu\text{g kg}^{-1}$. All samples contained the antimicrobially active isomer of chloramphenicol [42] and no correlation between the CAP concentration and the origin or type of straw was found.

Various hypotheses have been proposed to explain these results such as the illegal use of the drug in animal production and the natural production of CAP by soil bacteria naturally present in the environment [65]. Due to the variety of positive samples and the fact that recent findings of CAP in several products produced in different countries could not be explained by the use of the drug, additional investigations were urgent. A hypothesis, which to our knowledge has never been supported by solid scientific evidence is the potential accumulation by arable crops of CAP naturally produced in soil by biosynthesis by *actinomycetes* (figure 4.14). If proven correct, the CAP containing crops are processed to animal feed or used as stall bedding and consequently animals might ingest these products which may result in non-compliant CAP findings in products of animal origin.

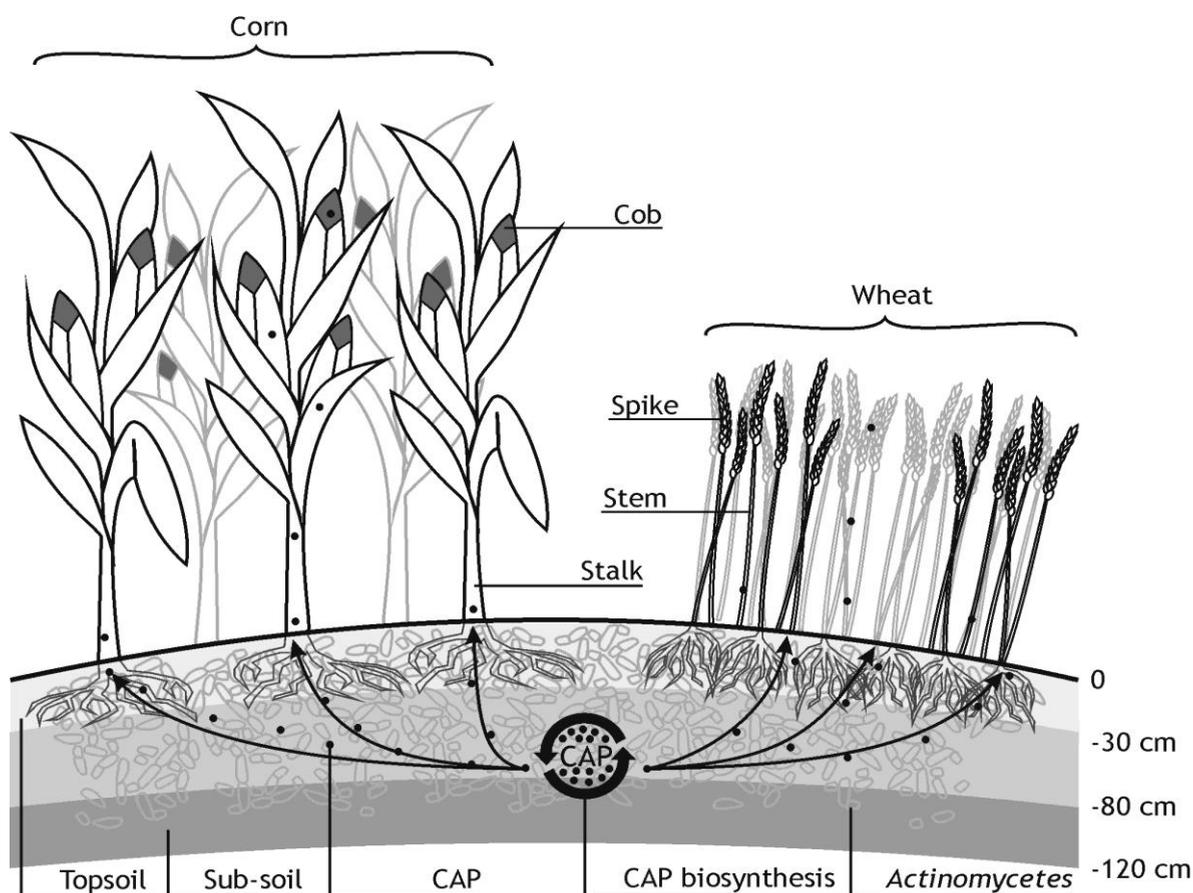


Figure 4.14. Graphical representation of tested hypothesis.

Two conditions should be fulfilled to confirm the posed hypothesis: (1) soil bacteria must be able to produce CAP in soil under natural conditions and (2) crops should be able to accumulate CAP from the soil in the above ground biomass. The adsorption, stability and production rate of CAP in soil was studied long ago under laboratory conditions [65,66]. After inoculation of sterile soils by CAP producing bacteria, CAP concentrations up to 1.1 mg kg^{-1} were found. Upon addition of an additional carbon source, CAP production even reached 25 mg kg^{-1} after 18 - 31 days of incubation [65]. In non-sterile soils, however, CAP production was not detected. In that specific study, the detection limit of the applied method was $50 \text{ } \mu\text{g kg}^{-1}$ and thus low, but relevant levels of CAP that may have been present could not have been detected at that time [67]. Transfer studies confirmed the presence of detectable levels of veterinary drugs in plants, among which tetracyclines [68,69], trimethoprim [70], sulphonamides [69-71], anticoccidials [72] and florfenicol [22]. A monitoring study, which focused on the detection of veterinary drugs in manure, groundwater, soil and plants, confirmed the presence of CAP in plants [73].

To verify the posed hypothesis, a series of three experiments were performed using state-of-the-art techniques. First, the stability of the antibiotic in soil was studied under sterile and non-sterile conditions. Second, the net production of CAP by *Streptomyces venezuelae* in sterile and non-sterile soil was investigated and third, the active uptake of free CAP by wheat and maize was quantitated in a controlled greenhouse experiment. Wheat and maize were selected because these are the major crops used as stall bedding and/or animal feed constituent. Results from these three experiments are combined to gain insight regarding the hypothesis that CAP contamination in crops can be explained by the natural production of CAP by soil bacteria.

Experimental

Reagents

ULC grade water and acetonitrile (ACN), HPLC grade methanol (MeOH) and ethyl acetate were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium formate, formic acid, acetic acid and 25 % ammonia were obtained from Merck (Darmstadt, Germany). Milli-Q water was prepared using a Milli-Q system at a resistivity of at least $18.2 \text{ M}\Omega \text{ cm}^{-1}$ (Millipore, Billerica, MA, USA). The reference standard of RR-*p*-CAP was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the internal standard RR-*p*-CAP- d_5 was obtained from Witega (Berlin, Germany). Stock solutions were prepared in MeOH at 100 mg L^{-1} and all dilutions were prepared in milli-Q water.

Transfer study

Soil used in the transfer study

The soil used in this study was a sandy soil (Gleyic Podzol, FAO) used for regular agriculture and originates from the Droevendaal experimental farm of Wageningen University. Fresh soil was collected prior to the experiment on May 10th 2012 from 2 depth layers, i.e. the 0 - 30 cm layer (topsoil) and the 80 - 120 cm layer (sub-soil). Two hundred kg of both soil types was transferred to the laboratory where it was homogenised and sieved ($< 2 \text{ mm}$ using stainless steel

sieve). The natural moisture content was determined by weight loss at 105 °C. To obtain a range in organic matter a third soil was created by mixing dried topsoil with an equivalent amount of sub-soil. This resulted in a series of soils with similar mineralogical properties and minor differences in pH. The latter varied from 5.0 (sub-soil) to 5.2 (topsoil) as determined in a 1:10 (soil:solution) extract using 0.01 M CaCl₂. The main variable of relevance is the soil organic matter (SOM) content which was determined in all soils by Loss on Ignition (LOI) at 550 °C. The SOM content ranged from 0.8 % in the sub-soil, 2.1 % in the mixed soil and 3.2 % in the topsoil, the latter being representative for normal arable sandy soils in the Netherlands.

Pot experiment to determine CAP transfer

Approximately 6.5 kilo of air dried top soil, mixed soil and sub-soil was used in 8 L ceramic pots. To obtain the desired moisture content at the start of the experiment, 370 mL of distilled water was added to each pot which is equivalent to 80 % of the water holding capacity for this soil type as determined experimentally. During the growth of the crops, the moisture content in the pot was maintained at 80 % of the water holding capacity by weight loss and correction for the total biomass present on the pot. Plants were watered daily during the growing season using normal tap water. In order to keep the growing conditions in all pots equal, a starting dose of N, P, K and Mg fertilizer was initially mixed with the soil. In total 1500 mg of N (25 % as Ca(NO₃)₂ 2M and 75 % as NH₄NO₃), 327 mg P (NaH₂PO₄), 241 mg Mg (MgSO₄) and 1245 mg K (KCL) were added to 6.5 kg of soil to overcome nutrient deficiencies. During the growth of the crops, aliquots of 50 mL of a nutrient solution based on the same ratio of N, P, K and Mg as listed here were added depending on the growing status of the plant. Two crops: maize (var. LG 30-208) and wheat (var. Lavett), three soil types: sub-soil, mixed soil and topsoil and three CAP treatment levels: blank, low and high, were used in the growing study, each carried out in duplicate (table 4.10). This resulted in a total of 36 pots. In each pot 10 maize or 20 wheat seeds were planted. To avoid any salt damage the seeds were placed in a layer of 0.5 cm of soil that was not amended with fertilizer. After mixing the bulk soil with the required amount of fertilizer, filling the pots with soil and installing the seeds in the top 0.5 cm layer of unamended soil, the 36 pots were transferred to the Nergena greenhouse facilities (Wageningen UR). The temperature and

humidity in the greenhouse were kept constant at 20 °C and 80 % respectively during the growth of the crop. After germination, the number of plants in each pot was reduced to 3 for maize and 10 for wheat. Daylight was maintained for 12 hours after September 15th 2012 using artificial light. The complete plants were harvested after ripening on October 2nd 2012 (wheat) and October 18th 2012 (maize). Wheat stems and spikes as well as maize stalks and cobs were separated. Samples were cut using a knife and subsequently minced under cryogenic conditions to obtain homogeneous samples and to improve extraction efficiency. Also soil samples, cleared of root material, were taken.

Table 4.10. Set-up of the pot experiment. Each experiment is carried out in duplicate resulting in 36 experiments.

	Topsoil	Mixed soil	Sub-soil
Wheat	0	0	0
	7.5 mg CAP pot-1	7.5 mg CAP pot-1	7.5 mg CAP pot-1
	75 mg CAP pot-1	75 mg CAP pot-1	75 mg CAP pot-1
Maize	0	0	0
	7.5 mg CAP pot-1	7.5 mg CAP pot-1	7.5 mg CAP pot-1
	75 mg CAP pot-1	75 mg CAP pot-1	75 mg CAP pot-1

Experimental design of the CAP additions

CAP was added to the plants after germination and an initial 2-week growth phase to avoid any CAP induced effects in the early growing stage. After reaching a plant height of approximately 20 cm for both crops, CAP was added weekly via 100 mL solution gifts containing the appropriate amount of CAP. In total 3 treatments levels were performed, including a 0-treatment receiving the same volume of deionised water, a low dose (7.5 mg CAP total addition per pot) and a high dose (75 mg CAP total addition per pot). A CAP stock solution was prepared by dissolving 112.5 mg of CAP in 500 mL of deionised water. After mechanical stirring for 30 minutes all CAP was dissolved and the solution transferred to brown 2L glass flasks. The total volume was brought to 1500 mL. One hundred mL of this stock solution equals an addition of 7.5 mg which was added weekly for 10 consecutive weeks to the 12 pots of the high dose treatment. From this stock solution 150 mL was diluted 10 times to a total volume of 1500 mL which served

as the low treatment dose. Again, 10 gifts of this solution were added to the low dose treatment pots during the growing phase of the plants. To ensure that all CAP accumulated by the plants occurred through root action and to avoid direct contact between CAP in the water and the above ground plant material, the CAP containing solutions as well as the deionised water (0-treatment) were added to the pots via a vertical plastic cylinder with a diameter of 4 cm and a total height of 10 cm which was installed in each pot. This cylinder was buried in the soil to a depth of 3 cm and the solutions could seep into the soil via small holes below the soil surface as is illustrated in figure 4.15. To avoid retention or degradation of CAP, the cylinder was filled with inert quartz sand with a high infiltration rate. After addition of 100 mL of the treatment solutions, 100 mL of deionised water was subsequently added to the cylinder to force the CAP into the soil and to minimise potential loss of CAP retained in the sand layer.

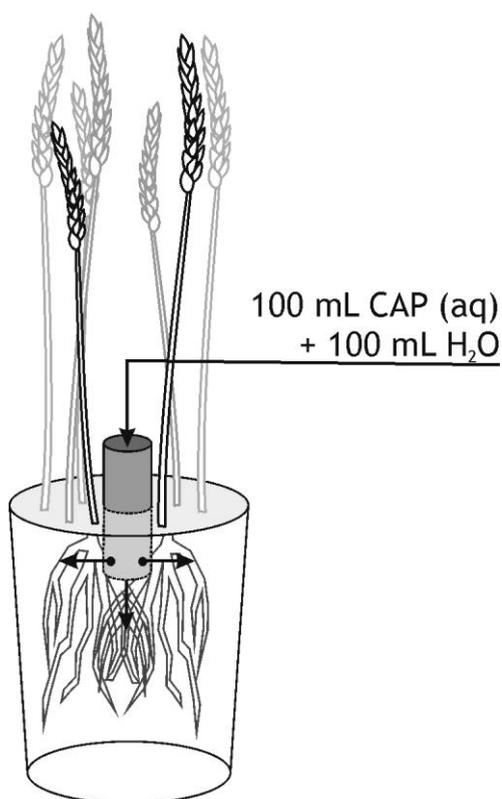


Figure 4.15. Schematic representation of the pots used in the study. All treatment solutions were added via the cylinder to avoid direct contact between the solutions and the plant material.

Stability study

Two gram aliquots of the dried soil material from the topsoil and sub-soil were transferred into individual polypropylene test tubes (n=24) and 0.5 mL of milli-Q water was added. Of both soil types, half of the soil containing test tubes were sterilised at 121 °C for 15 minutes during 2 consecutive days, the other tubes were stored at room temperature. At t = 0, an aqueous solution (125 µL) of CAP was added to two aliquots of each soil type resulting in a nominal concentration of 50 µg kg⁻¹. The spiked samples were shaken for 10 sec using a vortex mixer and placed at room temperature exposed to daylight. At t = 1, 2, 3 and 4 days this spiking procedure was repeated and at day 4, 5 µg kg⁻¹ of internal standard (RR-*p*-CAP-d₅) was added to all samples as an analytical reference, including the last set of blank samples, which were then analysed at random by chiral liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Chloramphenicol production in soil

To determine the natural production rate of CAP in soil by bacteria, eight 100 g batches of dried topsoil were transferred into 250 mL glass containers, which were covered with aluminum foil. Half of the containers were sterilised at 121 °C for 15 minutes during 2 consecutive days. *Streptomyces venezuelae* (DSM 40230, Leibniz Institute DSMZ, Braunschweig, Germany) was cultured in GYM (4 g L⁻¹ glucose, 4 g L⁻¹ yeast extract, 10 g L⁻¹ malt extract) for two days at 28 °C (200 rpm) until OD₆₀₀ of ~ 2.0 was reached. The culture (650 mL) was harvested by centrifugation (10 min. at 10,000 x g) and washed three times with 100 mL PBS (8 g L⁻¹ NaCl, 0.2 g L⁻¹ KCl, 1.44 g L⁻¹ Na₂HPO₄, 0.24 g L⁻¹ KH₂PO₄, pH 7.4) to remove any CAP that might have been produced during culturing. After the last washing step, the pellet was taken up in 65 mL of PBS and stored at 4 °C until further use. Enumeration of this inoculant yielded 2*10⁹ colony forming units (CFU) mL⁻¹. The inoculant was added to the sterile and non-sterile soil in duplicate at two concentrations: 2*10⁶ and 2*10⁸ CFU g⁻¹ soil. The moisture concentration was adjusted to 20 % for all containers. The beakers were covered with parafilm and placed into a humidity chamber at 28 °C. After 1, 8, 15 and 22 days the soil samples were homogenised by stirring with a wooden rod and 2.5 g aliquots were taken in duplicate. The aliquots were stored at < -70 °C until analysis.

Sample preparation for CAP analysis

The samples were prepared according to a previously reported and validated method [74] with minor adjustments making it suitable for plant material and soil. An additional validation was carried out for plant materials to ensure good method performance. Of the cryogenically minced crop samples, 2.5 g was extracted using 20 mL of ACN. After centrifugation (3500 g, 15 min) the organic phase was isolated, evaporated until dry (45 °C, N₂) and reconstituted in 5 mL of water. A Phenomenex (Torrance, CA, USA) Strata-X 200 mg / 6 mL solid phase extraction (SPE) cartridge was conditioned with 5 mL MeOH and 5 mL water. The sample extract was applied onto the cartridge and subsequently the cartridge was washed with 6 mL 40 % MeOH in water containing 1 % acetic acid followed by 6 mL 40 % MeOH in water containing 0.25 % ammonia. The cartridges were dried by applying vacuum for 3 minutes. CAP was eluted from the cartridge using 3 mL 80 % MeOH in water. The MeOH in the eluent was evaporated (45 °C, N₂). 2 mL of ethyl acetate was added to the remaining aqueous extract which was shaken for 1 minute using a rotary tumbler. After centrifugation (3500 g, 5 min) the ethyl acetate layer was isolated and evaporated (40 °C, N₂) until dry. The residue was redissolved in 500 µL water and transferred into an LC-MS/MS sample vial. Soil samples were analysed using the same method, but then the samples were extracted with 10 mL of water as was proven sufficient from previous experiments.

LC-MS/MS analysis

Samples of wheat stems and spikes, maize stalks and cobs, and soil were analysed separately. Quantitation was carried out by a calibration line in blank material, previously found to be free of CAP, ranging from 0 - 200 µg kg⁻¹. Internal standard (RR-*p*-CAP-d₅) was added to all individual samples before extraction at 10 µg kg⁻¹. All final extracts were injected as such and after 50-fold dilution in water to obtain a response within the calibration range.

The LC system consisted of a vacuum degasser, autosampler, and a Waters (Milford, MA, USA) model Acquity binary pump equipped with a Chromtech (Apple Valley, MN, USA) Chiral AGP (α1-acid glycoprotein) analytical column of 2.0 × 150 mm, 5 µm, placed in a column oven at 30 °C [74]. Isocratic elution was performed using a mobile phase consisting of 2 % ACN in 10 mM ammonium

formate buffer adjusted to pH=4.0 at a flow rate of 0.4 mL min⁻¹. The injection volume was 10 µL. Detection was carried out using a Waters model Xevo TQS triple quadrupole mass spectrometer in the negative electrospray ionisation (ESI) mode. The operating parameters were: capillary voltage, -1.5 kV; cone voltage, 20 V; source offset, 50 V; source temperature, 150 °C; desolvation temperature, 550 °C; cone gas flow, 150 L h⁻¹; and desolvation gas, 750 L h⁻¹. CAP and RR-*p*-CAP-d₅ were fragmented using collision induced dissociation (CID). The selected reaction monitoring (SRM) transitions are given in table 4.11.

Table 4.11. SRM transitions of CAP and the internal standard.

Compound	Precursor Ion (m/z)	Product ion (m/z)	Collision Energy (eV)
RR- <i>p</i> -CAP	321.0	152.0	17
		194.0	10
		257.0	10
RR- <i>p</i> -CAP-d ₅	326.0	199.0	10

Results and discussion

Stability in soil

The results of the stability study are presented in figure 4.16. CAP is rapidly degraded in non-sterile topsoil with a half-life of approximately one day. The rapid degradation is in line with results reported long ago [75,76] showing degradation kinetics depending on the soil composition. In sterilised topsoil, also a decrease of CAP concentrations was observed but at a significantly lower rate. At least part of the degradation of CAP can therefore be attributed to bacterial activity [65]. Sub-soil is expected to contain a much lower bacterial load and indeed the reduction of CAP concentrations in non-sterile sub-soil is less. For sterilised sub-soil no degradation of CAP seems to have occurred, but high standard deviations in the experiment with sterilised sub-soil prevent accurate comparison with the non-sterilised samples.

The instability of CAP in non-sterile soil explains the low number of positive CAP findings in soil samples [30]. Note that when CAP is produced in soil, especially in close proximity to the plant roots, it may very well be available for uptake by crops. This probably explains the relatively high number of positive plant samples compared to the number of positive soil samples as previously reported [30].

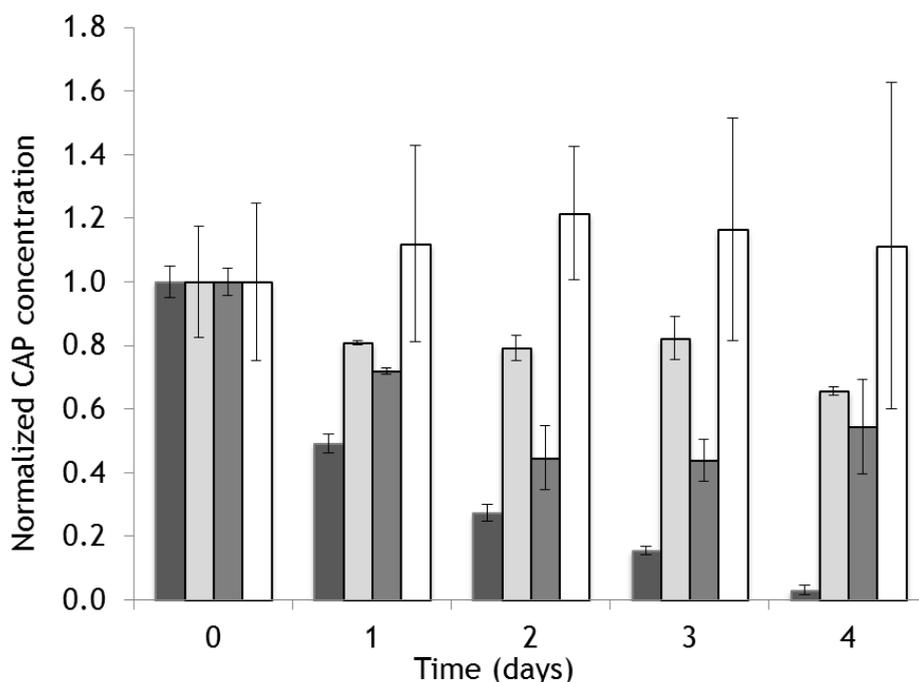


Figure 4.16. Normalised CAP concentration during incubation of CAP in (■) topsoil, (▒) sub-soil, (▓) sterilised topsoil and (□) sterilised sub-soil. Error bars represent the standard deviation ($n = 2$).

CAP production in soil

Both the inoculant used for this experiment and the topsoil were found not to contain any CAP. The results of the CAP production in sterile and non-sterile soil inoculated with *Streptomyces venezuelae* are presented in figure 4.17. Substantial CAP production is observed in the sterile soil samples. The concentration level of approximately $500 \mu\text{g kg}^{-1}$ that is reached during the first week, sustains throughout the incubation period and remarkably appears to be independent of the size of the inoculant. In non-sterile soil, inoculation with *Streptomyces venezuelae* yielded significantly lower CAP levels. The samples spiked with 2.0×10^8 inoculant showed a remarkable peak concentration at day 1 (47 and $140 \mu\text{g kg}^{-1}$ for the individual containers), suggesting that the presence of (competing) microbial flora triggers a specific physiological response. The subsequent collapse of the CAP concentration is in agreement with results for non-sterilised soil in the stability experiment. Furthermore, if CAP was only produced in the beginning of the production experiment, no CAP would be detectable after several days. Because also at 22 days of incubation CAP is detected, it is concluded that CAP is produced continuously during the experiment.

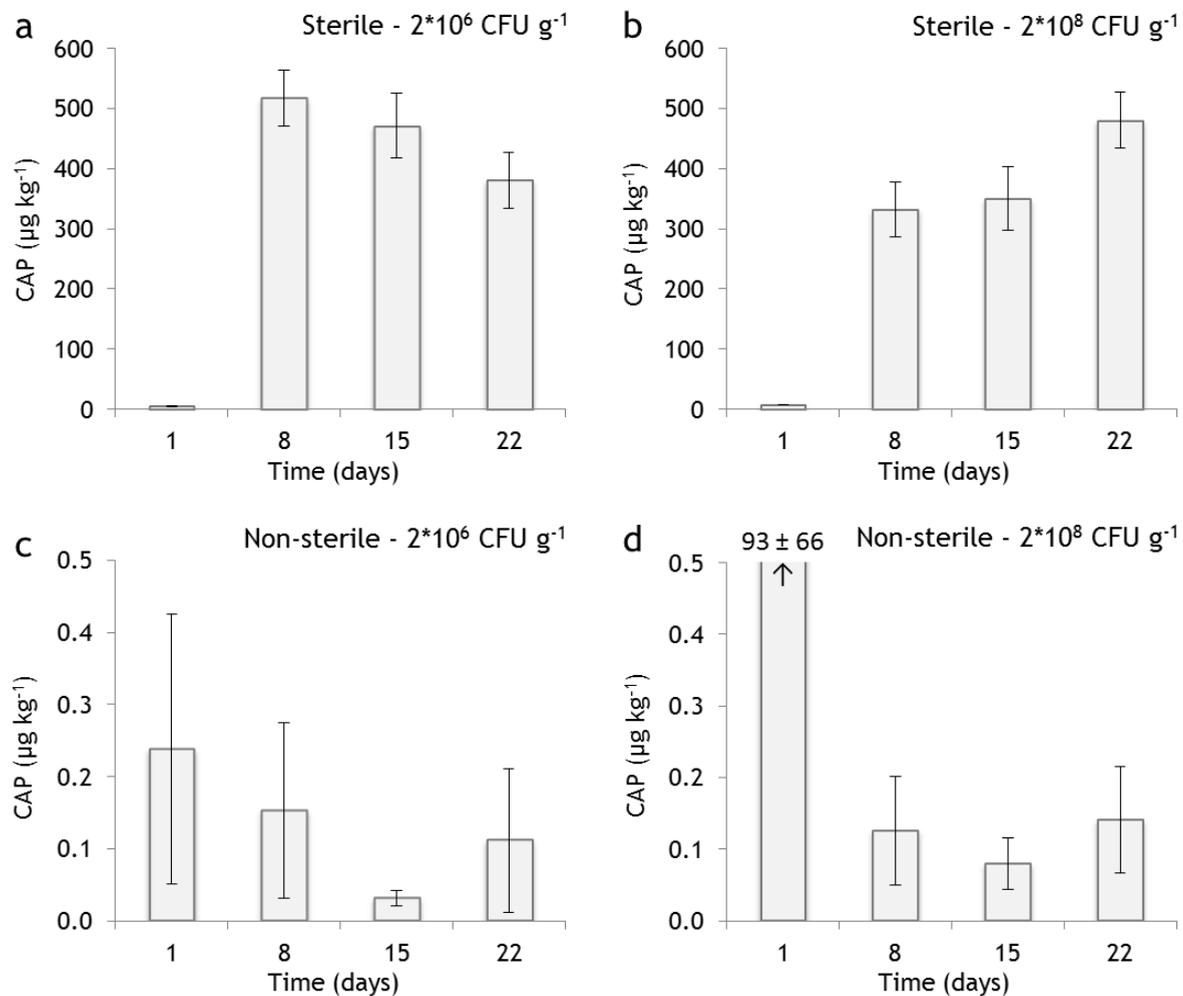


Figure 4.17. Average CAP concentration including standard deviation (error bars) in soil after incubation at 28 °C of (a) sterilised topsoil containing 2*10⁶ CFU g⁻¹, (b) sterilised topsoil containing 2*10⁸ CFU g⁻¹, (c) non-sterile topsoil containing 2*10⁶ CFU g⁻¹ and (d) non-sterile topsoil containing 2*10⁸ CFU g⁻¹, n = 4 (2 experiments analysed in duplicate).

From the stability and production experiments it is concluded that CAP can be produced in sterile as well as in natural soils and that biosynthesis and biodegradation occur simultaneously. CAP production and *Streptomyces venezuelae* growth rate are strongly related [77] and depend on many environmental factors. For instance, *Streptomyces venezuelae* growth at pH = 6.0 in MYM (maltose - yeast extract - malt extract) broth is optimal at 28 - 32 °C and is slightly lower at 22 °C. At pH = 7.5, the bacterial growth rate is reported to be optimal at 22 °C (lowest temperature tested) [78]. It is concluded that CAP can also be produced in the soil at lower temperatures, which are common in deeper soil layers. Furthermore, soil organic matter is of importance, showing

increased CAP production after addition of carbon and nitrogen sources, e.g. in fertilised soils [65,79,80]. Because of the simultaneously occurring processes and many environmental variables, the production experiment is considered to be proof of principle that CAP can be produced in natural soils rather than a precise quantitation of the CAP levels that can be produced in soil. Nevertheless, was shown that over $100 \mu\text{g kg}^{-1}$ CAP can be produced in non-sterile topsoil within a single day at high *Streptomyces venezuelae* inoculation levels. Note that the experimental setup was limited to detect free CAP and that the amounts of total CAP, e.g. present as conjugate or other metabolite, might be higher.

Transfer study

The transfer study was designed to study the influence of the soil, crop type (wheat versus maize) and administered CAP concentration. CAP solution was administered on a weekly basis so CAP was bioavailable during all stages of the growth. The determined average CAP concentrations ($n = 4$, 2 samples, both analysed in duplicate) in the wheat stems and spikes, maize stalks and cobs, and soil are presented in figure 4.18.

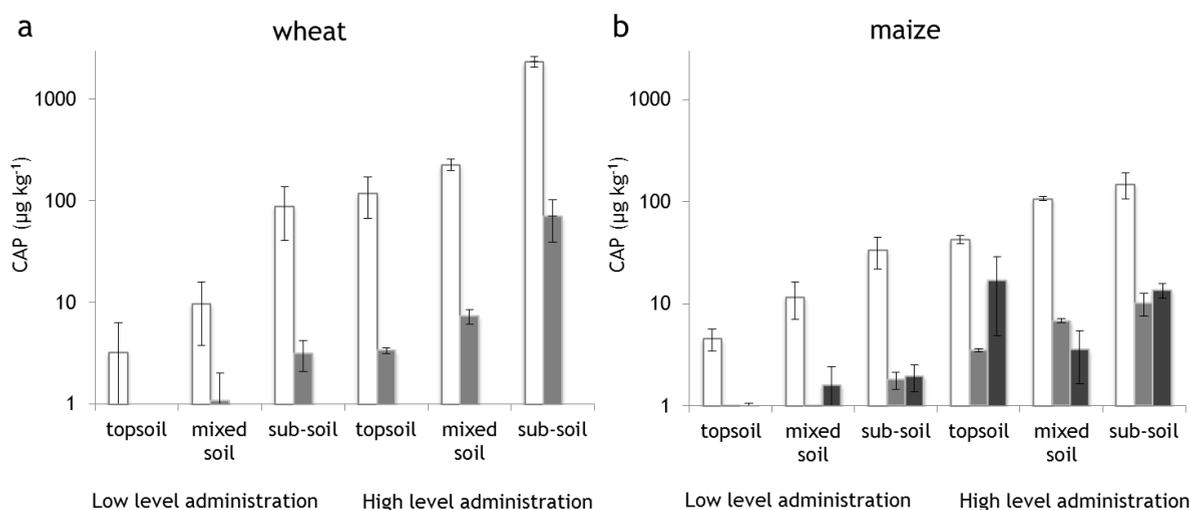


Figure 4.18. Average free CAP concentration including standard deviation (error bars) determined in the transfer study for (a) wheat: (□) stems, (■) spikes and (■) soil, and (b) maize: (□) stalks, (■) cobs and (■) soil at low and high level administration, $n = 4$ (2 samples, both analysed in duplicate). No CAP was detected in the soils used for wheat cultivation.

The fate of CAP in the system was assessed by adding up the absolute amounts of free CAP detected per pot (sum of soil and plant). This then was compared to the total amount of CAP administered over 10 weeks (7.5 mg pot^{-1} at the low level and 75 mg pot^{-1} at the high level). Thus calculated, the total amount of free CAP recovered was at $< 0.5 \%$, indicating that over 99% of free CAP disappeared during the experiment. The major cause of CAP loss is most likely the microbial degradation in soil. Also CAP conjugation, metabolism or degradation in the crops may occur, which may account for another part of the CAP loss.

The free CAP concentration in the wheat stems and the maize stalks is significantly higher than that in the wheat spikes or maize cobs. The average difference is approximately a factor 30 for wheat and 15 for maize which suggests a relatively higher transfer into maize cobs compared to wheat spikes. The data were studied in more detail using analysis of variance (ANOVA) in which the total amount of administered CAP and the soil type were considered as the factors under investigation. Although severe bio-variability is observed (variation between pots was significantly higher than variation between duplicate analyses of the same pot), for all plant materials, the effect of the administered CAP level, the soil type and the interaction of both were statistically significant ($\alpha < 0.001$). The administered CAP concentration and the soil type are both related to the theoretical bioavailability of the antibiotic and thus, it is most likely that the observed effects are related to a single parameter of importance, being the bioavailability of the antibiotic. The effects were more significant for wheat spikes and stems compared to maize stalks and cobs. As an example, the difference in CAP concentration in wheat grown on topsoil and sub-soil is a factor 20, whereas this is a factor 4 for maize.

For all experiments, the absolute amount of free CAP in the plants (total of stems and spikes for wheat, and stalks and cobs for maize) was divided by the total administered amount of CAP. The results for each experiment are presented in table 3. Note that the calculated transfer rates strongly depend on the experimental set-up, e.g. if CAP is administered continuously instead of weekly, the bioavailability of CAP would increase and a higher uptake is expected. Furthermore, only free CAP is taken into account, so if CAP is metabolised or conjugated in the crops, the actual transfer of CAP is higher than calculated here.

Table 4.12. Detected amount of free CAP in wheat and maize grown on topsoil, mixed soil and sub-soil versus administered CAP (n = 2).

Soil type	CAP administration (mg pot ⁻¹)	Detected CAP vs administered CAP (%)			
		Wheat (n=2)		Maize (n=2)	
		Pot 1	Pot 2	Pot 1	Pot 2
Topsoil	7.5	0.007	0.001	0.02	0.02
	75	0.02	0.009	0.02	0.02
Mixed soil	7.5	0.02	0.006	0.04	0.07
	75	0.006	0.07	0.04	0.05
Sub-soil	7.5	0.17	0.06	0.19	0.10
	75	0.19	0.10	0.05	0.07

A final observation from the transfer study is that CAP was detected in two of the wheat samples (up to 7 µg kg⁻¹) and two of the maize samples (up to 2 µg kg⁻¹) belonging to the untreated population. External contamination in the greenhouse was excluded by the set-up of the experiment and contamination in the laboratory was excluded by repeated analyses on another occasion and thus it was concluded that this most probably must have been the result of natural production of CAP in the pots.

Can CAP production in soil yield residues in crop?

The production and degradation of CAP in soil occur simultaneously, and both processes depend on many environmental parameters. Therefore, it is not possible to determine the total amount of free CAP produced in non-sterile soil that is available for uptake by the crops. Consequently it is also not possible to calculate what level of free CAP in crops can still be explained through natural CAP production by soil bacteria and subsequent uptake by crops. Based on the experimentally determined uptake rates (table 4.12) however, calculations were made to obtain an estimate of the required magnitude of CAP production in soil to explain the observed non-compliant findings in crops. Here it is calculated what level of CAP should be produced by soil bacteria to result in detection of 0.1 µg kg⁻¹ free CAP in crops.

The average mass of a single full grown wheat plant as determined in the transfer study is 16 g (fresh weight). Therefore, $0.1 \mu\text{g kg}^{-1}$ equals 1.6 ng CAP per wheat plant. Considering an average transfer rate for a wheat plant growing on topsoil of 0.009 % (average transfer rate using topsoil, table 4.12), a single plant should be exposed to 17 μg CAP during its lifetime. A regular field contains 200 wheat plants per square meter and thus on this square meter 3.4 mg of CAP should be produced to yield a level of $0.1 \mu\text{g kg}^{-1}$ of CAP in all of these wheat plants. Considering the availability of nutrients in a 30 cm layer of soil, having a density of 1.4 kg L^{-1} these crops have 420 kg of soil available and therefore CAP production should be $8 \mu\text{g kg}^{-1}$ soil in total during the growing period of the crops to yield $0.1 \mu\text{g kg}^{-1}$ of CAP in wheat. For maize a level of $2 \mu\text{g kg}^{-1}$ in the topsoil is required assuming a plant density of 10 maize plants per square meter and an average maize plant mass of 185 g (fresh weight). CAP levels as high as $10 \mu\text{g kg}^{-1}$ in wheat, being the highest concentration detected, can be explained if in total 800 μg of CAP is produced per kg soil when considering topsoil. Note that these levels do not indicate the level that should be present in soil at a certain time, but rather the total amount of CAP that should be produced per kg of soil during the whole crop production time of approximately 10 weeks.

Conclusions

The results from the CAP production experiment described here, showed that over $100 \mu\text{g kg}^{-1}$ CAP can be produced by *Streptomyces venezuelae* in non-sterile topsoil within a single day. This suggests that the low-ppb concentrations of CAP, and possibly also the high concentrations observed in crops in monitoring studies can be explained by the natural production of CAP by soil bacteria and the subsequent uptake of the drug by crops.

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Chapter 5

The Analysis of β -lactam antibiotics



B.J.A. Berendsen, M.L. Essers, P.P.J. Mulder, G.D. van Bruchem, A. Lommen, W.M. van Overbeek, A.A.M. Stolker, Newly identified degradation products of ceftiofur and cephapirin impact the analytical approach for quantitative analysis of kidney, *J. Chromatogr. A.* 1216, 46 (2009) 8177-8186.

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B.J.A. Berendsen, H.W. Gerritsen, R.S. Wegh, R. van Sebille, S. Lameris, A.A.M. Stolker, M.W.F. Nielen, Comprehensive analysis of β -lactam antibiotics including penicillins, cephalosporins and carbapenems in poultry muscle using liquid chromatography coupled to tandem mass spectrometry, *Anal. Bioanal. Chem.* (2013) In press, DOI: 10.1007/s00216-013-6804-6.

5.1. General introduction on β -lactam antibiotic analysis

β -lactam antibiotics

β -lactams are highly effective antibiotics in the treatment of bacterial infections [1]. The β -lactam antibiotics consist of several groups of which the penicillins, cephalosporins and carbapenems are the most important due to their activity and the number of compounds included.

The first penicillin was isolated from bacteria in 1928 [1,2]. Later many chemically altered semi-synthetic penicillins were developed. The penicillins derive their activity from the 6-aminopenicillanic acid nucleus which is effective against mainly Gram positive bacteria [1,3,4]. Amoxicillin, ampicillin, penicillin G (benzylpenicillin), penicillin V (phenoxymethylpenicillin), cloxacillin, dicloxacillin, oxacillin and nafcillin (figure 5.1) are registered for the treatment of food-producing animals: in fact penicillins are the most frequently used antibiotic in poultry production [5]. Maximum residue limits (MRLs) are established for the mayor species, including poultry (table 5.1) [6].

Cephalosporins were first isolated from bacteria in 1956 [7-9]. The six membered dihydrothiazine ring fused with a four membered β -lactam ring (figure 5.2) is responsible for the biological activity of this group of compounds. Cephalosporins are highly effective antibiotics in the treatment of bacterial infections of the respiratory tract [10]. As for the penicillins, many semi-synthetic cephalosporins were developed and in this four generations are distinguished based upon their time of discovery and their range of activity [11]. Cefacetril, cefalonium, cefazolin, cefalexin and cefapirin (all 1st generation), cefoperazone and ceftiofur (3rd generation), and cefquinome (4th generation) are all approved for treatment of mastitis infections in dairy cattle. Cefazolin is approved for the treatment of other ruminants (sheep and goat) as well. Furthermore, cefalexin and cefapirin are approved for the treatment of respiratory disease and foot rot in cattle, cefquinome is approved for the treatment of cattle, swine and horses, and ceftiofur for all food producing mammals [6,11]. For these species, MRLs are

established (Table 5.1). For cefapirin the MRL is defined as the sum of cefapirin and deacetylcefapirin and for ceftiofur as the sum of all active ceftiofur metabolites [6]. Molecular structures of these cephalosporins are presented in figure 5.2.

Table 5.1. Maximum residue limits for β -lactam antibiotics in muscle.

Compound	Species	MRL ($\mu\text{g kg}^{-1}$)
Amoxicillin	All food producing species	50
Ampicillin	All food producing species	50
Penicillin G	All food producing species	50
Penicillin V	Porcine, poultry	25
Cloxacillin	All food producing species	300
Dicloxacillin	All food producing species	300
Nafcillin	All ruminants	300
Oxacillin	All food producing species	300
Ceftiofur ^a	All mammalian food producing species	1000
Cefapirin ^b	Bovine	50
Cefquinome	Bovine, ovine, caprine	No MRL required
Cefalexin	Bovine	200
Cefazolin ^c	Bovine	No MRL required
Cefalonium ^d	Bovine	No MRL required
Cefacetrile	Bovine	No MRL required
Cefoperazone ^e	Bovine	No MRL required

^a Sum of all residues retaining the β -lactam structure expressed as desfuroylceftiofur.

^b Sum of cefapirin and desacetylcefapirin.

^c For intra-mammary use, except if the udder may be used as food for human consumption.

^d For intra-mammary use and eye treatment only.

^e For intra-mammary use in lactating cows only.

The first carbapenem was isolated from bacteria in 1971 [12]. The carbapenems are structurally very similar to the penicillins: the sulfur atom has been replaced with a carbon atom and an unsaturation has been introduced (figure 5.3) [13]. As a result the carbapenems possess the broadest antimicrobial activity amongst the β -lactams [13]. The most common carbapenems are imipenem, meropenem, ertapenem, doripenem and biapenem. The carbapenems are not registered for use in food-producing animals and are used off-label in companion animals [4]. A

β -lactam substance, related to the carbapenems is faropenem (figure 5.3). As the carbapenems, faropenem is active against ESBL producing bacteria [14].

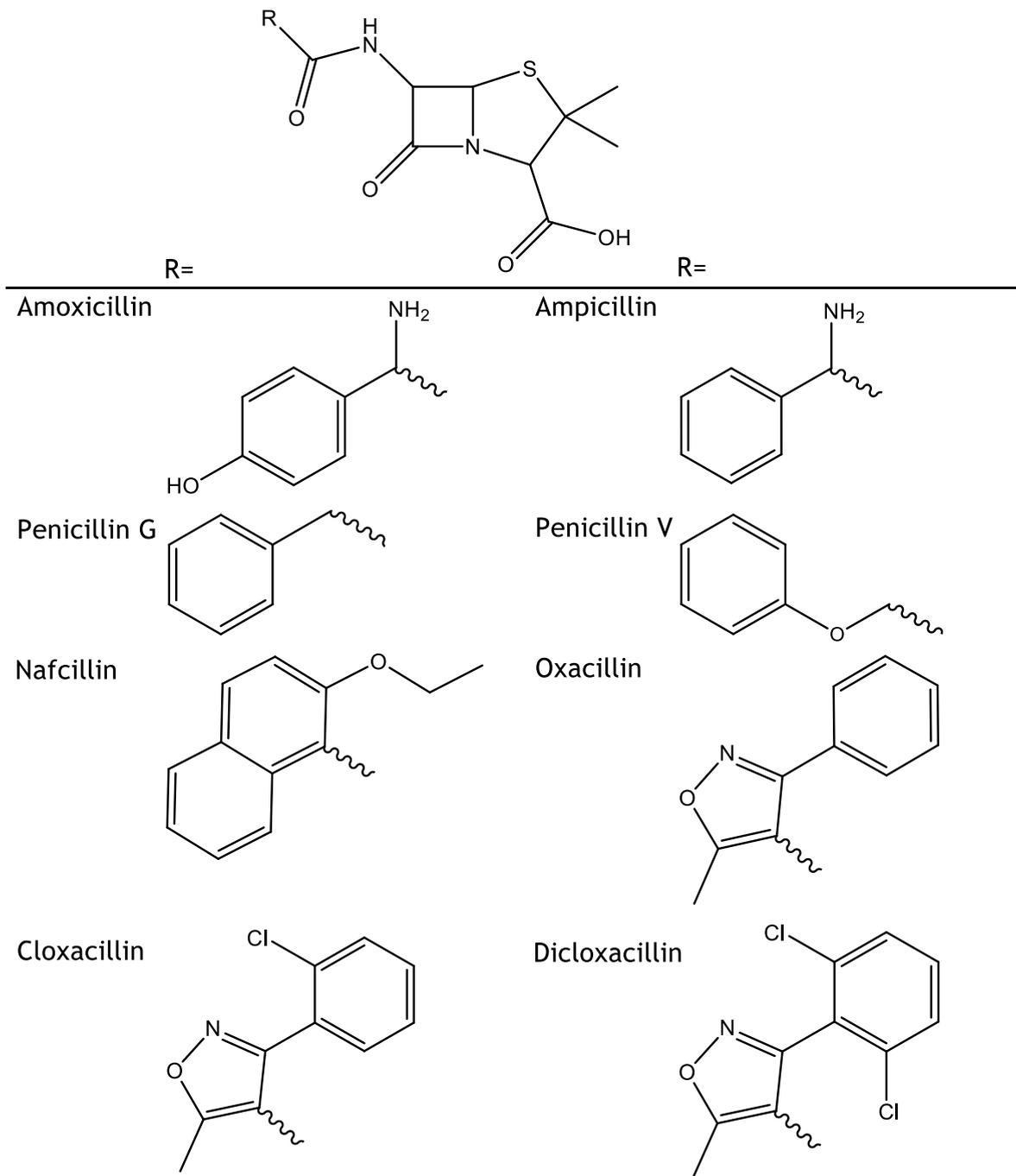


Figure 5.1. Molecular structure of the penicillins registered for use in animal practice.

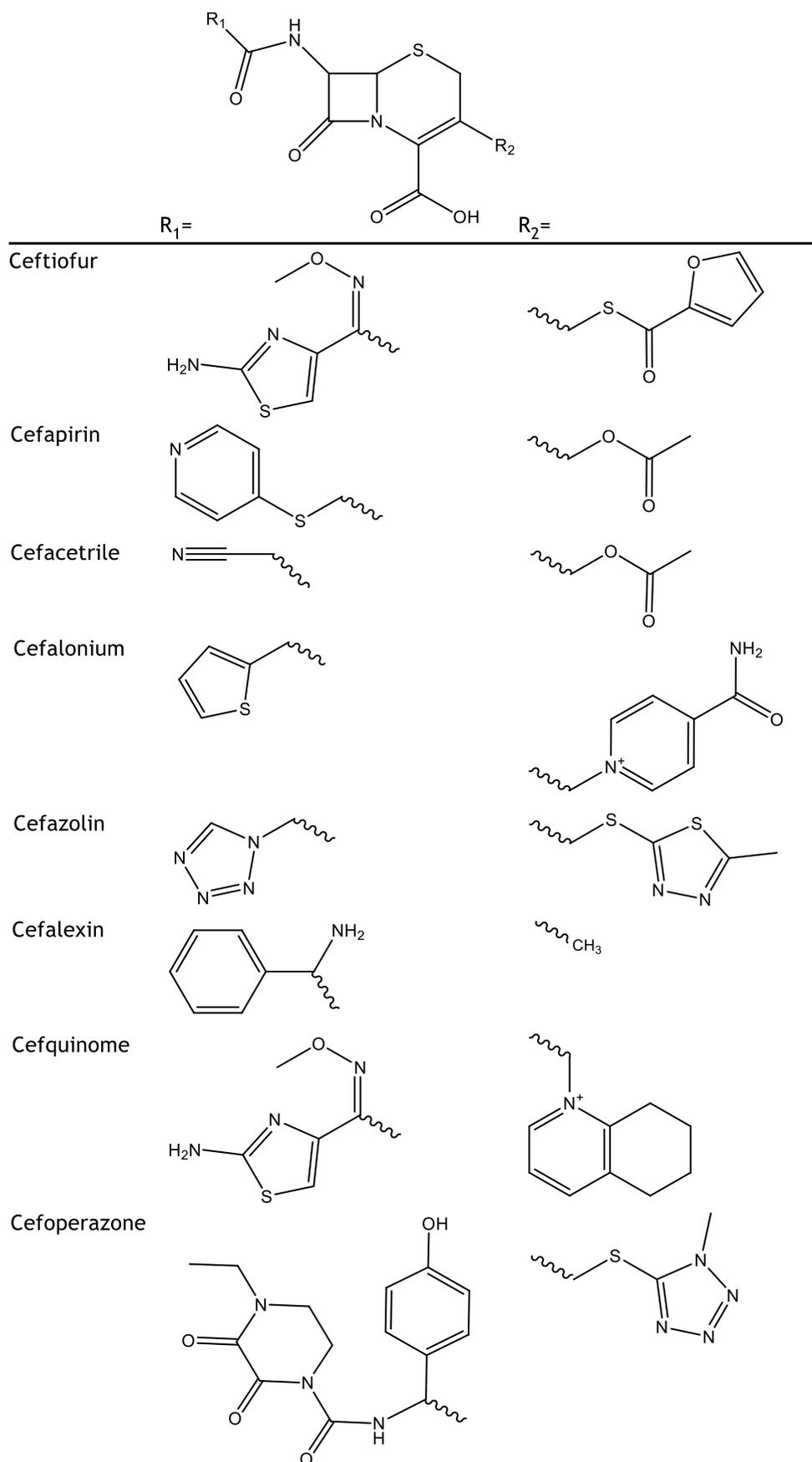


Figure 5.2. Molecular structure of the cephalosporins registered for use in animal practice.

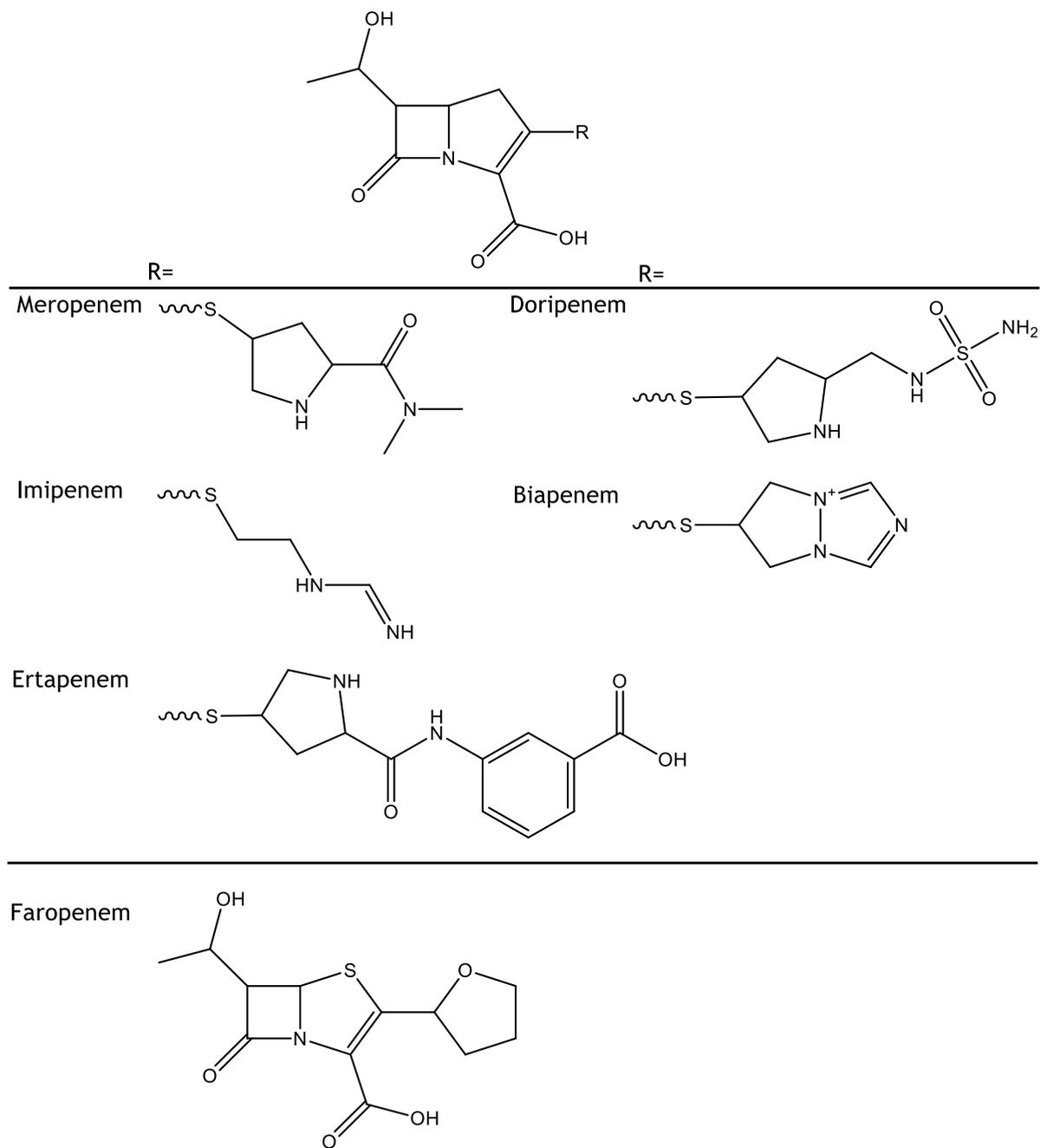


Figure 5.3. Molecular structure of the carbapenems: meropenem, imipenem, ertapenem, doripenem and biapenem and the penem faropenem.

β -lactam resistance

During the last decades, antimicrobial resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum- β -lactamase (ESBL) producing bacteria are emerging [1,15] which has become an important public health threat [15-19]. Most penicillins are found to be ineffective against ESBL producing bacteria [1,20-22]. Cephalosporins are more effective and, therefore, in 2007 they were assigned as critically important antimicrobials for human health [23]. Even though these compounds should only be used sparingly, resistance towards cephalosporins is emerging [1,16-18,22,24-29]. In contrast to penicillins and cephalosporins, carbapenems have proven to be highly effective in severe infections due to ESBL producing bacteria. As a result, due to the rising resistance to cephalosporins, carbapenem use has increased in the treatment of humans [30]. It is alarming that also carbapenem resistance is emerging [13,16,29-31].

Not only the antibiotic usage in humans contributes to rising bacterial resistance, also the regular use of antibiotics in veterinary practice contributes to the occurrence of resistant bacteria that can be transferred from animals to humans [32]. Furthermore, due to irresponsible or off-label use of antibiotics in veterinary practice, residues of these antibiotics can end up in the human food chain which contributes to increasing bacterial resistance as well [33]. β -lactams are among the most frequently sold antibiotics for animal breeding in The Netherlands [5] and their use in veterinary practice is likely to contribute to the emergence of resistant bacteria [15-19]. Penicillins are the most frequently sold antibiotics for treatment of broilers [34] and even though cephalosporins and carbapenems are not registered for use in animal production, due to their high effectiveness their veterinary use, including broilers, cannot be ruled out.

To prevent off-label use of β -lactams in animal breeding and thus to limit the dissemination of bacterial resistance, an effective control strategy for β -lactam usage in food-producing animals is needed including penicillins, cephalosporins and carbapenems. Therefore, the control of poultry muscle for penicillins at MRL level, and cephalosporins as well as carbapenems at levels as low as possible is of importance.

Cephalosporins in food products

The occurrence of residues of cephalosporins in the food chain due to their use as veterinary drug is not considered to be a potential risk, because levels of cephalosporins including known metabolites in edible tissues have been found to be low [35]. However, off-label use of cephalosporins should be restricted because of the risk of the development of bacterial resistance. Therefore the use of cephalosporins should be monitored and thus detection of these compounds at levels as low as reasonably possible is mandatory.

Ceftiofur and cefapirin are known to rapidly metabolise after intramuscular administration. For ceftiofur, reported metabolites include desfuroylceftiofur (DFC, figure 5.4a)), desfuroylceftiofur cysteine disulfide (figure 5.4b) and protein bound DFC [36-38] so the ceftiofur maximum residue limit (MRL) was defined as the sum of all residues retaining the β -lactam structure, expressed as DFC [39,40]. For cefapirin (figure 5.2), The main metabolite reported to date is desacetylcefapirin (DAC) [41] so the MRL was defined as the sum of cefapirin and DAC [39,42]. This is a complicating factor in cephalosporin analysis.

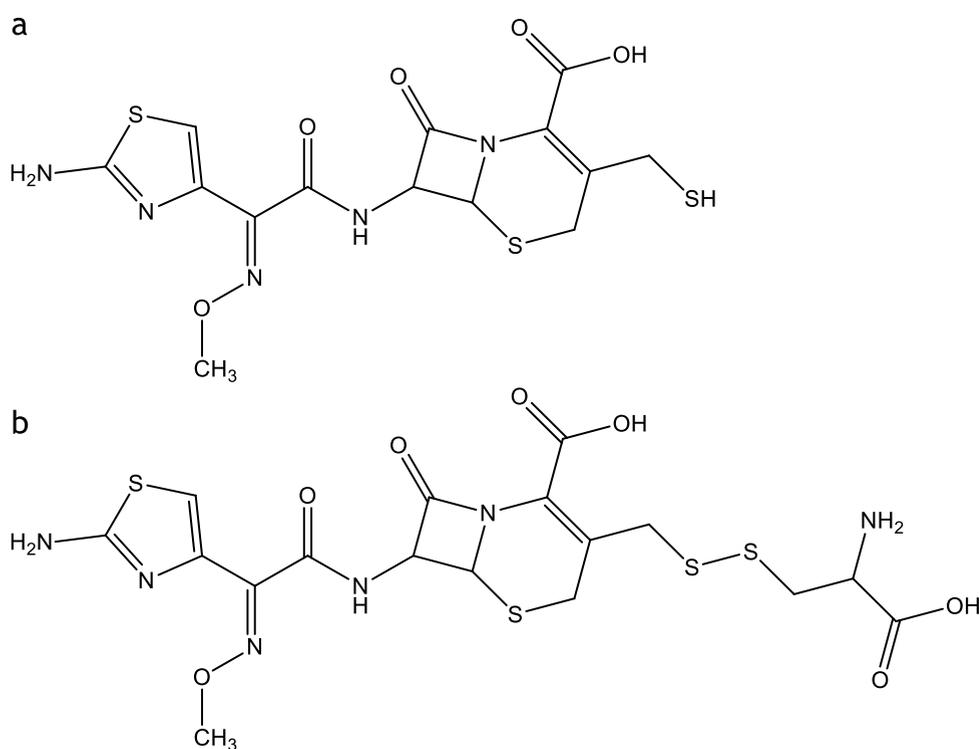


Figure 5.4 Molecular structure of DFC and DCCD.

In section 5.2 a combination of triple-quadrupole mass spectrometry, time of flight mass spectrometry and nuclear magnetic resonance is used to study the degradation of ceftiofur and cefapirin under different conditions. Based on the results a new approach was developed for the analysis of the total residue levels of ceftiofur and cefapirin in kidney.

In section 5.3 the new approach was incorporated in a new analytical procedure. To study the applicability of this new method it was compared to routinely applied methods for the analysis of ceftiofur in poultry tissues.

Other β -lactam antibiotics were included in the newly developed procedure which resulted, after some alterations of the method, in a multi- β -lactam method that includes penicillins, cephalosporins and carbapenems. This work is described in section 5.4.

5.2. Newly identified degradation products of ceftiofur and cefapirin impact the analytical approach for the quantitative analysis of kidney

Abstract

This section describes the research on the degradation of ceftiofur and cefapirin at physiological temperatures in kidney extract and in alkaline and acidic solution, conditions that regularly occur during sample preparation. Degradation products were identified using liquid chromatography combined with Time of Flight mass spectrometry, NMR and microbiological techniques. Additionally kinetics of the degradation processes were studied. A slight instability of cefapirin and desfuroylceftiofur was observed at elevated temperatures. Ceftiofur and cefapirin degraded immediately and completely in an alkaline environment, resulting in inactive degradation products. Ceftiofur and cefapirin also degraded immediately and completely in kidney extract resulting in both formerly reported metabolites as well as not previously reported products.

Our research shows that conditions often occurring during the analysis of ceftiofur or cefapirin result in rapid degradation of both compounds. From this it is concluded that underestimation of the determined amounts of ceftiofur and cefapirin is likely to occur when using conventional methods for the quantitative analysis of these compounds in tissue. Therefore, a new approach is needed for the analysis of both compounds including their degradation products.

Introduction

Cephalosporins, including ceftiofur and cefapirin (figure 5.2), are semi-synthetic antibiotics, consisting of a six membered dihydrothiazine ring fused with a four membered β -lactam ring which is responsible for the biological activity of the compounds. Because cephalosporins are highly effective antibiotics in the treatment of bacterial infections of the respiratory tract [10], the common use of cephalosporins in veterinary practice is expected. An effective monitoring of

cephalosporin use in animal breeding is mandatory to prevent excessive use, which will contribute to the emergence of bacterial resistance.

In monitoring food products, it is not clear that all relevant metabolites and degradation products, such as those produced by the degradation of ceftiofur and cefapirin during sample preparation, are taken into account when current methods are used to test for ceftiofur and cefapirin in tissue samples. The main causes of such degradation can be (1) the use of elevated temperatures, (2) the presence of tissue extract [43] and (3) an acidic or alkaline environment [44]. If degradation caused by these three aspects is not taken into account it is possible that ceftiofur and cefapirin residues are underestimated.

Ceftiofur and cefapirin are known to rapidly metabolise after intramuscular administration. For ceftiofur, reported metabolites include desfuroylceftiofur (DFC), desfuroylceftiofur cysteine disulfide and protein bound DFC [36-38] so the ceftiofur maximum residue limit (MRL) was defined as the sum of all residues retaining the β -lactam structure, expressed as DFC [6]. For cefapirin, The main metabolite reported to date is desacetylcefapirin (DAC) [41] so the MRL was defined as the sum of cefapirin and DAC [6].

Cephalosporin multi-methods that include both ceftiofur and cefapirin are lacking, although methods to detect cetiofur and cefapirin in tissue separately or in combination with a limited number of other cephalosporins have been reported [35,43,45-47].

Methods for the analysis of ceftiofur in tissue include a deconjugation and extraction of all protein bound DFC in slightly alkaline buffer (pH=9) using dithioerythritol followed by a derivatization of the resulting free DFC at pH=2.5 using iodoacetamide [35,45] and an extensive Solid Phase Extraction (SPE) procedure in which alkaline solutions are used. This method is not very robust so the procedure has to be closely followed to obtain good results and it is limited to the analysis of a few cephalosporins and thus unsuitable as a multi-method. Other methods focus only on one or a few metabolites of ceftiofur [43,46].

Methods for the analysis of cefapirin are based on the complete conversion of cefapirin to DAC within 5 minutes in kidney extract [43,47]. The degradation processes possibly occurring after this time are not taken into account.

Degradation of ceftiofur and cefapirin under three circumstances: physiological temperatures, the presence of kidney extract and acidic or alkaline environment, is reported in this section. Degradation products are detected and identified using liquid chromatography coupled to Time of Flight mass spectrometry (LC-ToF/MS) which is a high resolution 'full scan' technique. This means that by ToF/MS all ions are detected in contrary to triple quadrupole techniques in which only a predefined selection of ions is monitored [48,49]. Accurate mass determination and calculated elemental composition data can be used for structure elucidation. This makes ToF/MS very useful for the detection and identification of 'unknown' compounds. LC-ToF/MS is not previously reported for the identification of cephalosporin metabolites and degradation products. Additional structural information was obtained by NMR and microbiological experiments. Combining information from LC-ToF/MS, NMR and microbiological experiments made it possible to identify new degradation products of ceftiofur and cefapirin. The new identified products indicate that currently applied methods are likely to underestimate the residue levels of ceftiofur and cefapirin found in kidney samples. Furthermore, this research resulted in a new approach for the quantitative analysis of ceftiofur, cefapirin and other cephalosporins in tissue.

Experimental

Material

LC-MS grade water and methanol and HPLC grade methanol, HPLC grade acetonitrile, 2-propanol (Biosolve, Valkenswaard, The Netherlands), 25 % ammonia, acetic acid, 99.8 % methanol-d₄, sodium hydroxide, sodium formate, ortho-phosphoric acid (85 %), sodium hydrogendiphosphate, disodium hydrogenphosphate, trisodium phosphate (Merck, Darmstadt, Germany) and leucine-enkephalin (Sigma, St. Louis, MO, USA) were used. Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 10 M Ω ·cm (Millipore, Billerica, MA, USA). Cefapirin, desacetylcefapirin (DAC) and cefalothin lacton were a kind gift of Intervet (Boxmeer, The Netherlands). Ceftiofur was obtained from Pfizer (Capelle aan den IJssel, The Netherlands) and desfuroylceftiofur (DFC) was obtained from Toronto Research Chemicals (North York, Canada).

Stock solutions of ceftiofur, DFC, cefapirin and DAC were prepared in methanol at 100 µg/L. Sodium phosphate buffer 0.2 M at pH=2.5; 5.0; 7.0; 7.5; 9.0; 10.0; 11.0 and 12.0 was prepared by mixing 0.2 M aqueous solutions of ortho-phosphoric acid, sodium hydrogendiphosphate, disodium hydrogenphosphate and trisodium phosphate while determining the pH using a Schott CG840 pH-meter (Mainz, Germany) calibrated using certified pH 4.0, 7.0 and 10.0 buffers (Merck).

Preparation of kidney extract

A blank bovine kidney sample was defrosted and homogenised at room temperature, after which 5 g was transferred to a 50 mL test tube. An extract was made by adding 25 mL of Milli-Q water. After mixing for 15 min by using a rotary tumbler the extract was centrifuged at 3500 × g using a Falcon 6/300 centrifuge (London, UK). The supernatant was decanted in a new 50 mL test tube. Fresh kidney extracts were prepared daily.

LC-QqQ/MS

The LC instrumentation consisted of two Shimadzu (Nakagyo-ku, Kyoto, Japan) LC-20AD high pressure liquid delivery pumps, a SIL-HTc autosampler combined with a CTO-20A column oven. A chromatographic separation was established using a Symmetry C₁₈ analytical column, 150 x 3 mm, 5 µm (Waters, Manchester, UK). The gradient (mobile phase A, 0,05 % ammonia in water, pH adjusted to 8 with acetic acid; mobile phase B, 0.05 % ammonia in water/methanol (1:9, v/v), pH adjusted to 8 with acetic acid; flow rate 0.4 mL min⁻¹) was: 0 - 3 min, 15 % B; 3 - 8 min, linear increase to 100 % B; 8 - 9 min, 100 % B; 9 - 9.5 min, return to 15 % B. The flow was split 1:1 (MS:waste). Under these conditions, ceftiofur eluted after 8.3 minutes and cefapirin after 7.8 minutes.

Detection was carried out using a Waters Quattro Ultima mass spectrometer operating with electrospray ionisation (ESI) in positive mode. The operating parameters were: capillary voltage, 1.5 kV; cone voltage, 25 V; source temperature, 120 °C; desolvation temperature, 300 °C; cone gas flow, 200 L hr⁻¹; and desolvation gas, 500 L hr⁻¹. Ceftiofur, DFC, cefapirin and DAC were fragmented using collision induced dissociation (CID) with the settings presented in table 5.2. Data were acquired and processed using MassLynx 4.1 software (Waters).

Table 5.2. Precursor ions, collision energy and product ions of ceftiofur, DFC, cefapirin and DAC.

Compound	Precursor ion (m/z)	Collision energy (eV)	Product ion (m/z)
Ceftiofur	524	45	125
		17	241
DFC	430	25	126
		15	241
Cefapirin	424	13	292
		22	152
DAC	382	25	152
		15	292

LC-ToF/MS

The LC instrumentation used was an Acquity UPLC system and the separation was established on a Symmetry C₁₈ analytical column, 150 x 3 mm, 5 µm (Waters).

The gradient (mobile phase A, 0.05 % ammonia in LC-MS quality water, pH adjusted to 8 with acetic acid; mobile phase B, 0.05 % ammonia in LC-MS quality water/methanol (1:9, v/v), pH adjusted to 8 with acetic acid; flow rate 0.4 mL min⁻¹) was: 0 - 3 min, 15 % B; 3 - 13 min, linear increase to 100 % B; 13 - 18 min, 100 % B; 18 - 18.5 min, return to 15 % B. The flow was split 1:1 (MS:waste). Under these conditions ceftiofur eluted after 9.7 minutes and cefapirin after 8.6 minutes.

Detection was carried out using a Waters LCT Premier equipped with ESI. The instrument was operated in the positive W-mode (resolution ≥ 10,000) and was calibrated spanning a range of 90 to 1050 using a solution of sodium formate in 2-propanol to obtain a mass error below 5 ppm. Scans were performed in the continuum mode using a scan time of 0.2 seconds. The operating parameters were: capillary voltage, 2.7 kV; cone voltage, 20 V; source temperature, 150 °C; desolvation temperature, 300 °C; cone gas flow, 30 L hr⁻¹; desolvation gas, 400 L hr⁻¹; and aperture 1 voltage, 4. A solution of 1 µg mL⁻¹ leucine-enkephalin in water/acetonitrile (1:2), infused at a flow rate of 10 µL min⁻¹ was used as a reference, resulting in a lock mass of m/z = 557.2802 and an attenuated lock mass of m/z = 556.2771. The reference scan frequency was set at 10 scans, the reference cone voltage at 20 V and the reference aperture 1 voltage at 8. Data were acquired and processed using MassLynx 4.1 software (Waters).

Methods

Stability study

The stability of solutions of ceftiofur, DFC, cefapirin and DAC at elevated temperature was tested. Stock solutions were diluted to 200 ng mL⁻¹ in water of which 20 mL was transferred to a test tube and placed into a Julabo 25 water bath (Julabo, Seelbach, Germany) set at a temperature of 37 °C. After 0; 0.5; 1; 2; 4; 6; 24; 48; 72 and 144 hours, 1 mL of the solutions was transferred to an LC-MS vial and stored at < -70 °C. All solutions were defrosted at room temperature and 50 µL was injected onto the LC-QqQ/MS system to monitor ceftiofur, DFC, cefapirin and DAC.

The stability of solutions of ceftiofur, DFC, cefapirin and DAC at different pHs was also tested. Stock solutions were diluted in each of the phosphate buffer solutions to obtain 10 µg mL⁻¹ solutions at different pHs in the range of 2.5 through 12. After 0, 30, 60, 120 and 180 minutes at room temperature, 100 µL of these solutions was combined with 4.9 mL sodium phosphate buffer, 0.2 M, pH=7, conditions at which the compounds were considered stable, to quench possible degradation, after which 50 µL was injected onto the LC-QqQ/MS system to monitor ceftiofur, DFC, cefapirin and DAC. The compounds were considered unstable when the peak intensity decreased over 10 %.

Fractionation

The LC instrumentation used for fractionation was a 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a G1312B binary pump solvent delivery system, a G1322A degasser, a G1329A auto sampler with a G1330B thermostat and a G1316A column oven combined with a G1314B UV-detector and a G1364B fraction collector with a G1330B thermostat. A separation was established using an X-Bridge C₁₈ analytical column, 150 x 3 mm, 5 µm (Waters). The gradient (mobile phase A, 0,05 % ammonia in water; mobile phase B, 0.05 % ammonia in water/methanol (1:9, v/v); flow rate 0.4 mL min⁻¹) was: 0 - 3 min, 5 % B; 3 - 43 min, linear increase to 100 % B; 43 - 44 min, 100 % B; 44 - 44.5 min, return to 5 % B.

Concentrated solutions of $500 \mu\text{g mL}^{-1}$ cefapirin and 1 mg mL^{-1} ceftiofur were prepared in methanol. Both solutions and pure methanol were diluted tenfold in water to obtain solutions containing 10 % methanol after which 2 mL was transferred to different test tubes in duplicate, resulting in two identical sets, each set consisting of one blank tube, one containing $100 \mu\text{g}$ cefapirin, and one containing $200 \mu\text{g}$ ceftiofur. To the first set, 0.5 mL of 25 % ammonia was added. To the second set, 2.5 mL of kidney extract was added. Both sets were incubated for four hours in a water bath at $37 \text{ }^\circ\text{C}$. Of each of the incubates, 20 times $100 \mu\text{L}$ was injected on the fractioning system. Fractions of 30 sec were taken from a retention time of 6 min through 34 min. The different fractions of each of the 20 injections were combined in test tubes resulting in 4 mL fractions. From the collected fractions the methanol was evaporated at room temperature under a stream of nitrogen, $200 \mu\text{L}$ was transferred to an LC-MS vial and the remaining aqueous solvent in the test tubes was evaporated overnight at room temperature under nitrogen. The resulting residues were diluted in $700 \mu\text{L}$ deuterated methanol and subjected to analysis by NMR. From the LC-MS vials $25 \mu\text{L}$ was injected on the LC-ToF/MS system to identify the fractions containing relevant degradation products. Of the relevant fractions, the remaining solution in the LC-MS vial was subjected to the microbiological test.

Identification by LC-ToF/MS

Stock solutions of ceftiofur and cefapirin were diluted to $10 \mu\text{g mL}^{-1}$ in water. Four sets of five test tubes were prepared, each set consisting of a test tube containing 5 mL $10 \mu\text{g mL}^{-1}$ ceftiofur, 5 mL $10 \mu\text{g mL}^{-1}$ DFC, 5 mL $10 \mu\text{g mL}^{-1}$ cefapirin, 5 mL $10 \mu\text{g mL}^{-1}$ DAC and 5 mL 10 % methanol in water. The sets were treated according to the conditions presented in table 5.3. After the incubation time the solutions were stored at $< -70 \text{ }^\circ\text{C}$. All solutions were defrosted at room temperature and shaken by hand after which an aliquot of each solution was filtered using a 30 kD ultrafilter (3500 G, 15 minutes), transferred to an LC-MS vial and analysed by LC-ToF/MS.

Table 5.3. Incubation conditions for the identification experiment.

Set	Addition	Incubation temperature (°C)	Incubation time (h)
1	-	37	72
2	62.5 µL 25 % ammonia	Room temperature	2
3	2.5 mL kidney extract	37	72
4	2.5 mL kidney extract + 62.5 µL 25 % ammonia	37	72

MetAlign Software (www.metalign.nl, RIKILT Wageningen UR, The Netherlands) was used to detect degradation products. This software aligned chromatograms of the blank solution with the chromatograms of the ceftiofur and cefapirin spiked solutions, after which the differences between the two sets of chromatograms were determined. This procedure resulted in chromatograms containing accurate mass full scan data showing mainly degradation products. For each degradation product, the most likely molecular formula was selected, using the elemental composition option in MassLynx 4.1, combining the determined accurate mass and isotope ratio. The molecular formula of the degradation products was determined with a high certainty because ceftiofur and cefapirin contain sulfur atoms having a very specific isotope [M+2] and several nitrogen atoms. From these molecular formulas, possible molecular structures were generated.

NMR

¹H NMR experiments were performed on a Bruker Avance 400 MHz spectrometer. Presaturation was performed on the residual HDO resonance. A 90° pulse was used; the total relaxation delay was 3.7 s; the spectral width was 5000 Hz. The data were acquired in 16 K data points. Before Fourier transformation and phasing, a 1/3 shifted sine squared window multiplication was applied and a zero-filling to 128 K data points were applied. Calibration of spectra was achieved by setting the HCD2 resonance of deuterated methanol to 3.27 ppm.

Microbiology

Antimicrobial activity was determined using a modified *Bacillus stearothermophilus* var. *calidolactis* C953 disk assay method. Aliquots of 6 mL Plate Count Agar (Difco) inoculated with approximately 10^6 CFU mL⁻¹ spores were allowed to set in petri dishes with a diameter of 9 mm. Samples of 100 μ L were pipetted on filter paper sample disks (diameter 12.7 mm, Whatman, Schleicher & Schuell) and transferred to the center of a test plate using tweezers. The plates were incubated for 3.5 to 5 hours at 55 °C. Antimicrobial activity becomes visible as a zone of growth inhibition around the paper disk.

Kinetics

The kinetic experiment was carried out for ceftiofur and cefapirin separately. Stock solutions of ceftiofur and cefapirin were diluted to 10 μ g mL⁻¹ in water, and 10 mL of each was transferred into four 14 mL test tubes. Nothing further was added to the first test tube, 5 mL kidney extract was added to the second test tube, 125 μ L 25 % ammonia was added to the third test tube and 5 mL kidney extract and 125 μ L 25 % ammonia were added to the fourth test tube. The test tubes were placed in a water bath at 37 °C. After 0; 0.5; 1; 2; 4; 8; 24; 50; 72 and 144 hours, 1 mL of each of the solutions was transferred to a clean test tube and stored at < -70 °C. Just before analysis, all solutions were defrosted at room temperature, shaken by hand, filtered using a 30 kD ultrafilter (Millipore) (3500 G, 15 minutes), transferred to an LC-MS vial and analysed by LC-ToF/MS.

From the data obtained, the formation of the degradation products was studied in a qualitative way using the MetAlign Software. These data were unsuited for quantitation purposes, because no reference standards are available and because different degradation products do not necessarily show the same ionisation efficiency, resulting in a different sensitivity on the LC-MS system. In the figures displaying kinetic results the highest signal obtained is set at 100 % and all other signals are related to this without suggesting a quantitative relation exists.

Results & Discussion

An overview of suggested¹ degradation products is presented in table 5.4 (page 215) including retention time, accurate mass, molecular formula and suggested compound name. Molecular structures of the most relevant products are presented in figure 5.5 for ceftiofur and figure 5.6 for cefapirin.

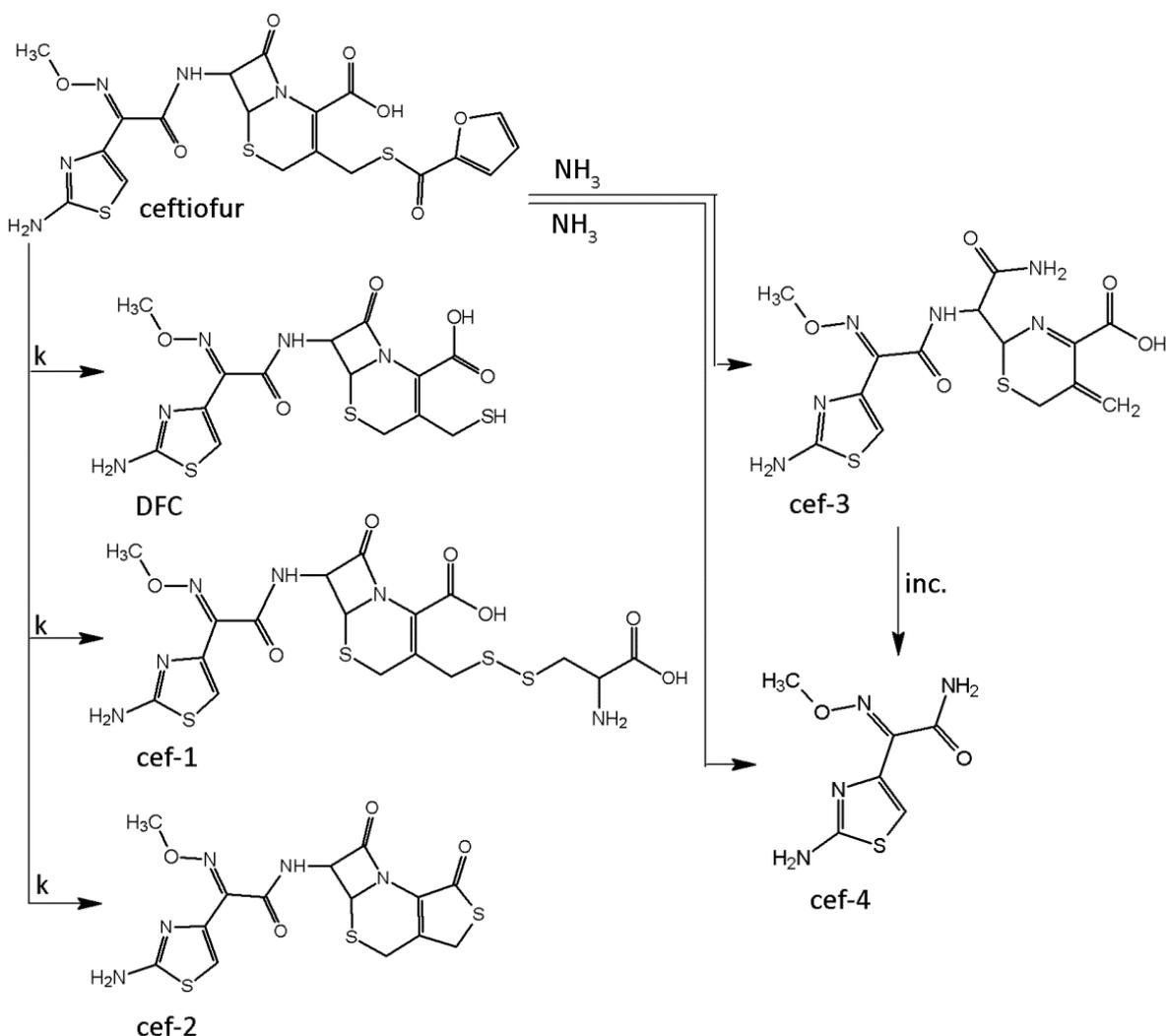


Figure 5.5. Overview of the suggested degradation products of ceftiofur (k) in kidney extract, (NH₃) after addition of ammonia and (inc.) after prolonged incubation at 37 °C. The corresponding retention times and molecular formulas are presented in table 5.4 (page 215).

Footnote

¹ All structures proposed for unknown degradation products are suggested based on the combination of data obtained from different techniques (LC-ToF, NMR and/or microbiological methods). Because of the lack of reference standards for the proposed degradation products, none of these structures can be considered as 100 % identified.

Physiological temperature

Stability

A representative LC-QqQ/MS chromatogram as obtained during the stability study is presented in figure 5.3. Due to the QqQ/MS technique applied, which is a targeted technique, only predefined compounds are monitored; possible other compounds, like degradation products, are not detected.

The stability of ceftiofur, DFC, cefapirin and DAC in aqueous solutions of 200 $\mu\text{g L}^{-1}$ at a temperature of 37 °C is presented in figure 5.7. Ceftiofur and DAC were stable for 24 hours at 37 °C, showing decreases in the signal of 2 % (ceftiofur) and 4 % (DAC), which are considered not to be significant.

Cefapirin is relatively unstable at 37 °C, showing 40 % degradation after 24 hours. DFC shows 25 % degradation after 24 hours and during incubation an additional peak appears in the DFC transition 430 > 126 eluting 0.3 minutes before DFC. The two peaks likely represent the syn- and anti-oxyimino isomers of DFC [50] for two reasons. First, the sum of the isomer and DFC results in 100 % of the initial amount of DFC. Second, from the results it is extrapolated that after 9 days of incubation 50 % of DFC is converted into the isomer and equilibrium is established.

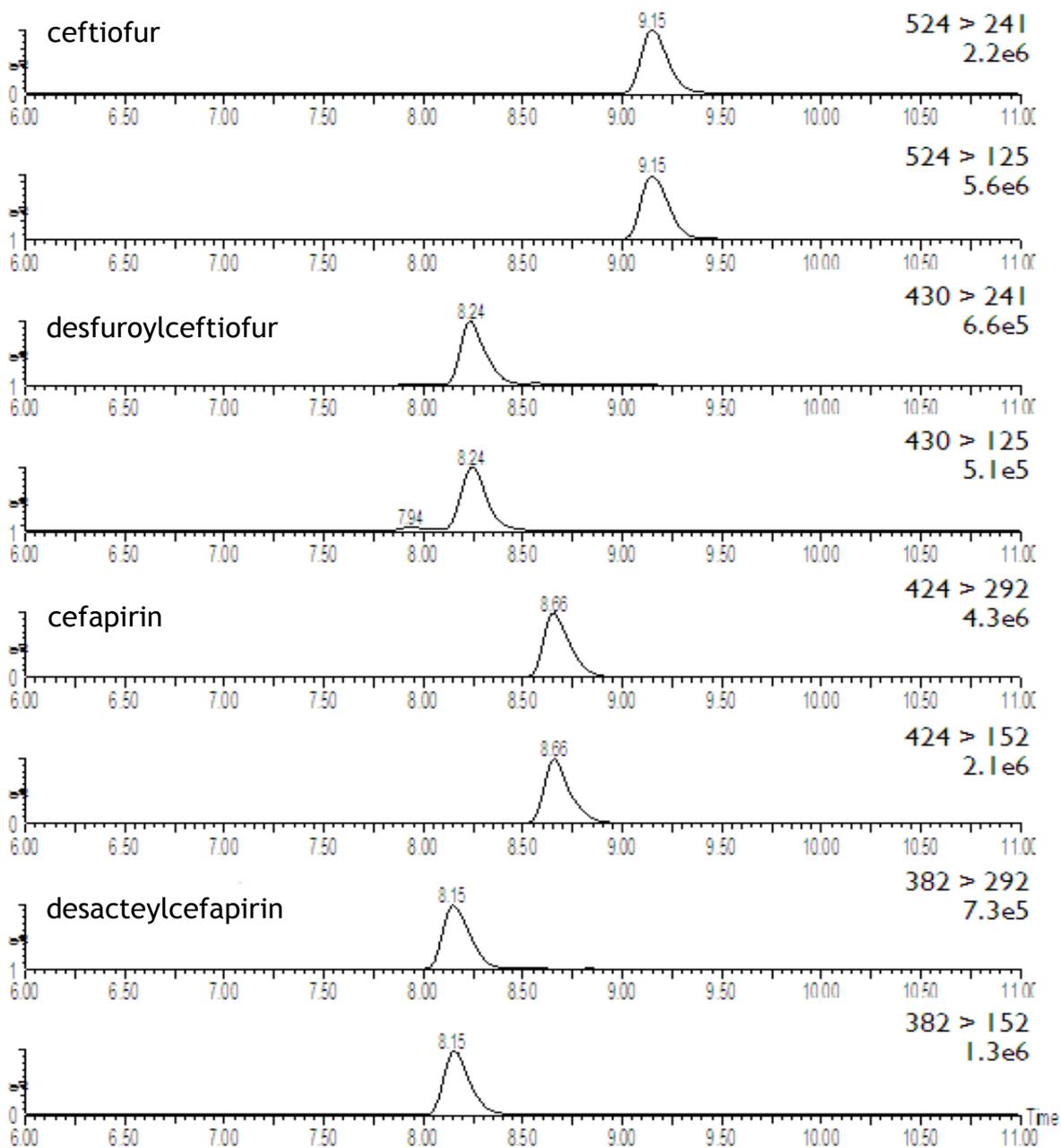


Figure 5.7. MRM chromatogram of a mixture of $200 \mu\text{g L}^{-1}$ ceftiofur, DFC, cefapirin and DAC.

Table 5.4. Overview of suggested degradation products of cefapirin under several conditions including retention time on LC-ToF/MS system, accurate mass and proposed molecular formula. Proposed structures are given in figure 5.5 and 5.6.

Code	RT (min)	Accurate mass [M+H] ⁺	Mol. Formula [M+H] ⁺	Suggested compound name
Ceftiofur	9.9	524.036	C ₁₉ H ₁₈ N ₅ O ₇ S ₃	
DFC	7.8	430.031	C ₁₄ H ₁₆ N ₅ O ₅ S ₃	
Cef-1	6.5	549.036	C ₁₇ H ₂₁ N ₆ O ₇ S ₄	DFC-cysteine disulfide
Cef-2	8.0	412.021	C ₁₄ H ₁₄ N ₅ O ₄ S ₃	DFC-thiolacton
Cef-3-NH ₃	6.5	413.070	C ₁₄ H ₁₇ N ₆ O ₅ S ₂	Ammoniated desthiofuoylceftiofur
Cef-4	2.7	201.045	C ₆ H ₉ N ₄ O ₂ S	2-amino- α -(methoxyimino)-4- thiazoleacetamide (AMTA)
Cefapirin	8.8	424.064	C ₁₇ H ₁₈ N ₃ O ₆ S ₂	
DAC	7.4	382.053	C ₁₅ H ₁₆ N ₃ O ₅ S ₂	
Cefp-1a	8.4	396.068	C ₁₆ H ₁₈ N ₃ O ₅ S ₂	Methoxy DAC
Cefp-1b	9.3	396.068	C ₁₆ H ₁₈ N ₃ O ₅ S ₂	DAC methyl ester
Cefp-2	8.1	364.043	C ₁₅ H ₁₄ N ₃ O ₄ S ₂	Desacetoxy cefapirin
DAC-NH ₃	6.6	399.080	C ₁₅ H ₁₉ N ₄ O ₅ S ₂	Ammoniated DAC
Cefp-2-NH ₃	7.6	381.069	C ₁₅ H ₁₇ N ₄ O ₄ S ₂	Ammoniated desacetoxy cefapirin
DAC-NH ₃ d	7.3	397.064	C ₁₅ H ₁₇ N ₄ O ₅ S ₂	Ammoniated desacetyl dehydrocefapirin
Cefp-2-NH ₃ d	8.2	379.054	C ₁₅ H ₁₅ N ₄ O ₄ S ₂	Ammoniated desacetoxy cefapirin
Cefp-3	6.5	169.044	C ₇ H ₉ N ₂ OS	2-(pyridine-4-ylthio)acetamide (PTA)

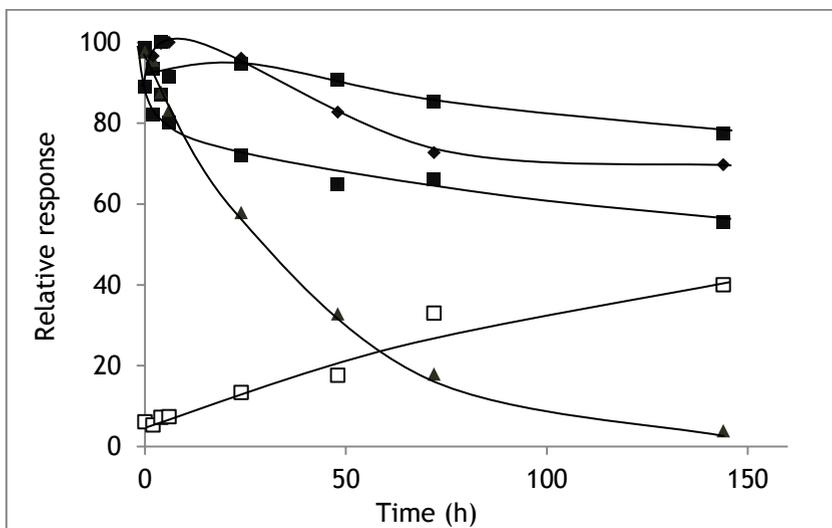


Figure 5.8. The kinetics for (●) ceftiofur, (■) DFC, (□) a conformational isomer of DFC, (▲) cefapirin and (◆) DAC during incubation at 37 °C.

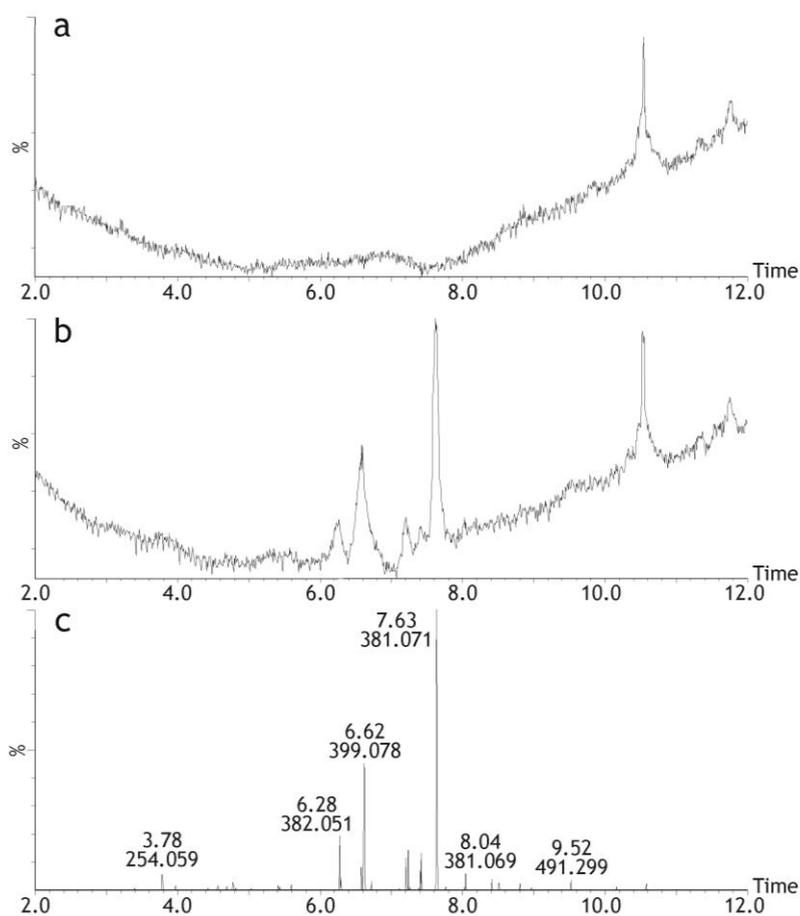


Figure 5.9. LC-ToF/MS chromatogram of (a) 10 % methanol + 62.5 μL 25 % ammonia, (b) 10 $\mu\text{g L}^{-1}$ cefapirin + 62.5 μL 25 % ammonia and (c) the resulting MetAlign output.

Kinetics

An example of a typical LC-ToF/MS chromatogram including the MetAlign™ output as obtained during the identification of unknown degradation products is presented in figure 5.9. Because ToF/MS was used, which is a non-targeted technique, all products, including interferences and new degradation products, are detected.

Ceftiofur remains stable during incubation at 37 °C. For cefapirin at 37 °C in 10 % methanol, three major degradation products, including DAC were detected. The accurate masses of the other products were 396.068 (cefp-1), showing two peaks and 364.043 (cefp-2) (table 5.4, page 215). The formation of these degradation products and the decay of cefapirin during incubation at 37 °C are presented in figure 5.6. DAC is immediately produced and the concentration increases during 24 hours. For cefp-1 two peaks are found at retention times of 8.4 and 9.3 min which might be caused by the presence of two different forms (a and b). The production of cefp-1a and b is complete after 75 hours. Cefp-2 reaches a maximum intensity after 4 hours of incubation, after which it remains constant.

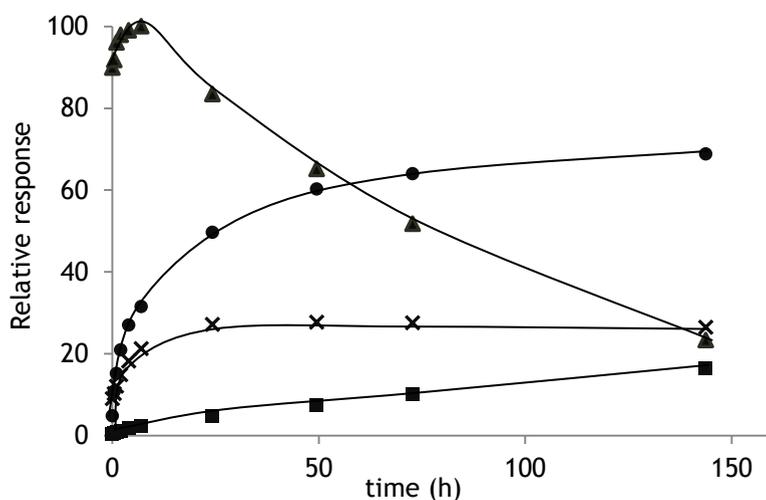


Figure 5.10. The kinetics for (▲) cefapirin, (x) DAC, (●) cefp-1 and (■) cefp-2 during incubation of an aqueous cefapirin solution at 37 °C.

Kidney extract

Kinetics

For ceftiofur, three major degradation products were detected, with accurate masses of 430.031 (DFC), 549.035 (cef-1) and 412.021 (cef-2). DFC is produced immediately after addition of kidney extract but is unstable under these conditions. The products cef-1 and cef-2 show a maximum intensity after 4 hours of incubation (figure 5.11a).

For cefapirin, three major degradation products were detected: DAC, cefp-1b and cefp-2. DAC is produced immediately after addition of kidney extract as has also been reported [43,47] but is unstable and therefore not the only degradation product (figure 5.11b). Cefp-1b and cefp-2 are also produced during degradation at elevated temperature only. However, for both cefp-1b and cefp-2 effects of the kidney extract are observed. In contrast to the process in aqueous solution at physiological temperature, in kidney extract both cefp-1b and cefp-2 are produced immediately after addition. Furthermore, cefp-1b is detected at a relatively high intensity.

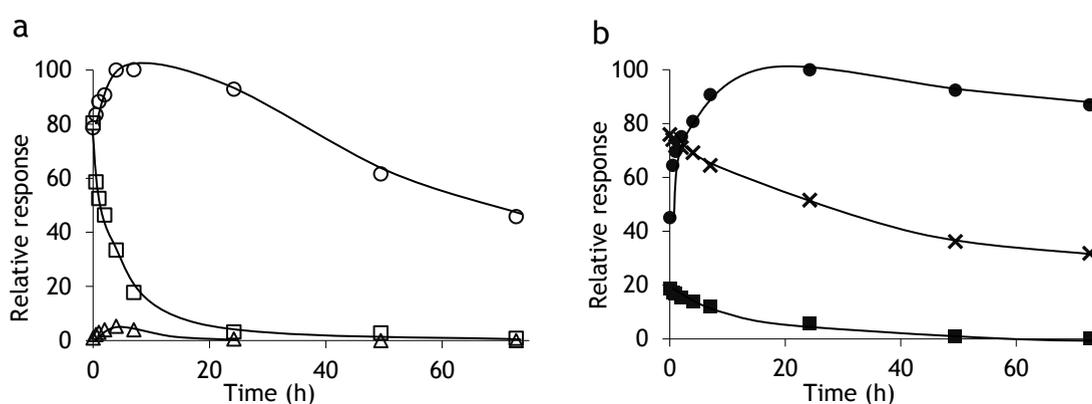


Figure 5.11. (a) The kinetics for (□) DFC, (○) cef-1 and (Δ) cef-2 during incubation at 37 °C after addition of ceftiofur to of kidney extract. Ceftiofur was not detected. (b) The kinetics for (x) DAC, (•) cefp-1b and (■) cefp-2 during incubation at 37 °C after addition of cefapirin to kidney extract. Cefapirin was not detected.

*Identity*¹

For each of the unknown degradation products of ceftiofur and cefapirin a molecular formula and possible molecular structures were determined from the accurate mass and isotope ratio (table 5.4 (page 215), figures 5.5 and 5.6 (page 211 and 212)).

For cef-1, one likely structure is proposed: DFC cysteine disulfide reported in milk and plasma of lactating cows [38] and in swine tissues [35,46]. For cef-2 one likely structure is proposed: ceftiofur thiolacton. The presence of ceftiofur thiolacton was first reported in acidified plasma and urine of ceftiofur-treated cows [37] and was reported as a hydrolysis product of ceftiofur [51]. To the best of our knowledge, this is the first time ceftiofur thiolacton has been reported in kidney extract.

For cefp-1b suggested structures are methoxy DAC and DAC methyl ester. A third candidate structure which does not obtain an intact β -lactam ring, was ruled out because the microbiological experiment indicated antimicrobial activity for this product, showing that it contains an intact β -lactam ring. Methoxy DAC is a somewhat more polar compound compared to DAC methyl ester and therefore elutes first. It is proposed that cefp-1a is methoxy DAC and cefp-1b is DAC methyl ester. No NMR spectrum was obtained for cefp-1b due to its low concentration. In the standard solution of cefapirin a trace (0.5 %) of cefapirin methyl ester was detected which could have degraded to DAC methyl ester in analogy to the degradation of cefapirin to DAC. However, the production of the cefp-1b cannot be fully explained by the degradation of a trace of cefapirin methyl ester, because in the kinetic experiment the signal of cefp-1b is higher than the signal of DAC. This is very unlikely to be caused by a difference in sensitivity only. It is concluded that cefp-1b is a major degradation product of cefapirin in the presence of kidney extract and is suggested to be DAC methyl ester. It is stated that the presence of methanol in the solution could result in esterification of cefapirin or degradation products of cefapirin.

For cefp-2 three suggested structures are cefapirin lacton, formerly reported by Heller *et al.* [52] to occur in milk of cefapirin treated cows, DAC methyl ester and desacetoxy cefapirin. Unfortunately no NMR spectrum was obtained for this compound due to its low concentration. However, this product shows analogy

with the product cefp-2-NH₃ detected for cefapirin in the presence of ammonia of which NMR signals were observed. Therefore it is suggested that cefp-2 is desacetoxy cefapirin.

Impact

During incubation at 37 °C in aqueous kidney extract, ceftiofur immediately degrades to several products including the unstable DFC. The main products formed are DFC-cysteine disulfide and DFC-thiolacton. To the best of our knowledge, DFC-thiolacton has not been previously reported in kidney extract.

Possibly DFC-thiolacton is not taken into account when applying the derivatization procedure using dithioerythritol and iodoacetamide to produce DFC from all active metabolites present [35], because it does not contain the complete DFC structure. In the kinetic experiment the intensity of DFC thiolacton was rather low. However, in the LC-ToF/MS identification experiment the intensity of DFC-thiolacton was found to be 40 % of DFC and therefore it is concluded that the derivatization procedure can result in an underestimation of the total amount of active ceftiofur compounds present in kidney.

During incubation at 37 °C in aqueous kidney extract cefapirin immediately degrades into several products among which the unstable DAC. The main products formed are cefp-1b which is likely DAC methyl ester and cefp-2 which is likely desacetoxy cefapirin. To the best of our knowledge these products have not been formerly reported. Using the generally applied method in which the sum of cefapirin and DAC is determined [43,47], cefapirin methyl ester as well as desacetoxy cefapirin which are both antimicrobially active products are not taken into account.

pH**Stability**

Ceftiofur is unstable in alkaline phosphoric buffer showing complete degradation within 30 min at pH = 12, 80 % degradation within 3 hours at pH = 11 and no degradation between pH = 2.5 through 10 (figure 5.12a). Surprisingly, compared to ceftiofur, DFC is more stable at high pH but less stable at low pH (figure 5.12b). At pH = 12, about 70 % of DFC is degraded after 3 hours, at pH = 11 this is 30 % and no degradation occurs between pH = 5 and 10. At pH = 2.5, 60 % of DFC degrades within 3 hours. In this experiment ceftiofur was less unstable than stated by Sunkara *et al.* [44] who reported a stable pH range for ceftiofur from 5.0 through 6.8 and complete degradation of ceftiofur within 10 min at pH = 10. The present study shows a stable pH range from 2.5 through 10 expanding the applicable pH range. These differences could be caused by a difference in ionic strength or quenching procedure.

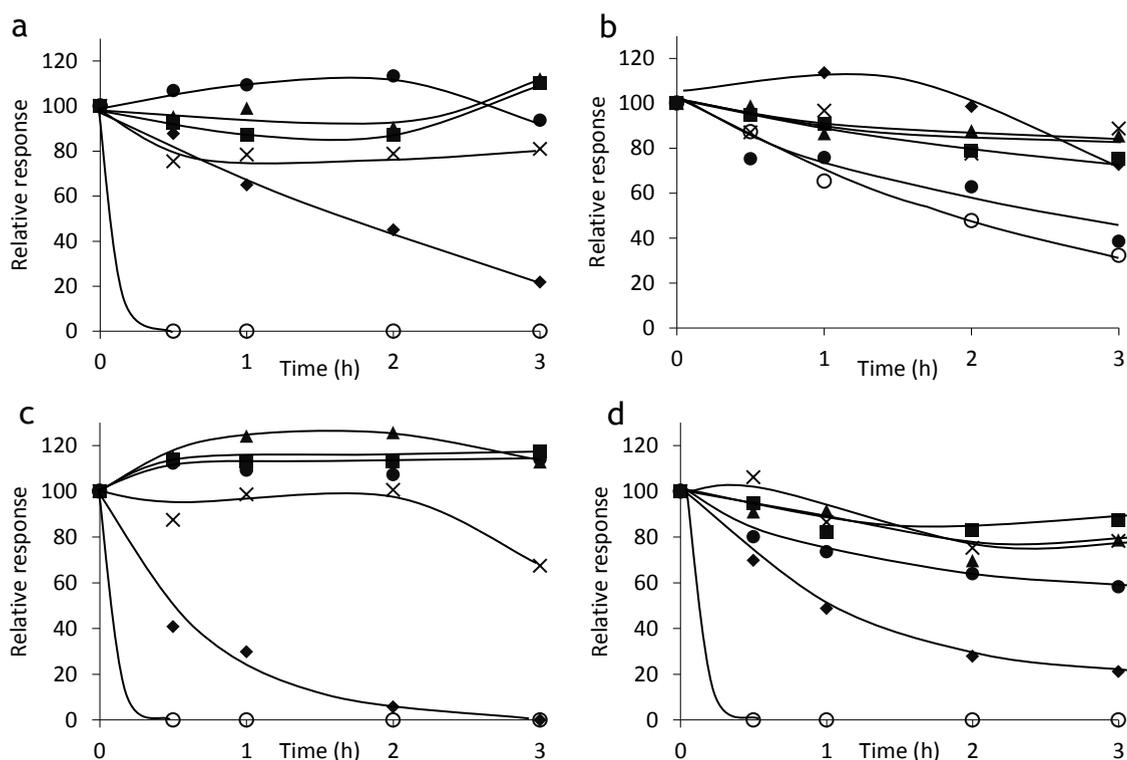


Figure 5.12. The degradation of (a) ceftiofur, (b) DFC, (c) cefapirin and (d) DAC in aqueous solution at pH (●) 2.5, (■) 5.0, (▲) 7.5, (×) 10.0, (◆) 11.0 and (○) 12.0.

Cefapirin and DAC show similar results to ceftiofur and DFC. Cefapirin is unstable in alkaline solution showing complete degradation within 30 min at pH = 12, complete degradation within 3 hours at pH = 11, slight degradation at pH 10 and no degradation between pH = 2.5 through 7.5 (figure 5.12c). Compared to cefapirin, DAC is more stable at high pH but less stable at low pH (figure 5.12d). DAC completely degrades within 30 min at pH = 12, but at pH = 11 degradation is slower compared to cefapirin: 80 % within 3 hours. DAC is stable from pH= 5 through 10 and at pH = 2.5 it degrades for 40 % within 3 hours.

Kinetics

After addition of ammonia to a ceftiofur solution two major degradation products were detected with accurate masses of $m/z = 413.070$ (cef-3-NH₃) which shows a maximum intensity after 4 hours of incubation, and 201.045 (cef-4) which is produced slowly at 37 °C (figure 5.13a).

For cefapirin two major degradation products were detected with accurate masses of $m/z = 399.080$ (DAC-NH₃) and 381.069 (cefp-2-NH₃). DAC-NH₃ and cefp-2-NH₃ are immediately produced after addition of ammonia and show maximum intensity after 2 and 24 hours of incubation respectively (figure 5.13b).

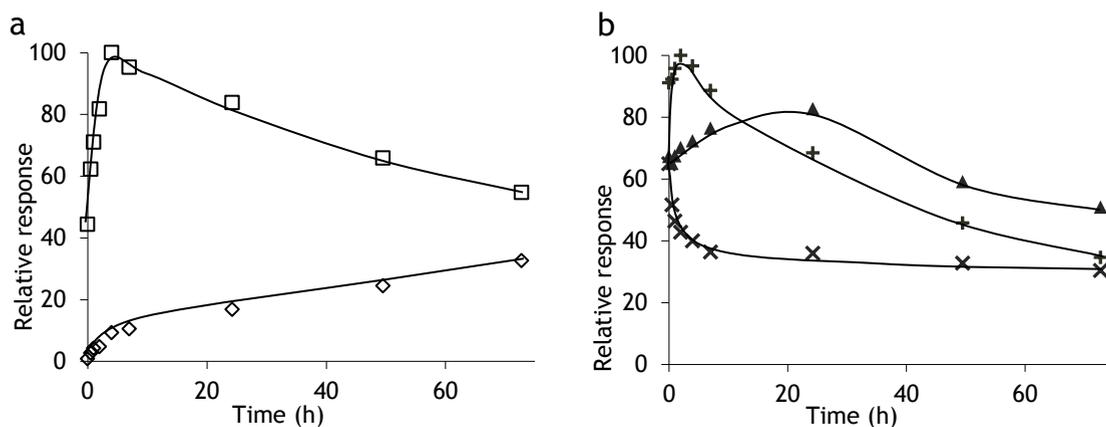


Figure 5.13. (a) The kinetics of (□) cef-3-NH₃ and (◇) cef-4 produced from ceftiofur in diluted ammonia during incubation at 37 °C. No ceftiofur was detected. (b) The kinetics of (x) DAC, (+) DAC-NH₃, (▲) and cefp-2-NH₃ produced from cefapirin in alkaline environment during incubation at 37 °C. No cefapirin was detected.

*Identity*¹

For each of the detected degradation products of ceftiofur and cefapirin in ammonia a molecular formula and possible molecular structures were determined (table 5.4 (page 215), figures 5.5 and 5.6 (page 211 and 212)) from the accurate mass and isotope ratio.

The isolated ceftiofur degradation products obtained after addition of ammonia showed slight microbiological activity which is explained by the occurrence of low amounts (< 1 %) of compounds containing an intact β -lactam ring in these fractions. The isolated cefapirin degradation products do not show any antimicrobial activity and thus it is concluded that the degradation products become antimicrobially inactive by addition of ammonia. This is in agreement with the NMR data in which no β -lactam ring structure (geminal protons at both 4.9 and 5.6 ppm) was observed for any of the products after addition of ammonia. By combining the LC-ToF/MS, NMR and microbiological data it is concluded that the β -lactam ring, responsible for the antimicrobial activity is ammoniated at the carbonyl group producing an amide moiety.

For cef-3-NH₃ two possible molecular structures are ammoniated ceftiofur lacton and ammoniated desthiofuroylceftiofur that can be produced because DFC contains a sulfide group, which is a good leaving group [53]. The production of ammoniated desthiofuroylceftiofur is more likely, since for ceftiofur lacton, two sets of geminal protons would be expected in the NMR spectrum which were not observed. For this product two chromatographic peaks were observed, suggesting the formation of conformational isomers, e.g. the syn- and anti-oxyimino isomers.

Cef-4 is likely the inactive part of ceftiofur containing the aromatic ring, the oxyimino moiety and an amide group: 2-amino- α -(methoxyimino)-4-thiazoleacetamide (AMTA). From the LC-ToF/MS data and the knowledge that ammonia causes opening of the β -lactam ring, it is suggested that DAC-NH₃ is ammoniated DAC.

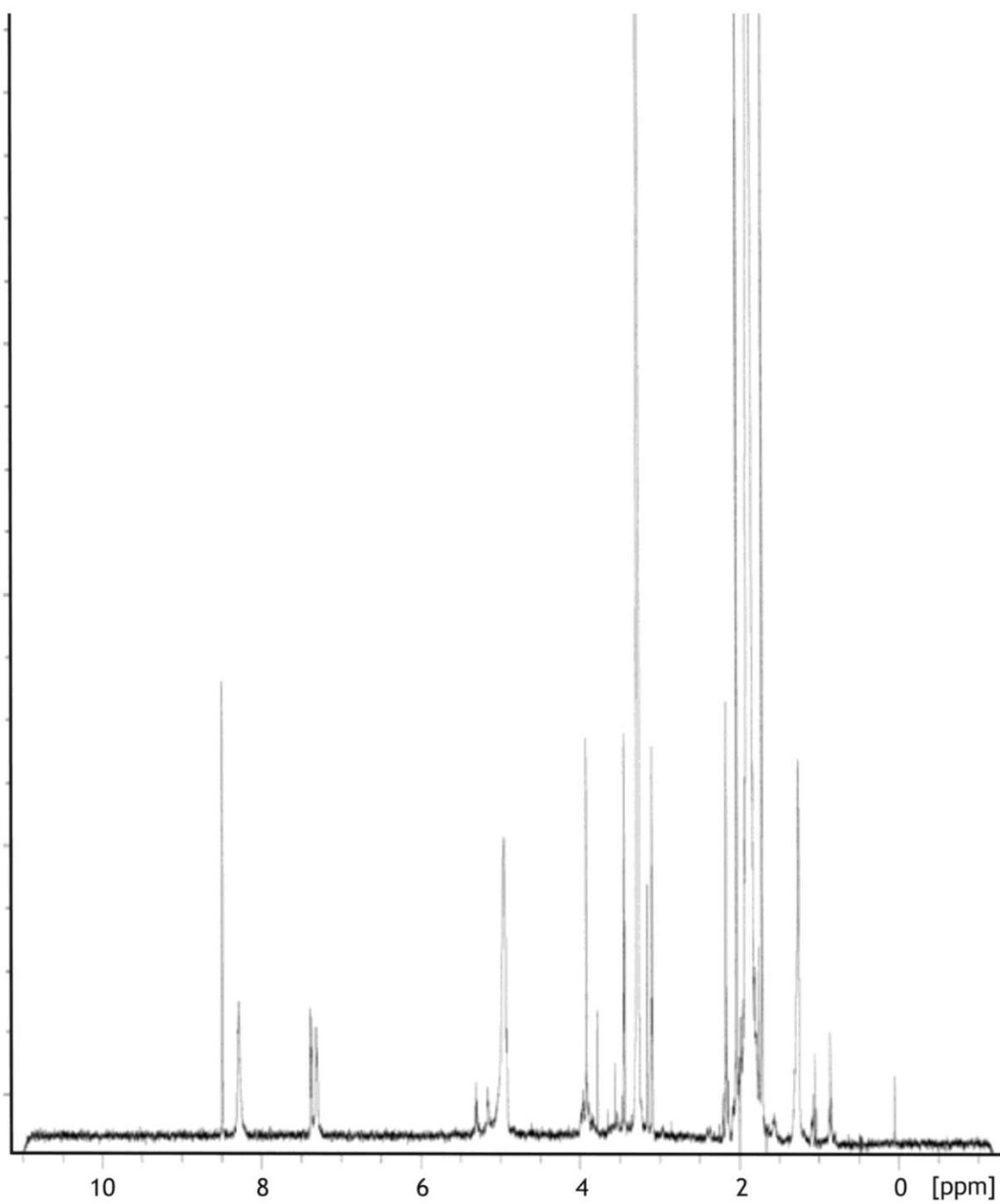


Figure 5.14. The NMR spectrum of cefp-2-NH3.

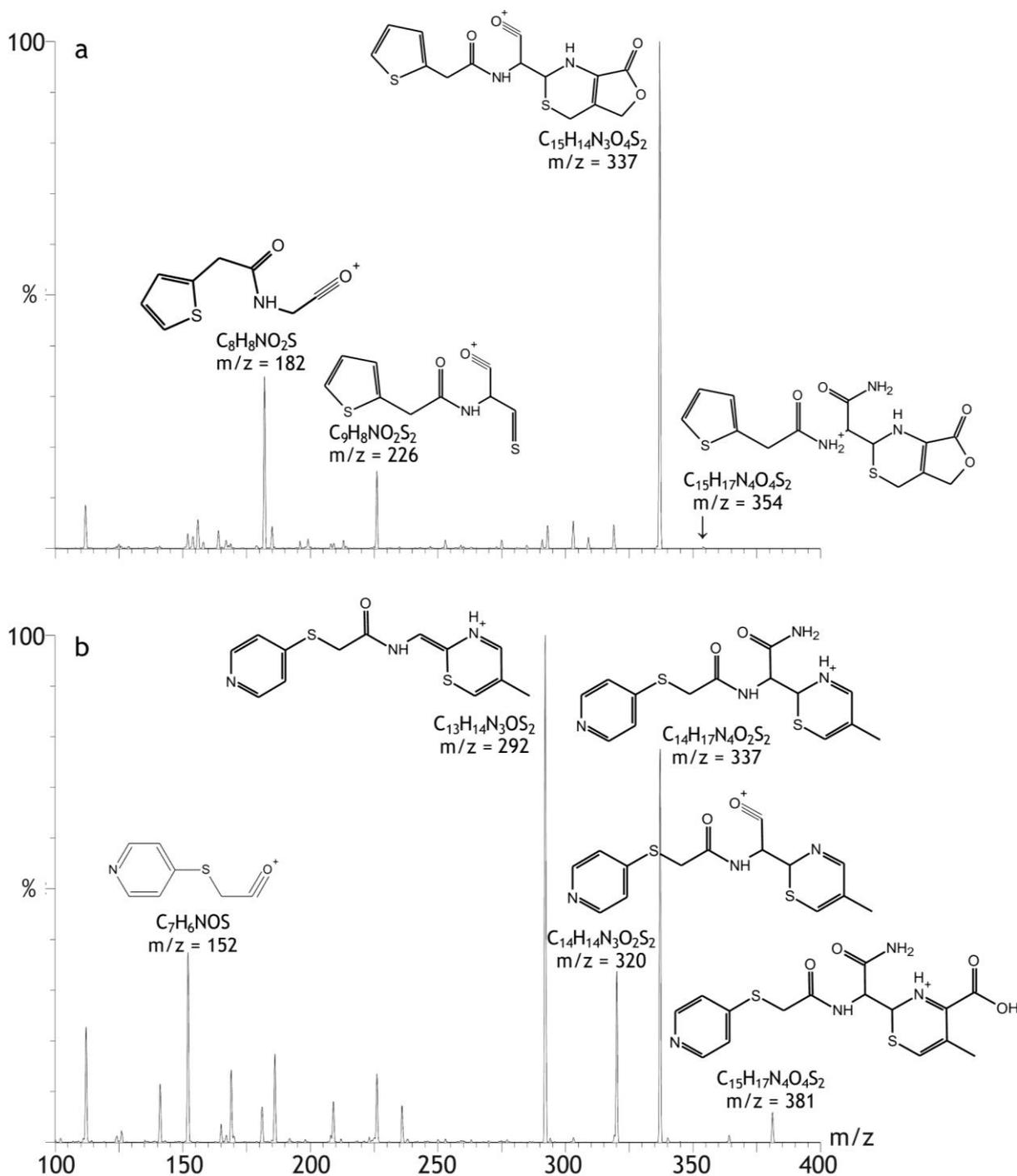


Figure 5.15. Spectra of (a) ammoniated cefalothin lacton and (b) ceft-2-NH₃, including proposed molecular formulas and structures.

For cefp-2-NH₃ three possible molecular structures are ammoniated cefapirin lacton, ammoniated DAC methyl ester and ammoniated desacetoxy cefapirin. The NMR spectrum (figure 5.14) does not show the expected couple of geminal protons that would be observed for cefapirin lacton. An NMR signal that is likely to originate from the methyl group in ammoniated desacetoxy cefapirin is observed at 2.2 ppm. The LC-MS/MS fragmentation pattern of cefp-2-NH₃ and ammoniated cefalothin lacton, obtained by adding ammonia to a standard solution of cefalothin lacton, were compared (figure 5.15) to obtain additional information. Cefalothin lacton was used because of the lack of a reference standard of cefapirin lacton. The fragmentation pattern of cefp-2-NH₃ consists of the loss of CO₂ (m/z = 337), the loss of both CO₂ and NH₃ (m/z = 320) and the loss of CO₂, NH₃ and CO (m/z = 292). Ammoniated cefalothin lacton (m/z = 354) did show the loss of NH₃ (m/z = 337), but did not show an abundant peak corresponding with the loss of CO₂. This indicates that, in contrast to cefalothin lactone, cefp-2-NH₃ does not contain a lactone ring. Based on the combination of LC-ToF/MS, NMR, microbiological and LC-MS/MS data it is likely that cefp-2-NH₃ is ammoniated desacetoxy cefapirin.

For both DAC-NH₃ and cefp-2-NH₃ four chromatographic peaks were observed indicating the presence of several conformational isomers. After isolation of a specific isomer by fractionation and injection on the LC-ToF/MS system, again four peaks were observed, indicating the slow formation of an equilibrium among the isomers.

Impact

From these results it is concluded that for ceftiofur and cefapirin high alkaline conditions result in immediate degradation. When using ammonia the main process occurring is ammoniation of the β-lactam ring resulting in microbiologically inactive products. DFC and DAC are stable in the pH range from 3 - 10. At more extreme conditions severe degradation of both compounds occurs. Therefore, for analysis involving ceftiofur, DFC, cefapirin and DAC, the pH during sample preparation and analysis should be well controlled. If the pH gets below 3 or above 12, as might occur during adjustment of the pH, rapid degradation occurs resulting in an underestimation of the residue level of ceftiofur and cefapirin.

Alkaline kidney extract

Kinetics

When ammonia is added to ceftiofur spiked kidney extract, DFC and cef-2, found in aqueous kidney extract at neutral pH, are not detected. Cef-1 is detected but degrades almost completely within 4 hours (figure 5.16a). This is expected because high levels of ammonia immediately ammoniate the β -lactam ring. By comparing the processes in the presence and absence of kidney extract (figure 5.13a vs 4.16a) it is concluded that the degradation of cef-3-NH₃ is accelerated in kidney extract. Cef-4, found in alkaline solutions, is also found in alkaline kidney extract, showing the same kinetics obtaining a stable concentration after 60 hours of incubation at 37 °C.

When ammonia is added to cefapirin spiked kidney extract, DAC and cefp-1b found in aqueous kidney extract at ambient pH, are detected but degrade rapidly due to the ammoniation of the β -lactam ring. Cefp-2 was not detected in this experiment. Cefp-2-NH₃, found in the presence of ammonia, is also found in alkaline kidney extract showing comparable kinetics to the degradation process in absence of kidney extract (figure 5.16b). DAC-NH₃ was detected in this experiment but decreased more rapidly compared to the degradation process in absence of kidney extract. It is concluded that the degradation of DAC-NH₃ is catalysed by active compounds in kidney extract.

Some unknown products with accurate masses of $m/z = 397.064$ (DAC-NH₃d), 379.054 (cefp-2-NH₃d) and 169.044 (cefp-3) are formed in alkaline kidney extract during incubation. After about 50 hours of incubation at 37 °C the production of these products is complete after which the intensity remains stable.

Identity¹

For each of the unknown degradation products of ceftiofur and cefapirin a molecular formula and possible molecular structures were determined (table 5.4 (page 215), figures 5.5 and 5.6 (page 211 and 212)) from the accurate mass and isotope ratio.

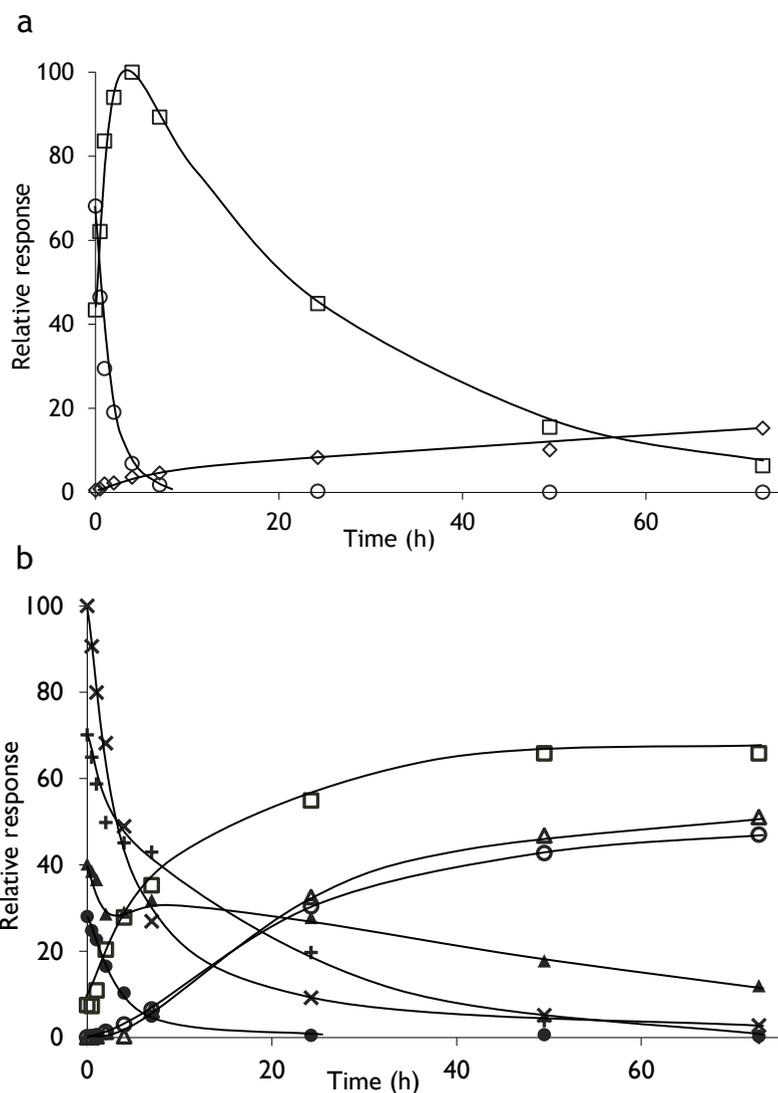


Figure 5.16. (a) The kinetics of (○) *cef-1*, (□) *cef-3-NH₃* and (◇) *cef-4* produced from ceftiofur in alkaline kidney extract during incubation at 37 °C. No ceftiofur was detected. (b) The kinetics of (x) DAC, (●) *cefp-1b*, (+) DAC-NH₃, (▲) *cefp-2-NH₃*, (○) DAC-NH₃d, (Δ) *cefp-2-NH₃d* and (□) *cefp-3* produced from cefapirin in alkaline kidney extract during incubation at 37 °C.

It is noteworthy that DAC-NH₃ and DAC-NH₃d as well as *cefp-2-NH₃* and *cefp-2-NH₃d* only differ two hydrogen atoms. This indicates that these products are related and differ only one double bond. It is suggested that during incubation an aromatic system is formed in the six membered thiazine ring resulting in a more unsaturated compound.

The products *cef-4* and *cefp-3* are suggested to be analogs being AMTA and 2-(pyridine-4-ylthio)acetamide (PTA).

Impact

From these results it is concluded that both active compounds in kidney extract and ammonia influence the degradation process of ceftiofur and cefapirin. For ceftiofur, the highly polar cef-4 is the only detected stable product in alkaline kidney extract. For cefapirin, DAC-NH₃d, cefp-2-NH₃d and the polar cefp-3 are found to be stable products. The formation of these products is only complete after 50 hours of incubation at 37 °C. At 80 °C the formation of AMTA is complete after 8 hours and the formation of the PTA after 2 hours. Further research is needed to determine if these products can result in a new approach for the quantitative analysis of ceftiofur and cefapirin in kidney.

Conclusions

Ceftiofur and cefapirin are stable in aqueous solution within a pH range 2.5 - 10. Above pH = 10, the ceftiofur and cefapirin β-lactam ring is degraded and at pH = 12 this is almost an immediate process. It is concluded that the pH has to be well controlled during analysis to prevent the occurrence of such degradation.

In kidney extract as well, ceftiofur and cefapirin are highly unstable. For ceftiofur the suggested main degradation products are DFC, DFC-cysteine disulfide and DFC-thiolacton. For cefapirin the main degradation product is DAC which degrades further to a product that is suggested to be DAC methyl ester and a product that is suggested to be desacetoxy cefapirin. To the best of our knowledge DFC-thiolacton, DAC methyl ester and desacetoxy cefapirin have not been previously reported to be found in kidney extract.

Due to the processes occurring in kidney extract and the fact that the pH has to be well controlled, severe degradation during sample preparation is likely to occur. Furthermore, the identification of the degradation products indicate that reported methods do not include all relevant analytes. Both aspects result in an expected underestimation of the residue levels of ceftiofur and cefapirin.

Additional research to determine whether newly identified degradation products can result in a new approach for the quantitative determination of ceftiofur, cefapirin and other cephalosporins in tissue is reported in section 5.3.

5.3. Assessment of liquid chromatography tandem mass spectrometry approaches for the analysis of ceftiofur metabolites in poultry muscle

Abstract

The use of cephalosporin antibiotics in veterinary practice is likely to play an important role in the development of β -lactam resistant bacteria. To detect off-label cephalosporin antibiotic usage an analytical method is needed that, besides the native compound, also detects their active metabolites. In this section the applicability of three approaches for the quantitative analysis of ceftiofur using liquid chromatography coupled to tandem mass spectrometry is assessed, viz. (A) the analysis of ceftiofur, desfuroylceftiofur and/or desfuroylceftiofur cystein disulfide, (B) the derivatization of ceftiofur metabolites to desfuroylceftiofur acetamide and (C) the chemical hydrolysis using ammonia in order to produce a marker compound for ceftiofur. It was found that approach A is not suited for quantitative analysis of total ceftiofur concentration nor for effectively detecting off-label use of ceftiofur. Approach B resulted in adequate quantitative results, but is considered to be a single compound method because it depends on the cleavage of a thioester group which is present in only a limited number of cephalosporin antibiotics. Approach C showed adequate quantitative results as well. In contrast to approach B, this approach is applicable to a range of cephalosporin antibiotics and therefore applicable as a broad quantitative screening of cephalosporin compounds in poultry tissue samples to indicate off-label use of cephalosporins in poultry breeding. Based on the research presented here, it is concluded that the multi-method following approach C is the most suited to detect off-label use of a range of cephalosporin antibiotics.

Introduction

Cephalosporins are semi-synthetic β -lactam antibiotics, consisting of a six membered dihydrothiazine ring fused with a four membered β -lactam ring which is responsible for the biological activity of the compounds. Nowadays, five generations of cephalosporins are distinguished reflecting their spectrum of activity, structural similarity and time of introduction [11]. Ceftiofur (figure 5.17a) is a third generation cephalosporin which is highly effective in the treatment of bacterial infections of the respiratory tract [10]. Ceftiofur is registered for use in food-producing mammals [6] but occasionally off-label use occurs in poultry breeding. This is likely to contribute to the emergence of resistant bacteria like extended-spectrum- β -lactamase (ESBL) producing bacteria [15-19]. Cephalosporins are assigned as critically important antimicrobials for human health [23] and should therefore be used sparingly in veterinary practice to prevent the occurrence of bacterial resistance [24-27,54].

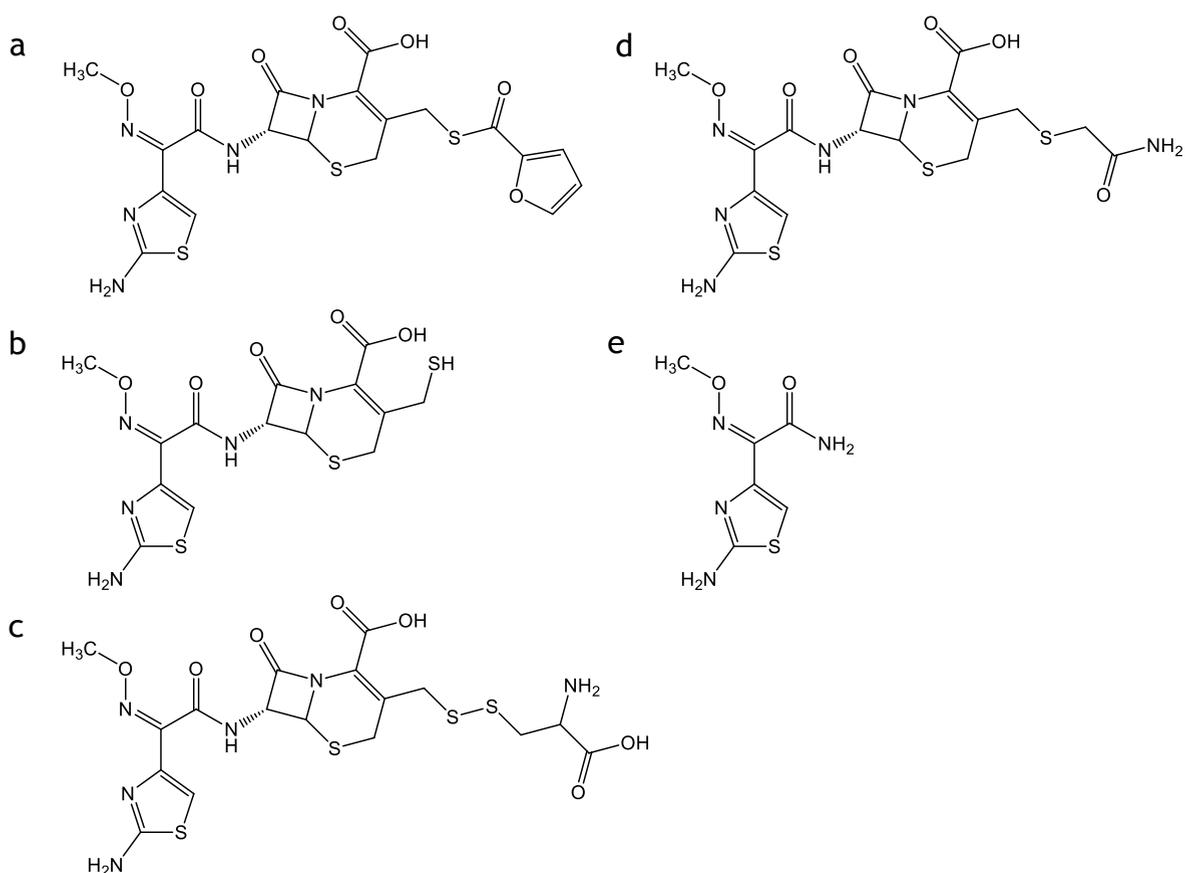


Figure 5.17. Molecular structure of (a) ceftiofur, (b) desfuroylceftiofur (DFC), (c) desfuroylceftiofur cysteine disulfide (DCCD), (d) desfuroylceftiofur acetamide (DCA) and (e) 2-amino-a-(methoxyimino)-4-thiazoleacetamide (AMTA).

Ceftiofur is known to metabolise rapidly after intramuscular administration. Reported metabolites include desfuroylceftiofur (DFC, figure 5.17b), desfuroylceftiofur cysteine disulfide (DCCD, figure 5.17c), protein bound DFC and desfuroylceftiofur thiolacton [36-38,55]. Although no maximum residue limit (MRL) has been established for poultry muscle, it is important to realise that ceftiofur MRLs for other species are defined as the sum of all residues retaining the β -lactam structure, expressed as DFC [6]. This implies that all active metabolites should be included in the analysis of ceftiofur in food products.

Two main approaches for the analysis of ceftiofur residues in animal tissue and plasma have been reported. The first approach focuses on the analysis of one or more ceftiofur metabolites. LC-UV methods for the analysis of ceftiofur itself were reported [56,57] as well as an LC-UV method including ceftiofur and DFC [58] and one including ceftiofur, DCCD and DFC dimer [46]. Furthermore, a very straightforward LC-MS/MS method monitoring DCCD as the marker compound in kidney was reported [59].

The second approach includes an extraction and deconjugation of all protein bound DFC using dithioerythritol (DTE) followed by a derivatization of the resulting free DFC using iodoacetamide to obtain desfuroylceftiofur acetamide (DCA) as a marker residue (figure 5.17d). This approach was first introduced for the analysis of plasma in combination with UV detection [60] and was later optimised for the analysis of muscle [35,61]. This second approach was validated by a multi-laboratory trial demonstrating its applicability [62]. However, these methods are very laborious including several solid phase extraction (SPE) steps. Simplified methods applying the same approach were reported for the analysis of plasma and synovial fluid [63] and for the analysis of several matrices among which serum and endometrial tissue [45,64].

Based on the results presented in section 5.2, it is expected that currently applied methods are likely to underestimate the residue levels of ceftiofur and cephalosporin found in kidney samples because they do not include all active metabolites. Furthermore, it is noted that cephalosporin multi-methods that include unstable cephalosporins (like ceftiofur and cefapirin) and are able to detect the active metabolites of these compounds, are still lacking. Therefore, a new approach for the analysis of ceftiofur including its metabolites was suggested [55]. This third approach is based on an alkaline hydrolysis of ceftiofur

and its metabolites to produce 2-amino- α -(methoxyimino)-4-thiazoleacetamide (AMTA, figure 5.17e) as a marker residue for the total amount of active ceftiofur metabolites. This method is also applicable for other cephalosporins, including cefcapene, cefapirin and cefquinome.

Based on incurred ceftiofur poultry muscle material a critical assesment of the three approaches is presented in this section: (A) the analysis of ceftiofur, DFC and DCCD using the method reported by Mastovska *et al.* [59], (B) the derivatization of ceftiofur metabolites to DCA as reported by Witte *et al.* [45] and (C) a newly developed method applying an alkaline hydrolysis of ceftiofur, of which the preliminary concept was reported in section 5.2.

Experimental

Reagents

HPLC grade methanol, HPLC grade acetonitrile, hexane (Biosolve, Valkenswaard, The Netherlands), 25 % ammonia, 99 % acetic acid, sodium chloride, sodium hydroxide, ammonium acetate, 85 % phosphoric acid (Merck, Darmstadt, Germany), disodium tetraborate, dithioerythol (DTE) and iodoacetamide (IAA) (Sigma-Aldrich, St. Louis, MO, USA) were used. Ceftiofur, DFC, DCCD and ceftiofur- d_3 were obtained from Toronto Research Chemicals (North York, Ontario, Canada).

Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18.2 M Ω ·cm (Millipore, Billerica, MA, USA). Bakerbond[®] Octadecyl 40 μ m was obtained from J.T. Baker (Phillisburg, NJ, USA). The solid phase extraction (SPE) cartridges used were Mega Bond-Elut C₁₈ 1 g / 6 mL, Bond Elut SCX 100 mg / 3 mL (Agilent Technologies, Santa Clara, CA, USA) and Oasis HLB 60 mg / 3 mL (Waters, Milford, MA, USA).

Borate buffer pH 9 was prepared by dissolving 10.0 g disodium tetraborate and 29.2 g sodium chloride in 1 L of water. A 1 M ammonium acetate solution was prepared by dissolving 7.71 g of ammonium acetate in 100 mL water. This was diluted tenfold in water to obtain a 0.1 M ammonium acetate solution. A 17 % phosphoric acid solution was prepared by diluting 10 mL 85 % phosphoric acid to

50 mL with water. A 2 % acetic acid solution was prepared by diluting 10 mL 99 % acetic acid to 500 mL with water. A 5 M sodium hydroxide solution was prepared by dissolving 20 g sodium hydroxide in 100 mL water. A 20 mg mL⁻¹ DTE solution was prepared by dissolving 2.0 g of DTE in 100 mL borate buffer pH 9. A 0.2 M IAA solution was prepared by dissolving 3.7 g IAA in 100 mL 0.1 M ammoniumacetate buffer. Individual stock solutions of ceftiofur and ceftiofur-d₃ were prepared in methanol and stock solutions of DFC and DCCD in water at 100 µg L⁻¹.

Methods

Three sample preparation methods (described below) were implemented and tested at our laboratory for the analysis of ceftiofur and its metabolites in poultry muscle. All final extracts were analysed using the LC-MS/MS system described.

Method A: individual analysis of ceftiofur, DFC and DCCD

This method focuses on the individual analysis of ceftiofur, DFC and DCCD and was based on Mastovska *et al.* [59]. One gram of poultry muscle was weighed into a centrifuge tube and ceftiofur-d₃ was added as the internal standard. 2 mL of water and 8 mL of acetonitrile was added to the sample and the tube was shaken using a rotary tumbler (5 min). After centrifugation (3500 g, 15 min) the supernatant was decanted into a new centrifuge tube containing 0.5 g of octadecyl sorbent. After vortex shaking (30 s) and centrifugation (3500 g, 5 min) 5 mL of the extract was transferred into a 12 mm centrifuge tube and evaporated (45 °C, N₂) until the volume was below 1 mL. The concentrated extract was transferred into an autosampler vial.

Method B: derivatization to DCA

This method includes a deconjugation and derivatization of ceftiofur metabolites to DCA and was based on Witte *et al.* [45]. One gram of poultry muscle was weighed into a centrifuge tube and ceftiofur-d₃ was added as the internal standard. Deconjugation of DFC conjugates resulting in free DFC was carried out by adding 5 mL of DTE solution followed by shaking using a rotary tumbler (15 min). The extract was incubated in a water bath set at 50 °C for 15 minutes and afterwards DFC was stabilised by adding 5 mL IAA solution followed by shaking

using a rotary tumbler (15 min) resulting in DCA. The derivatization was completed by incubating the extract for 30 min at room temperature. After the derivatization the pH of the extract was adjusted to pH 3 by adding droplets of 17 % phosphorous acid solution. After centrifugation of the extract (4000 g, 30 min) the supernatant was isolated and the pH was adjusted to pH 5 by adding droplets of a 5M sodium hydroxide solution. A 1g, 6 mL Mega Bond Elut C₁₈ SPE cartridge was conditioned using 5 mL of methanol followed by 5 mL 0.1M ammonium acetate solution. The entire extract was applied onto the SPE cartridge which was subsequently washed with 5 mL 0.1 M ammonium acetate solution and 5 mL 2 % acetic acid in water. DCA was eluted from the SPE cartridge using 5 mL 2 % acetic acid in water : acetonitrile (8:2, v/v). A 100 mg, 3 mL SCX SPE cartridge was conditioned with 3 mL of methanol followed by 3 mL 2 % acetic acid in water. The eluent of the C₁₈ cartridge was applied onto the SCX cartridge which was subsequently washed with 2 mL of methanol. After drying the cartridge under vacuum for 5 min, DCA was eluted using 1 mL 1M ammonium acetate in water : acetonitrile (85:15, v/v). The extract was transferred into an autosampler vial.

Method C: hydrolysis to AMTA

This method includes an alkaline hydrolysis of ceftiofur metabolites to AMTA and was derived from Berendsen *et al.* [55]. 2.5 grams of poultry tissue was weighed into a centrifuge tube and ceftiofur-d3 was added as the internal standard. 10 mL of borate buffer pH 9 and 250 µL of 25 % ammonia were added to the sample. After shaking using a rotary tumbler (5 min) the extract was incubated in a water bath of 60 °C for 20 hours. After hydrolysis, 10 mL hexane was added and the extract was shaken using a rotary tumbler (5 min) and centrifuged (3500 g, 15 min). A 60 mg, 3 mL OasisTM HLB SPE cartridge was conditioned by subsequently 5 mL of methanol and 5 mL of water. 5 mL of the clear aqueous layer of the extract was transferred onto the SPE cartridge, which was subsequently washed with 3 mL of water. After drying the cartridge under vacuum for 5 min, the derivate AMTA was eluted using 5 mL methanol:acetonitrile (1:1, v/v). The eluent was evaporated until dryness (45 °C, N₂) and the residue was redissolved in 500 µL of water, filtered using a 0.45 µm acrodisc[®] PVDF membrane filter (Pall corporation, Port Washington, NY, USA) and transferred into an autosampler vial.

LC-MS/MS

The LC instrumentation consisted of a vacuum degasser, autosampler and a binary pump (Acquity Waters, Milford, MA, USA) equipped with a Waters X-Bridge C₁₈ (3.5 µm) analytical column of 2.1 × 100 mm placed in a column oven at 50 °C. The gradient (solvent A, 0.2 % ammonia in water adjusted to pH 8 using acetic acid; solvent B, 0.2 % ammonia in water adjusted to pH 8 using acetic acid : methanol (1:9, v/v)) was: 0 - 1.0 min, 0 % B, 1.0 - 5.0 min, linear increase to 50 % B, 5.0 - 6.0 min, linear increase to 100 % B with a final hold of 0.5 min, operating at a flow rate of 0.4 mL min⁻¹. The injection volume was 20 µL.

Detection was carried out using a Waters Quattro Ultima mass spectrometer operating with electrospray ionisation (ESI) in positive mode. The operating parameters were: capillary voltage, 2.7 kV; cone voltage, 20 V; source temperature, 120 °C; desolvation temperature, 450 °C; cone gas flow, 200 L hr⁻¹; and desolvation gas, 550 L hr⁻¹. Ceftiofur, ceftiofur-d₃, DFC, DCCD, DCA, DCA-d₃, AMTA and AMTA-d₃ were fragmented using collision induced dissociation (CID) with the settings presented in table 5.5. Data were acquired and processed using MassLynx 4.1 software (Waters).

Table 5.5. Precursor ions, product ions and collision energy of ceftiofur, DFC, DCCD, DCA, AMTA and the internal standards.

Compound	Precursor ion (m/z)	Collision energy (eV)	Product ion (m/z)
Ceftiofur	524	40	125
		17	241
DFC	430	45	125
		17	241
DCCD	549	25	183
		20	241
DCA	487	25	167
		20	241
AMTA	201	23	125
		15	126
Ceftiofur-d ₃	527	17	244
DCA-d ₃	490	20	244
AMTA-d ₃	204	15	126

Controlled treatment study

Following ethical approval, ceftiofur incurred poultry tissue samples were obtained from Schothorst feed research (Lelystad, The Netherlands). Fifteen one day old broilers were held under controlled conditions (water, feed and housing) for 22 days. At day 22, the broilers were weighed and 12 broilers were injected in the breast with a 4 mg mL⁻¹ solution of Excenel (Pfizer Animal Health, New York, NY, USA). The other three animals were considered the untreated control group. The volume of the injected solution was adapted to the body weight in such a way that each animal received 3 mg ceftiofur per kg. The animals were euthanised via cervical dislocation, three animals each at 1, 2, 4 and 8 hours after injection. From each animal breast and thigh muscle, liver and kidneys were removed and stored at -80 °C. After transportation to the laboratory on dry ice, the samples were thawed, minced using a laboratory mincer and stored at -80 °C immediately afterwards to prevent degradation.

Derivatization/hydrolysis yield

For method B and C the efficiency of the conversion of ceftiofur, DFC and DCCD to DCA and AMTA respectively were tested. Ceftiofur, DFC and DCCD were each added to six aliquots of blank poultry muscle at a concentration of 1000 µg kg⁻¹. The poultry muscle samples were analysed according to method B and C and the peak areas of DCA and AMTA were determined. Peak areas of ceftiofur and DCCD were corrected to 1000 µg kg⁻¹ DFC to facilitate a correct comparison of the results by: $(Y/M_{DFC}) * M$, in which Y is the response of DCA or AMTA of the samples spiked with ceftiofur or DCCD, M_{DFC} is the molecular mass of DFC and M is the molecular mass of ceftiofur or DCCD. The corrected areas of the samples fortified with the different metabolites were compared using a Students' t-test ($\alpha = 0.05$).

Quantitative analysis

First, the limit of detection (LOD) of the methods were determined by extrapolation of the chromatogram of the lowest calibration point (10 µg kg⁻¹) of the least abundant product ion monitored to a signal to noise ratio of 3. Next, all of the incurred poultry breast muscle samples were analysed in duplicate using the three described methods. The samples were quantitated using a matrix

matched calibration line. For method A, blank samples were spiked with 0 to 500 $\mu\text{g kg}^{-1}$ ceftiofur, DFC and DCCD. For method B and C blank samples were spiked with 0 to 3000 $\mu\text{g kg}^{-1}$ ceftiofur only. For all methods the total amount of ceftiofur metabolites was calculated and expressed in $\mu\text{g kg}^{-1}$ DFC by: $(X/M)*M_{\text{DFC}}$, in which X is the calculated concentration of ceftiofur or DCCD in the samples, M is the molecular mass of ceftiofur or DCCD and M_{DFC} is the molecular mass of DFC. For each animal the results obtained by applying different methods were compared using a Students' t-test ($\alpha = 0.05$).

Applicability to other matrices.

The applicability of the hydrolysis approach (method C) was additionally tested for other matrices than poultry breast muscle by studying the concentration of total ceftiofur residues in the thigh muscle, kidney and liver samples obtained from the ceftiofur treated broilers. The ceftiofur metabolites in each matrix were quantitated using matrix matched standards prepared from the materials obtained from the broilers in the control group.

Results and discussion

General considerations

Method A and B were adopted from literature and method C was based upon the concept of the alkaline hydrolysis of ceftiofur to AMTA as reported previously [55] (section 5.2). To study the applicability of this method C concept, a straightforward extraction and sample clean-up procedure was developed. It was found to be crucial to carry out the hydrolysis during extraction (in the presence of the muscle matrix) to obtain good hydrolysis yields for ceftiofur, DFC and DCCD. If ammonia in pure water (without addition of salts) is used as the extraction solvent, muscle proteins denature during incubation, resulting in extreme gelation of the extract. To prevent gelation, sodium borate buffer pH=9 and sodium chloride had to be used as the extraction solvent to facilitate alkaline hydrolysis in the presence of the muscle matrix.

Critical assessment of approaches

Linearity and detection limits

For method A, B and C a chromatogram of a blank poultry muscle sample, spiked at $10 \mu\text{g kg}^{-1}$ with the relevant compounds and internal standard ceftiofur- d_3 , is presented in figure 5.18.

In the range from 0 to $3000 \mu\text{g kg}^{-1}$ good linearity ($r > 0.998$) was observed for all three methods indicating that they are suited for the quantitation of ceftiofur and (the selected) metabolites in poultry muscle. Method A resulted in an LOD of $1 \mu\text{g kg}^{-1}$ for ceftiofur, $2 \mu\text{g kg}^{-1}$ for DFC and $3 \mu\text{g kg}^{-1}$ for DCCD. Method B resulted in an LOD of $1 \mu\text{g kg}^{-1}$ for the total ceftiofur content which is superior to previously reported LODs for this approach ($10 \mu\text{g kg}^{-1}$ for swine muscle [35], $100 \mu\text{g kg}^{-1}$ for endometrial tissue [45] and uterine tissue [64]). Method C resulted in an LOD of $0.5 \mu\text{g kg}^{-1}$ for the total ceftiofur content indicating that this method is very adequate for detecting residues of ceftiofur metabolites.

Derivatization/hydrolysis yield

For a method that includes the production of one marker compound from several individual compounds, the conversion is important: each metabolite should be converted to the marker compound with the same yield to obtain a correct total concentration. The average response and the standard deviation of six poultry muscle samples spiked at $1000 \mu\text{g kg}^{-1}$ with ceftiofur, DFC and DCCD individually, analysed using both method B and C, were calculated. The results are presented in figure 5.19a and b.

From the results it is observed that, when using method B, the derivatization of ceftiofur and DCCD to DCA occurs with the same yield. However, the derivatization of DFC only results in 20 % DCA compared to ceftiofur and DCCD. DFC is the most reactive metabolite having a free sulfide group and quickly degrades under the formation of e.g. protein bound DFC [65] and DFC thiolactone [55] and might therefore not be effectively derivatised to DCA. From this, it is concluded that when using DFC for preparation of matrix matched calibrants, an incorrect quantitation of the total amount of ceftiofur metabolites is obtained.

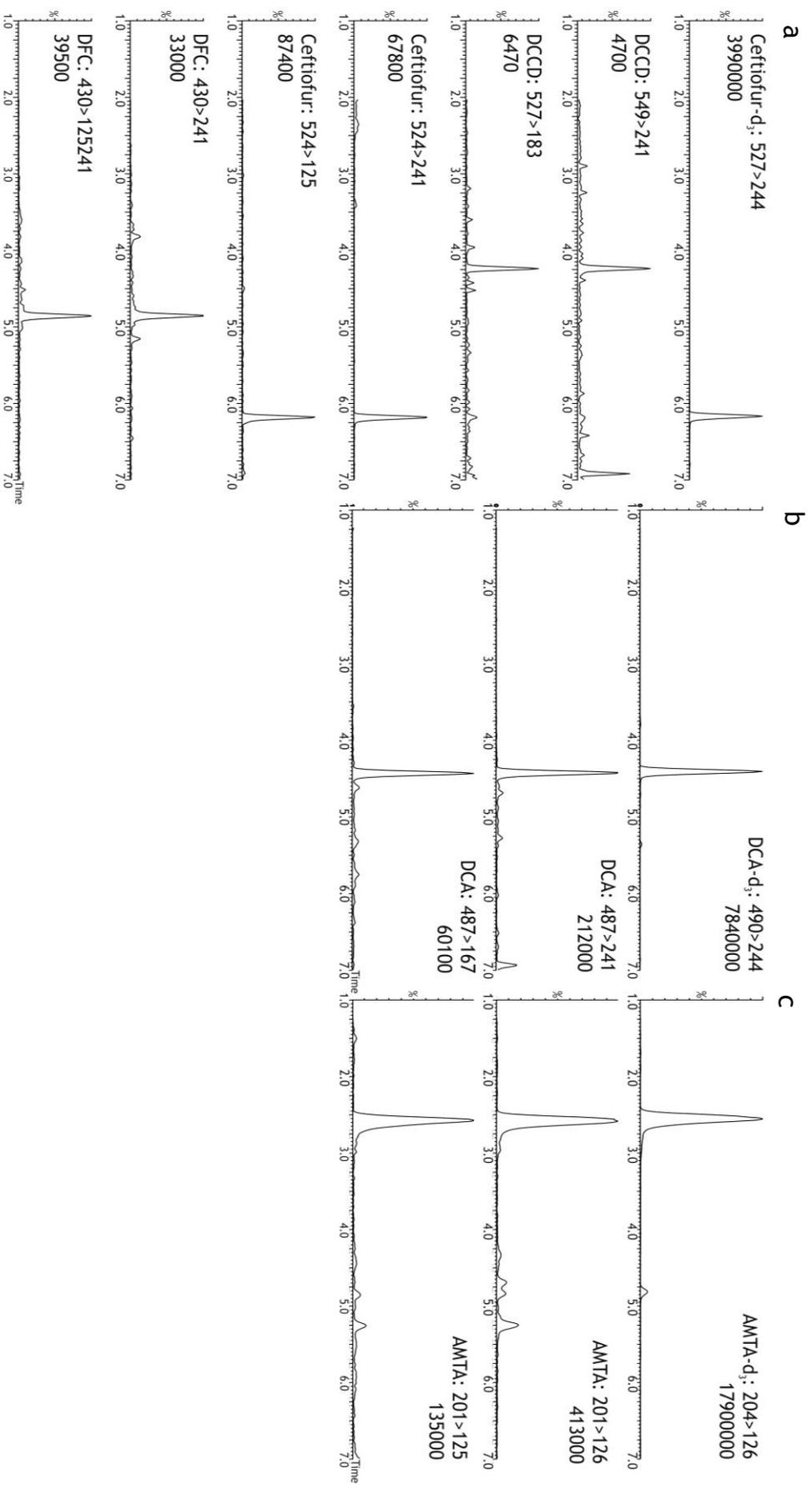


Figure 5. 18. A blank poultry muscle sample spiked at 10 µg kg⁻¹ with (a) ceftiofur, DFC, DCCD and ceftiofur-d₃ analyzed using method A, (b) ceftiofur and ceftiofur-d₃ analyzed as DCA and DCA-d₃ using method B and (c) ceftiofur and ceftiofur-d₃ analyzed as AMTA and AMTA-d₃ using method C.

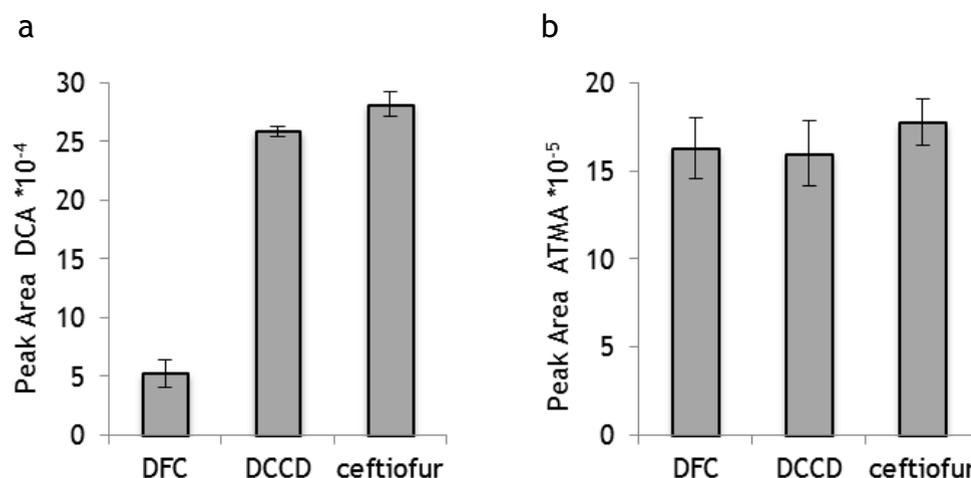


Figure 5.19. Average peak area including standard deviation ($n=6$) of poultry muscle samples spiked at $1000 \mu\text{g kg}^{-1}$ with ceftiofur, DFC and DCCD individually, analysed using (a) method B and (b) method C.

From figure 5.19b it is observed that the hydrolysis of ceftiofur, DFC and DCCD results in the same amount of AMTA: no significant differences among the groups is observed. It is concluded that the yield of the hydrolysis of various ceftiofur metabolites to AMTA is comparable and an accurate quantitation of the total amount of ceftiofur metabolites is realistic using the method C approach.

Quantitative analysis of incurred poultry samples

The individual levels of ceftiofur, DFC and DCCD found in the incurred poultry breast muscle samples using method A are presented in table 5.6. It is observed that a significant concentration of ceftiofur is present 1 hour after administration, whereas it is not detectable 5 hours after treatment. DFC is the main metabolite observed, but also this metabolite is metabolised/excreted rapidly and from extrapolation it is estimated that it will no longer be detectable 24 hours after administration of a single dose of ceftiofur. DCCD, which is suggested to be a suitable biomarker for detecting ceftiofur [43] is present at concentrations lower than DFC and is excreted rapidly as well: it is no longer detectable approximately 24 hours after administration.

The determined levels of total ceftiofur metabolites, expressed as DFC, using method A, B and C are presented in figure 5.20. Method A clearly results in the lowest concentration of ceftiofur metabolites for all broilers. It is previously reported that a significant amount of protein-bound metabolites are formed after

ceftiofur administration [36,65,66]. This is in agreement with the observation that when using method A, which only detects selected metabolites, a lower total ceftiofur concentration is found compared to methods B and C, which do include protein-bound metabolites. It is concluded that method A results in an underestimation of the total amount of ceftiofur metabolites. From this and the observation that ceftiofur cannot be detected approximately 24 hours after administration of a single dose it was concluded that the analysis of individual metabolites of ceftiofur is not a suitable approach for detecting the off-label use of ceftiofur in the poultry-breeding.

For 10 out of the 12 samples, method C results in an average higher total ceftiofur concentration compared to method B, however, except for animal number 4 and 8 no statistically significant difference was found between method B and C using a Students' t-test. Note that method C also includes the minor metabolite desfuroylceftiofur thiolacton [55]. It is concluded that method C is comparable to or better than method B regarding its quantitative aspect and that the method C approach is adequate for the quantitative analysis of ceftiofur metabolites in poultry muscle.

Table 5.6. Concentration of ceftiofur, DFC and DCCD, expressed as DFC, detected in poultry breast muscle samples from a single dose controlled treatment using method A. Data between brackets: standard deviation (n=2).

Broiler no.	Time after administration (h)	Ceftiofur ($\mu\text{g kg}^{-1}$)	DFC ($\mu\text{g kg}^{-1}$)	DCCD ($\mu\text{g kg}^{-1}$)
1	1	167 (2)	386 (31)	246 (22)
2	1	267 (42)	561 (147)	187 (10)
3	1	157 (41)	349 (58)	115 (36)
4	2	2 (1)	78 (68)	79 (47)
5	2	13 (2)	121 (61)	69 (32)
6	2	4 (1)	53 (13)	70 (2)
7	4	ND	26 (5)	14 (0)
8	4	ND	41 (3)	12 (3)
9	4	ND	24 (17)	34 (7)
10	8	ND	17 (2)	11 (1)
11	8	ND	15 (6)	6 (2)
12	8	ND	11 (6)	15 (4)

ND = Not detected

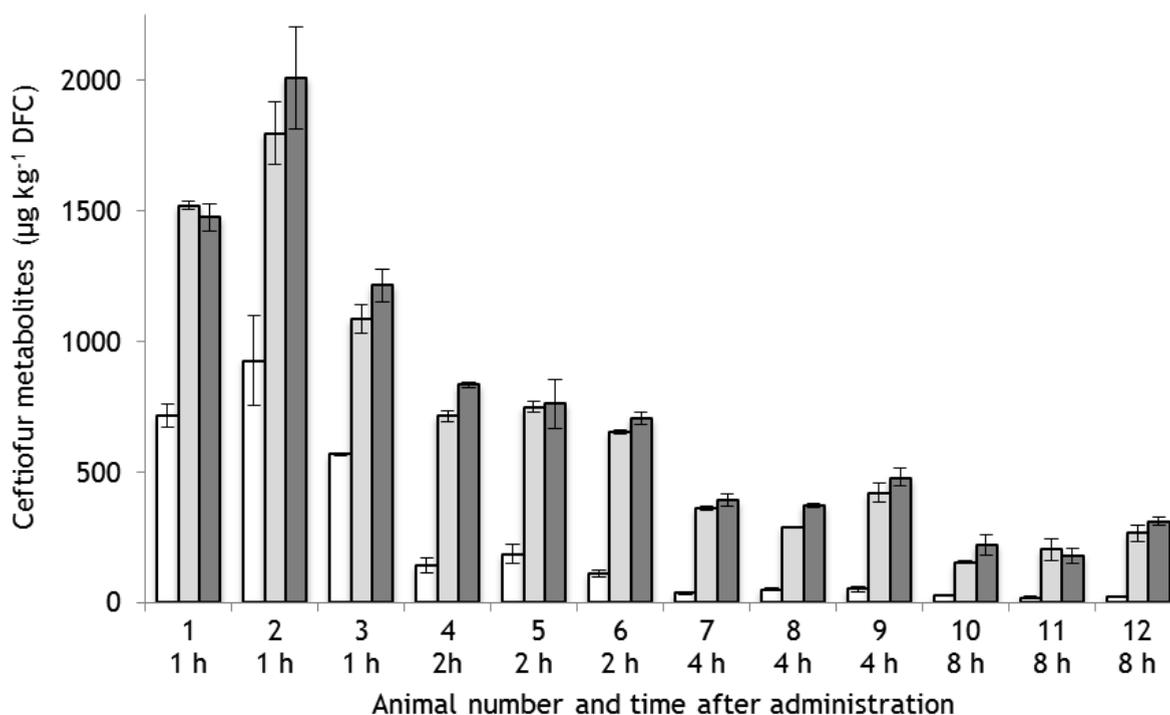


Figure 5.20. The determined levels of total ceftiofur metabolites (expressed as DFC) in poultry breast muscle samples from a single dose controlled treatment using method (□) A, (▨) B and (■) C (n=2).

Applicability to cephalosporin antibiotics other than ceftiofur

The approach applied in method B depends on the cleavage of a thioester bond using DTE. None of the other registered cephalosporins (for use in cattle and swine breeding) contains the thioester bond and therefore it is expected that method B is only applicable to a very small range of cephalosporins. This was confirmed by Beconi-Barker *et al.* [35] who demonstrated that cefquinome, cefacetrile and cefoperazone (figure 5.21) were not detectable using method B, when applying UV detection, whereas a signal possibly corresponding to cefapirin (figure 5.21b) was detected. In this work this derivatization procedure was tested for cefcapene, a cephalosporin antibiotic being an ester instead of a thioester (figure 5.21a). After applying the derivatization procedure used in method B, neither cefcapene acetamide nor any other representative marker for cefcapene was found by direct infusion analysis. It is concluded that method B is not a suitable approach for detecting a broad range of cephalosporins as was also stated by Fagerquist *et al.* [67].

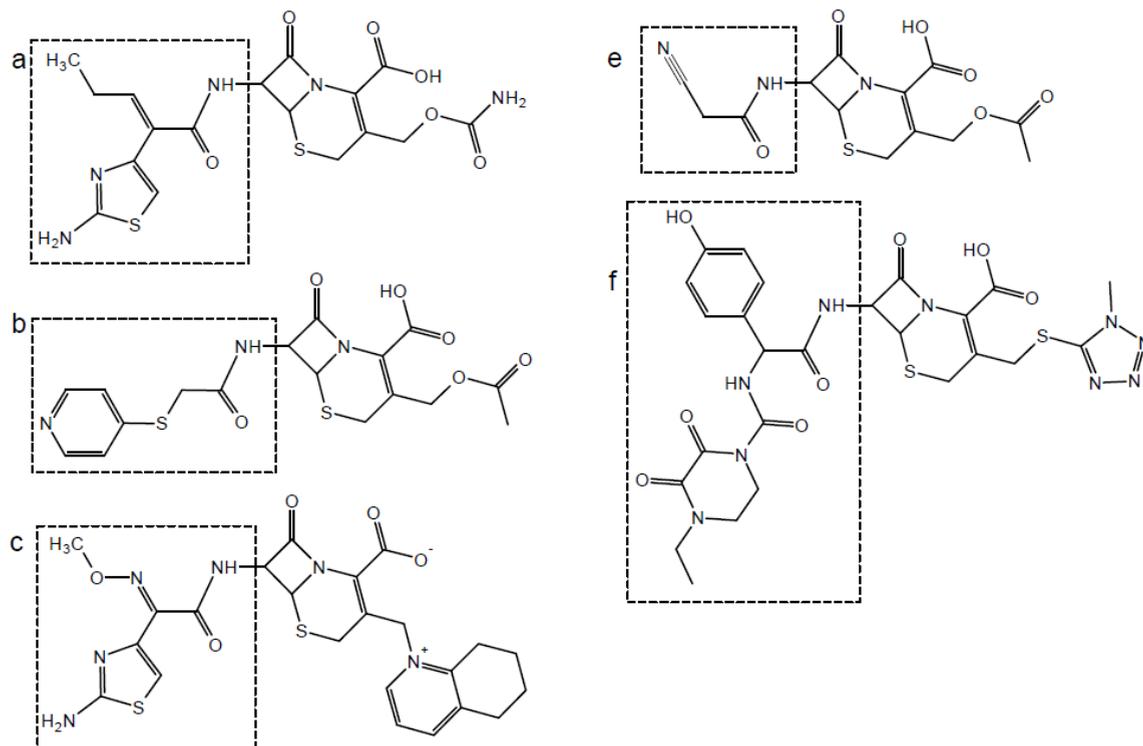


Figure 5.21. Molecular structure of (a) cefcapene, (b) cefapirin, (c) cefquinome, (d) cefacetrole and (e) cefoperazone. The AMTA analogue is indicated by the dotted square.

In theory, the approach used in method C is more generic and should be applicable to a broad range of cephalosporin antibiotics. Previously it was reported that an AMTA analogue was detected after hydrolysis of cefapirin [55] (section 5.2). The broad applicability was further demonstrated by derivatization of cefcapene in the presence of poultry muscle, resulting in the marker compound analogous to AMTA. A chromatogram of a blank poultry muscle sample spiked with 1 mg kg^{-1} of ceftiofur and cefcapene is presented in figure 5.22. From this it is concluded that, in contrast to method B, the method C hydrolysis approach is applicable as a multi-cephalosporin method that includes active metabolites, provided that not only AMTA but also the AMTA analogues are recovered during the sample clean-up and detected by selected reaction monitoring (SRM).

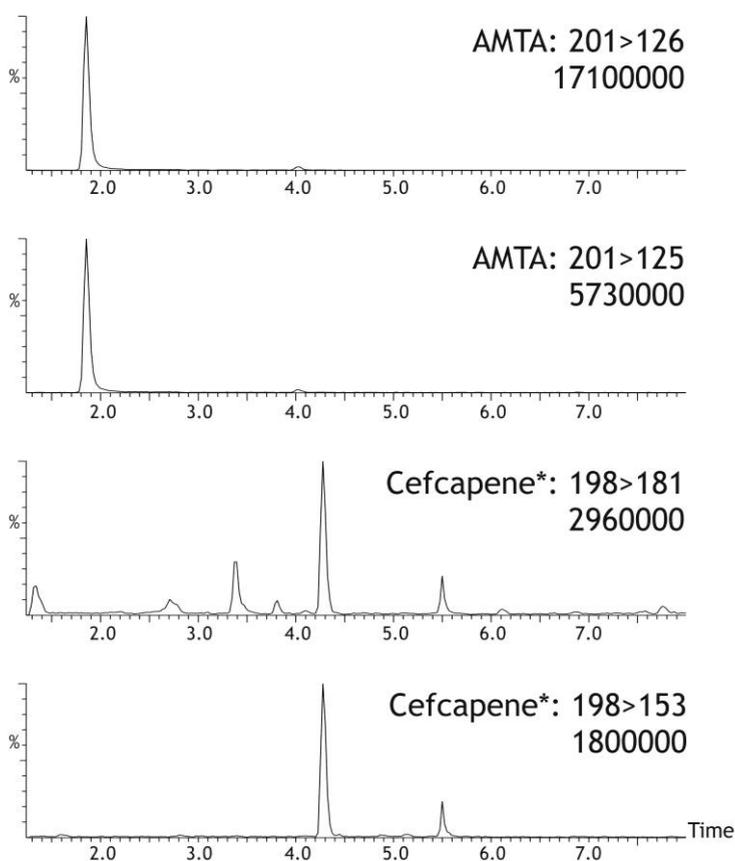


Figure 5.22. A blank poultry muscle sample spiked at 1 mg kg^{-1} with ceftiofur and cefcapene analysed using method C. Cefcapene* indicates the hydrolysis product of cefcapene.

The broad applicability of the new approach might also be considered as a weakness. Only a minor moiety of ceftiofur is used as the marker compound and therefore the selectivity of AMTA as a bio-marker for ceftiofur metabolites might be questioned. The Pubchem database [68] was searched for molecules containing the AMTA substructure resulting in many hits, amongst which 18 antibacterial substances, all being cephalosporines (eg. cefquinome, figure 5.21c). This demonstrates that the new approach is not specific for ceftiofur. However, if the AMTA analogues are analysed as well, the new approach is very useful as a broad quantitative screening of cephalosporin compounds in tissue samples to effectively indicate off-label use of cephalosporins in poultry breeding. Subsequently, for the determination of the identity of the actual cephalosporin administered, another method is needed monitoring intact cephalosporins or metabolites thereof.

Application to other matrices

The applicability of the method C hydrolysis approach was tested for other matrices than poultry breast muscle. This was done by studying the concentration of total ceftiofur residues in the thigh muscle, kidney and liver samples obtained from the ceftiofur treated broilers. For each of the matrices a calibration line with a coefficient of correlation above 0.991 was obtained, indicating that the quantitative aspect of the method is adequate for the analysis of different kinds of tissue matrix. The total amount of ceftiofur metabolites, expressed as DFC, in thigh muscle, kidney and liver, determined using method C, is presented in table 5.7.

Table 5.7. Concentration of total ceftiofur, expressed as DFC, detected in poultry thigh muscle, kidney and liver samples from a single dose controlled treatment using method C. Data between brackets: standard deviation (n=2), not duplicate value available for kidney because of the lack of sample material.

Broiler number	Time after administration (h)	Thigh muscle ($\mu\text{g kg}^{-1}$)	Kidney ($\mu\text{g kg}^{-1}$)	Liver ($\mu\text{g kg}^{-1}$)
1	1	488 (20)	3042	1906 (97)
2	1	595 (9)	2722	1755 (286)
3	1	458 (27)	2894	1855 (24)
4	2	442 (77)	1802	821 (1)
5	2	545 (10)	2240	796 (44)
6	2	476 (8)	2111	797 (28)
7	4	475 (22)	1277	524 (109)
8	4	300 (20)	854	285 (39)
9	4	392 (5)	1123	356 (91)
10	8	145 (8)	705	221 (8)
11	8	196 (29)	942	317 (77)
12	8	222 (5)	939	358 (3)

The results indicate that ceftiofur metabolites are rapidly distributed throughout the body. Levels of ceftiofur metabolites in the breast muscle are equal to the concentrations in liver following intramuscular breast injection. Levels in the thigh muscle were lower, whereas the highest concentrations were detected in the kidneys. This is in agreement with previous findings after intramuscular treatment of swine [36,66] and subcutaneous injection of ducks [69]. It is

concluded that, from an analytical point of view, poultry kidney is potentially better suited for detecting off-label use of ceftiofur. However, only a very limited amount of kidney (approximately 5 g per broiler) is available complicating its use for monitoring purposes. From the results it is concluded that the hydrolysis approach is applicable to different kinds of poultry tissues illustrating its versatility and ruggedness.

Conclusions

Three LC-MS/MS approaches for the analysis of ceftiofur and its active metabolites in poultry muscle were critically compared. The first approach focused on the analysis of ceftiofur, DFC and DCCD. The detection limits of this method are 1 $\mu\text{g kg}^{-1}$ for ceftiofur, 2 $\mu\text{g kg}^{-1}$ for DFC and 3 $\mu\text{g kg}^{-1}$ for DCCD. Although this approach is the simplest one to apply, it results in a serious underestimation of the total ceftiofur residue concentration and off-label use of ceftiofur is only detectable for approximately 24 hours after a single dose treatment. Therefore, it is concluded that the analysis of ceftiofur, DFC and/or DCCD is inadequate for detecting off-label use of ceftiofur in the poultry-breeding sector. The second approach included a derivatization to produce DCA, as a marker for ceftiofur and its metabolites. The LOD of this method was 1 $\mu\text{g kg}^{-1}$ and this approach resulted in significantly higher total ceftiofur concentrations compared to the first approach. However, this second method should be considered a single compound method. The third approach included a hydrolysis using ammonia to produce AMTA as a marker for ceftiofur including its metabolites. The detection limit was 0.5 $\mu\text{g kg}^{-1}$, and this approach included protein-bound ceftiofur and the minor metabolite desfuroylceftiofur thiolacton, resulting in comparable or slightly higher total ceftiofur concentrations compared to the second approach. Only the third approach is applicable to a broad range of cephalosporin antibiotics. It is fair to state that the third approach is not entirely specific for ceftiofur because some chemicals, other than ceftiofur, might yield the same AMTA marker. Nevertheless, the method can be very useful as a broad quantitative screening of any cephalosporin compound in poultry tissue samples to indicate off-label use of cephalosporins in poultry breeding. It is expected that such a multi-method that includes the active metabolites of all unstable cephalosporins will help to detect off-label use of cephalosporin antibiotics.

5.4. Comprehensive analysis of β -lactam antibiotics including penicillins, cephalosporins and carbapenems in poultry muscle using liquid chromatography coupled to tandem mass spectrometry

Abstract

A comprehensive method for the quantitative residue analysis of trace levels of 22 β -lactam antibiotics, including penicillins, cephalosporins and carbapenems in poultry muscle by liquid chromatography in combination with tandem mass spectrometric (LC-MS/MS) detection is reported. The samples analysed for β -lactam residues are hydrolysed using piperidine, in order to improve compound stability and to include the total residue content of the cephalosporin ceftifour. The reaction procedure was optimised using a full experimental design. Following detailed isotope labeling tandem mass spectrometry studies and exact mass measurements using high resolution mass spectrometry reaction schemes could be proposed for all β -lactams studied. The main reaction occurring is the hydrolysis of the β -lactam ring under formation of the piperidine substituted amide. For some β -lactams multiple isobaric hydrolysis reaction products are obtained, in accordance with expectations, but this did not hamper quantitative analysis. The final method was fully validated as a quantitative confirmatory residue analysis method according to Commission Decision 2002/657/EC and showed satisfactory quantitative performance for all compounds with trueness between 80 and 110 % and within-laboratory reproducibility below 22 % at target level, except for biapenem. For biapenem the method proved to be suitable for qualitative analysis only.

Introduction

β -lactams are highly effective antibiotics in the treatment of bacterial infections [1]. The β -lactam antibiotics consist of several groups of which the 8 penicillins, 8 cephalosporins and 6 carbapenems were selected because of their important (biological) activity.

The first penicillin was isolated from bacteria in 1928 [2]. Later, many chemically altered semi-synthetic penicillins were developed. The penicillins derive their activity from the 6-aminopenicillanic acid nucleus which is effective against mainly Gram positive bacteria [3,4]. Amoxicillin, ampicillin, penicillin G (benzylpenicillin), penicillin V (phenoxymethylpenicillin), cloxacillin, dicloxacillin, oxacillin and nafcillin (chemical structures included in online resource 1) are registered for the treatment of food-producing animals: in fact penicillins are the most frequently used antibiotics in poultry production [33]. Maximum residue limits (MRLs) are established for the major species, including poultry (table 5.8) [6].

Cephalosporins were first isolated from bacteria in 1956 [8]. The six membered dihydrothiazine ring fused with a four membered β -lactam ring is responsible for the biological activity of this group of compounds. As for the penicillins, many semi-synthetic cephalosporins were developed. Several generations of cephalosporins are distinguished based upon their time of discovery and their range of activity [11]. Cefacetril, cefalonium, cefazolin, cefalexin and cefapirin (all 1st generation), cefoperazone and ceftiofur (3rd generation), and cefquinome (4th generation) are all approved for treatment of mastitis infections in dairy cattle (chemical structures included in online resource 2). Cefazolin is approved for the treatment of other ruminants (sheep and goat) as well. Furthermore, cefalexin and cefapirin are approved for the treatment of respiratory disease and foot rot in cattle, cefquinome is approved for the treatment of cattle, swine and horses, and ceftiofur for all food producing mammals [6,11]. For cefapirin the MRL is defined as the sum of cefapirin and deacetylcefapirin and for ceftiofur as the sum of all active ceftiofur metabolites (table 5.8) [6].

The first carbapenem was isolated from bacteria in 1971 [12]. The carbapenems are structurally very similar to the penicillins: the sulfur atom has been replaced by a carbon atom and an unsaturation has been introduced [13]. As a result the carbapenems possess the broadest antimicrobial activity amongst the β -lactams [13]. The most common carbapenems are imipenem, meropenem, ertapenem, doripenem and biapenem (chemical structures included in online resource 3). The carbapenems are not registered for use in food-producing animals and are used off-label in companion animals [4]. Another β -lactam substance, related to the carbapenems is faropenem (chemical structures included in online resource 3). As the carbapenems, faropenem, a related compound, is active against ESBL producing bacteria [14].

During the last decades, antimicrobial resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum- β -lactamase (ESBL) producing bacteria are emerging [15,19] which has become an important public health threat [15-19]. Most penicillins are found to be ineffective against ESBL producing bacteria [20-22]. Cephalosporins are more effective and, therefore, in 2007 they were assigned as critically important antimicrobials for human health [23]. Even though these compounds should only be used sparingly, resistance towards cephalosporins is emerging [16-18,22,24-29]. In contrast to penicillins and cephalosporins, carbapenems have proven to be highly effective in severe infections due to ESBL producing bacteria. As a result, due to the rising resistance to cephalosporins, carbapenem use has increased in the treatment of humans [30]. It is alarming that also carbapenem resistance is emerging [13,16,29,30].

Not only the antibiotic usage in humans contributes to rising bacterial resistance, also the regular use of antibiotics in veterinary practice contributes to the occurrence of resistant bacteria [15-19,24] that can be transferred from animals to humans [32]. Furthermore, due to off-label use of antibiotics in veterinary practice, residues of these antibiotics can end up in the human food chain which contributes to increasing bacterial resistance as well [33]. Penicillins are the most frequently sold antibiotic group for treatment of broilers [34]. Cephalosporins and carbapenems are not registered for use in poultry production within Europe, but due to their high effectiveness, their use in broilers cannot be ruled out. To prevent off-label use of β -lactams in animal breeding and thus to

limit the dissemination of bacterial resistance, an effective monitoring strategy is needed for β -lactam usage analysis in food-producing animals. Therefore, the monitoring of poultry muscle for penicillins, cephalosporins as well as carbapenems at levels as low as reasonably possible is of importance.

Table 5.8. MRLs and target values for the validation of the β -lactams included in the develop dmethod.

Component	MRL ($\mu\text{g}/\text{kg}$) [6]			Study's target level ($\mu\text{g kg}^{-1}$) ¹
	Poultry muscle	Beef	Milk	Poultry muscle
Amoxicilline	50			50
Ampicilline	50			50
Penicilline G	50			50
Penicilline V	25			25
Cloxacilline	300			300
Dicloxacilline	300			300
Nafcilline	300			300
Oxacilline	300			300
Ceftiofur	n.a.	1000	100	10
Cefquinome	n.a.	50	20	10
Cefapirine	n.a.	50	60	10
Cefalexin	n.a.	200	100	10
Cefalonium	n.a.		20	50
Cefazolin	n.a.		50	25
Cefacetrile	n.a.		125	50
Cefoperazone	n.a.		50	50
Biapenem	n.a.			100
Doripenem	n.a.			50
Ertapenem	n.a.			50
Imipenem	n.a.			100
Meropenem	n.a.			25
Faropenem	n.a.			25

¹ Target value is the MRL or in case no MRL has been established, as low as reasonably possible.

n.a.: not available, substance not registered for use in poultry.

Many multi-analyte methods for the analysis of β -lactams in muscle using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have been reported [70]. Most of them include penicillins only [71-75] or a combination of penicillins and some cephalosporins [43,47,59,67,74]. The multi-analyte methods that include ceftiofur, an important member of the cephalosporin group, target for ceftiofur itself or include desfuroylceftiofur (DFC) and/or DFC cysteine disulfide (DCCD) [43,59,67] but do not detect protein bound metabolites, which is the largest fraction of the ceftiofur related metabolites after intra-muscular administration. As a result, these methods result in an underestimation of the total amount of active ceftiofur metabolites [76] (section 5.3). To the best of our knowledge, no methods for the analysis of carbapenems in food products have been reported at all. In other words, methods that comprehensively cover the broad range of β -lactam antibiotics and that do offer an effective monitoring strategy for ceftiofur are lacking.

In this section a comprehensive β -lactam LC-MS/MS method is presented that is suitable for the simultaneous analysis of eight penicillins, eight cephalosporins, five carbapenems and faropenem in poultry muscle that does include the active metabolites of ceftiofur.

Experimental

Reagents and equipment

ULC/MS grade water and acetonitrile (ACN), and HPLC grade methanol (MeOH) were obtained from Biosolve (Valkenswaard, The Netherlands). Acetic acid, formic acid, 25 % ammonia, 32 % ammonia (GPR Rectapur), sodium chloride and n-hexane were obtained from VWR International (Darmstadt, Germany). Piperidine (99 %) and disodium tetraborate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Piperidine- d_{11} was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Milli-Q water was prepared using a Milli-Q system at a resistivity of at least $18.2 \text{ M}\Omega \text{ cm}^{-1}$ (Millipore, Billerica, MA, USA). The reference standard of amoxicillin, ampicillin, penicillin G Na, penicillin V K, cloxacillin Na H_2O , dicloxacillin Na H_2O , nafcillin Na H_2O , oxacillin Na H_2O , ceftiofur HCl, cefapirin HCl, cefalonium H_2O and cefoperazone Na were obtained from Sigma-

Aldrich. Cefalexin H₂O, cefazolin Na, cefacetrile, ampicillin-d₅, dicloxacillin-¹³C₄ Na H₂O, cefalexin-d₅ H₂O, cephalirin-d₄, ceftiofur d₃ HCl and meropenem-d₆ were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Cefquinome sulphate was obtained from AK scientific (Union City, CA, USA). Biapenem, ertapenem Na, imipenem H₂O, faropenem and meropenem were obtained from Ontario Chemicals (Guelph, ON, Canada). Doripenem, penicillin-V-d₅, amoxicillin-d₄ and cefazolin-¹³C₂-¹⁵N were obtained from Cachesyn (Mississauga, ON, Canada). Penicillin-G-d₇ ethylpiperidinium was obtained from Witega (Beril, Germany). Imipenem-d₄ and ertapenem-d₄ were obtained from Alsachim (Illkirch Graffenstaden, France).

Stock solutions of the penicillins, ceftiofur, cefapirin, cefalonium, cefacetrile and the corresponding isotopically labeled analogues were prepared in MeOH at 100 mg L⁻¹. Stock solutions of cefquinome, cefalexin, cefazolin, cefoperazone, the carbapenems, faropenem and the corresponding isotopically labeled analogues were prepared in water at 100 mg L⁻¹. All dilutions were prepared fresh daily in Milli-Q water.

Borate solution was prepared by dissolving 10 g disodium tetraborate (0.05 M) and 29.2 g sodium chloride (0.5 M) in 1 L of water, stirred using a magnetic bar for 30 min (this is a saturated solution).

Sample preparation

The poultry muscle samples were homogenised using a laboratory blender, 2.5 g was weighted into a 50 mL poly propylene (PP) centrifuge tube and 200 µL internal standard solution was added to obtain a concentration of the internal standard of 2 * target level (table 5.8). 10 mL of borate solution and 500 µL piperidine were added to the sample. The sample was vigorously shaken by hand for 5 sec and next incubated in a water bath (1 h, 60 °C). After incubation the samples were cooled at room temperature for 10 min and next 10 mL of n-hexane was added to the reaction mixture. After shaking using a rotary tumbler (5 min) and centrifugation (3500 g, 15 min) the aqueous layer was transferred into a clean test tube and neutralised (pH 7.2) by adding acetic acid (25 %) and/or ammonia (2.5 %) and the sample was centrifuged again (3500 g, 15 min). A Phenomenex (Torrance, CA, USA) Strata-X 200 mg / 6 mL reversed phase solid phase extraction (SPE) cartridge was conditioned with 5 mL MeOH and 5 mL

water. The clear neutralised extract was applied onto the SPE cartridge which was subsequently washed with 5 mL of MeOH/water (1:9 v/v) and dried by applying vacuum for 5 min. The β -lactam hydrolysis products were eluted from the cartridge using 5 mL MeOH/ACN (50:50, v/v) followed by evaporation of the solvent (45 °C, N₂). The residue was redissolved in 500 μ L 1 % piperidine in water and transferred into an LC-MS/MS sample vial.

Liquid chromatography - tandem mass spectrometry

The LC system consisted of a Waters (Milford, MA, USA) model Acquity with a Waters Acquity UPLC CSH C₁₈ analytical column of 2.1 \times 100 mm, 1.7 μ m, placed in a column oven at 50 °C. The gradient (solvent A, 0.0032 % ammonia in water; solvent B, 0.0032 % ammonia in water/acetonitrile (1:9 v/v)) was: 0 - 1.0 min, 0 % B, 1.0 - 9.0 min, linear increase to 40 % B, 9.0 - 10.0 min, linear increase to 100 % B with a final hold of 0.5 min, operating at a flow rate of 0.4 mL min⁻¹. The injection volume was 10 μ L. Detection was carried out by LC-MS/MS using a Waters Xevo TQS or a AB Sciex (Ramingham, MA, USA) Q-Trap 6500 mass spectrometer in the positive electrospray ionisation (ESI) mode. All compounds were analysed on both instruments. The operating parameters of the Xevo TQS were: capillary voltage, 2.0 kV; cone voltage, 25 V; source offset, 20 V; source temperature, 150 °C; desolvation temperature, 550 °C; cone gas flow, 150 L h⁻¹; and desolvation gas, 600 L h⁻¹. The selected β -lactam hydrolysis products were fragmented using collision induced dissociation (argon) and the Selected Reaction Monitoring (SRM) transitions are presented in table 5.9. Data were processed using QuanLynx software V4.1 (Waters). The operating parameters of the QTrap 6500 were: capillary voltage, 2.0 kV; cone voltage, 25 V; source offset, 20 V; source temperature, 150 °C; desolvation temperature, 550 °C; cone gas flow, 150 L h⁻¹; and desolvation gas, 600 L h⁻¹. The selected β -lactam hydrolysis products were fragmented using collision induced dissociation (nitrogen) and the Selected Reaction Monitoring (SRM) transitions are presented in table 5.9. Data were processed using Multiquant software V2.1.1 (AB Sciex).

Table 5.9. SRM transitions of the selected β -lactam hydrolysis products using the Xevo TQS and the QTrap 6500.

Product of	Precursor ion (m/z)	Product ion (m/z)	Xevo TQS		QTrap 6500	
			Cove (V)	Collision energy (eV)	DP	Collision energy (eV)
Penicilline G	420.2	261.2 ^b	25	15	40	15
		176.0	25	20	40	20
		160.0 ^a	25	20	40	20
Penicilline G-d ₇	427.2	160.0	25	20	40	20
Ampicilline	435.2	286.0 ^{a b}	15	20	40	20
		276.2	15	20	40	20
Ampicilline-d ₅	440.2	291.0	25	20	40	20
Penicilline V	436.0	277.0 ^{a b}	25	115	40	20
		192.0	25	20	40	20
		160.0	25	20	40	20
Penicilline-V-d ₅	441.0	160.0	25	20	40	20
Amoxicilline	451.2	275.2 ^a	25	15	40	15
		302.2 ^b	25	15	40	15
Amoxicilline-d ₄	455.2	279.2	25	15	40	15
Oxacilline	487.0	160.0 ^a	25	20	40	17
		328.2 ^b	25	15	40	15
		243.1	25	20	40	20
Nafcilline	500.0	256.2 ^{a b}	16	15	40	15
		199.1	16	22	40	22
Cloxacilline	521.2	160.0 ^a	25	20	40	20
		362.2 ^b	25	15	40	15
Dicloxacilline	555.0	160.0 ^a	25	20	40	20
		396.0 ^b	25	15	40	15
Dicloxacilline- ¹³ C ₄	559.0	160.0	25	20	40	20
Cefalexine	276.2	143.1	58	12	40	22
		259.1 ^{a b}	58	10	40	22
		231.2	58	16	40	22
Cefalexine-d ₅	281.2	264.1	58	10	40	22
Cefapirine	294.1	152.0 ^{a b}	25	22	40	24
		181.0	25	20	40	22
		143.1	25	22	40	22
Cefapirine-d ₄	298.1	156.0	25	22	40	24

Ceftiofur / cefquinome	326.1	169.0 ^{a b}	25	20	40	19
		241.0	25	15	40	17
		213.0	25	20	40	22
Cefquinome by- product	134.0	106.0	25	25	40	37
		78.0 ^a	25	35	40	49
Ceftiofur-d ₃	329.1	169.0	25	20	40	17
Cefacetrole	468.2	339.2 ^a	20	15	20	19
		424.2 ^b	20	15	20	17
Cefazolin	493.2	294.1 ^a	25	20	40	22
		336.2 ^b	25	20	40	22
Cefazolin- ¹³ C ₂ - ¹⁵ N	496.2	296.1	25	20	40	22
Cefalonium	507.2	378.2	20	16	45	24
		463.4 ^{a b}	20	10	45	19
Ceforperazone	718.3	386.3 ^a	50	25	40	27
		630.4 ^b	50	20	40	22
Imipenem	358.1	314.1 ^{a b}	25	10	35	17
		172.1	25	20	35	24
		154.2	25	25	35	37
Imipenem-d ₄	362.1	318.1	25	10	35	17
Faropenem	371.1	154.2 ^{a b}	25	15	55	21
		172.1	25	15	55	17
		257.1	25	15	55	23
Biapenem	399.2	154.1 ^{a b}	25	25	50	35
		172.1	25	20	50	31
Meropenem	469.2	207.1 ^a	25	18	20	27
		425.2 ^b	25	10	20	13
Meropenem-d ₆	475.2	207.1	25	18	20	27
Doripenem	506.0	207.1	25	20	30	29
		462.2 ^{a b}	25	10	30	19
Ertapenem	560.9	517.1 ^b	25	20	40	23
		207.2 ^a	25	25	40	31
Ertapenem-d ₄	564.9	207.2	25	25	40	31

^a Most abundant product ion in Xevo TQS

^b Most abundant product ion in QTrap 6500

LC-Orbitrap-MS

The LC system consisted of a Thermo Fisher Scientific (San Jose, CA, USA) Accela HPLC system. The LC conditions and column were the same as for the triple quadrupole MS analysis. Detection was carried out using a Thermo Fisher Scientific Exactive single-stage Orbitrap MS operating with a heated electrospray ionisation (HESI) II source in positive mode. The operating parameters were: electrospray voltage, 2.8 kV; sheath gas, 19 arbitrary units; auxiliary gas, 7 arbitrary units; source heater, 300 °C; heated capillary, 360 °C. Data were acquired in full scan from m/z 55 - 1000 (scan time 0.5 sec) at a resolving power of at least 50,000. Internal mass calibration within each scan was done using an ion that is always present in the background (m/z 218.1387, not identified). Data were processed using Xcalibur software V2.2 (Thermo Fisher Scientific).

Identification of reaction products upon piperidine treatment

All β -lactams were hydrolysed by adding 250 μ L piperidine to 5 mL of an aqueous standard solution of the individual β -lactams (10 μ g mL⁻¹). After incubation in a water bath (60 °C, 1 h) the solution was transferred onto a conditioned Strata-X RP 200 mg / 6 CC SPE cartridge (Phenomenex). The cartridge was washed with 3 mL water to remove excessive piperidine and dried by applying vacuum for 5 min. The β -lactam reaction products were eluted with 5 mL MeOH/ACN (1:1 v/v) followed by evaporation of the solvent (45 °C, N₂). The residue was redissolved in 2 mL water. The same procedure was followed using piperidine-d₁₁ and, if available, isotopically labeled analogues of the β -lactams. These solutions were infused into the triple quadrupole MS at a rate of 20 μ L min⁻¹ and full scan spectra were obtained in the range of m/z 50 - 1000. All selected reaction products were subjected to collision induced dissociation (CID) to obtain information on their identity. The exact mass of the marker compounds was determined by injecting the solutions into the LC-Orbitrap-MS system.

Optimization of the reaction conditions in poultry muscle

The hydrolysis was optimised using a full experimental design consisting of three factors: temperature at 40, 60 and 80 °C; piperidine concentration at 2.5, 5 and 10 %; and time at 10, 20, 30 and 60 min. The experiment consisted of 36 poultry muscle samples, spiked at 500 μ g kg⁻¹ with all β -lactams. Data were evaluated

using single factor analysis of variance (ANOVA) in Genstat (VSN International, Hemel Hempstead, UK).

Method Validation

A full validation was carried out according to Commission Decision 2002/657/EC [77]. The following parameters were determined: linearity, trueness, repeatability, within-laboratory reproducibility, decision limit ($CC\alpha$), detection capability ($CC\beta$), selectivity, ruggedness and stability. For each compound a target level was set: for the penicillins this was the MRL [6] and for the other compounds this was as low as reasonably possible using the presented method (table 5.8). The validation was carried out at three different levels (0.5, 1.0 and 1.5 * target level), on three different occasions, by two different technicians, including two different mass spectrometers and including poultry muscle samples from different origin (n=21). For the penicillins, an additional validation was carried out at a target level of $10 \mu\text{g kg}^{-1}$ on a single day, including seven different poultry muscle samples to determine the performance of the method for the quantitation of penicillins at levels well below the MRL. Because ceftiofur and cefquinome result in the same hydrolysis product, they interfere in each other's quantitation. To be able to determine method characteristics for both compounds, separate samples for ceftiofur and cefquinome were prepared.

Quantitative results were obtained using isotopically labelled internal standards. If available correction were carried out using the corresponding labelled internal standard. Nafcillin, cloxacillin and oxacillin were all corrected using dicloxacillin- $^{13}\text{C}_4$, cefquinome using ceftiofur- d_3 , cefacetrile, cefazolin, cefalonium and cefoperazone were all corrected using cefazolin- $^{13}\text{C}_2^{15}\text{N}$, faropenem using penicillin G- d_7 , biapenem using ertapenem- d_4 and doripenem using amoxicillin- d_4 .

Linearity

On four different days a matrix matched calibration line was prepared including 0, 0.5, 1, 2, 3 and 5 * target level by adding solutions of the β -lactams to blank poultry muscle sample aliquots. Calibration lines were constructed by plotting the peak areas, corrected by the corresponding internal standard, versus the added level and carrying out least squares linear regression. The linearity was considered acceptable if the coefficient of correlation was at least 0.990.

Trueness, repeatability and within-lab reproducibility

On each of the days seven different blank poultry muscle samples were selected and analysed as such and spiked at 0.5, 1.0 and 1.5 * target level. For each sample the β -lactams concentration was calculated using the calibration line constructed on the same day. The trueness was calculated by dividing the average calculated level by the nominal concentration. According to 2002/657/EC [77] the trueness should be between 70 and 110 % for validation levels between 1.0 and 10.0 $\mu\text{g kg}^{-1}$ and between 80 and 110 % for validation levels exceeding 10.0 $\mu\text{g kg}^{-1}$.

The repeatability and within-lab reproducibility were calculated using ANOVA. According to 2002/657/EC [77] the relative within-lab reproducibility (RSD_{RL}) and relative repeatability (RSD_{r}) are considered acceptable if below the value calculated from the Horwitz equation [78] respectively two thirds of this value, i.e. below 31.7 % and 21.1 % respectively at a validation level of 10 $\mu\text{g kg}^{-1}$. Thompson [79] however, demonstrated that the Horwitz equation is not applicable to the lower concentration range ($< 120 \mu\text{g kg}^{-1}$) and suggested a complementary model. Based on this model the RSD_{RL} and RSD_{r} are acceptable if below 22 % and 14.7 % respectively. The latter, more stringent criteria were adopted for this validation study for all validation levels $< 120 \mu\text{g kg}^{-1}$. Note that because different muscle samples were used within one day, the repeatability is not the pure repeatability as stated in 2002/657/EC [77]. However, using different muscle samples a better understanding of the between sample variation is obtained and the validation better reflects a routine analysis situation.

Decision limit and detection capability

For the penicillins $\text{CC}\alpha$ and $\text{CC}\beta$ were calculated according to the procedure described in 2002/657/EC [77] for MRL substances. Because $\text{CC}\alpha$ and $\text{CC}\beta$ are calculated from the within-laboratory reproducibility, no specific criteria are set for these parameters. In addition, for all compounds, $\text{CC}\alpha$ and $\text{CC}\beta$ were calculated based upon the zero tolerance approach using the linearity approach as stated in ISO 11843 [80], to reflect the lowest levels that can still be determined. This approach tends to give correct results only if the spiking levels are near the $\text{CC}\beta$ of the method. Because the validation levels of the penicillins are far above the detection limit, additional blank poultry muscle samples ($n=7$)

were spiked at 5, 10 and 15 µg/kg with the penicillins and analysed according to the presented procedure. The results were assessed using the results of the samples spiked at the lowest validation level. In at least 95 % of these samples a peak for the β-lactams should be observed for the least abundant product ion.

Selectivity

The selectivity of the method was studied based on a practical and a theoretical study. First, the 21 analysed blank samples were checked for interferences at the retention times corresponding to the β-lactam hydrolysis reaction products. Second, the procedure previously described for validation of selectivity was applied to estimate the probability of interfering substances [81] (chapter 3).

Ruggedness

The ruggedness became clear during the optimisation of the method. Additionally some minor changes to the method were assessed in triplicate being: (1) hydrolysis for an additional 60 min, (2) evaporation of the eluent until dryness and leaving the test tubes in the evaporator for 30 min, and (3 and 4) adjustment of the pH of the extract to pH 6.0 and 8.0. The trueness and repeatability of these tests were compared to the characteristics of the method as described.

Stability

Stability data for the β-lactams in solutions and final extracts were obtained from literature for the penicillins [82] and studied for the cephalosporins and carbapenems. The stability was tested for 10 mg L⁻¹ academic solutions in methanol according to a previously described procedure [82]. Mixed reference solutions of 10 mg L⁻¹ of the studied compounds were placed at < -70 °C, -18 °C and 4 °C. After 1 and 2 months two containers of both -18 °C and 4 °C were placed at < -70 °C. At the day of analysis, all solutions were defrosted, hydrolysed and analysed in quadruplicate in random order.

The stability of final extracts was determined by storing these extracts at -18 °C after analysis. After 7 days, the matrix matched calibration standards and the samples spiked at target level (n=7) were defrosted and injected in the LC-MS/MS system again. The linearity, the trueness and the repeatability of this series was

evaluated based on the established validation criteria to determine whether quantitative analysis is still possible after storing the sample extracts at $-18\text{ }^{\circ}\text{C}$ for one week.

Application to routine samples

The method was assessed by analysing ceftiofur incurred poultry muscle samples obtained from ceftiofur treated chicks obtained 4h, 8h and 24h after treatment (n=3). These samples were analysed using the presented method and the method previously presented using a hydrolysis with ammonia which was found to have comparable results to other routinely applied methods for ceftiofur analysis [76] (section 5.3). The results were compared using a Students't-test. Finally the presented method was used to monitor 25 poultry muscle samples obtained from different local super markets.

Results & Discussion

Characterization of the hydrolysis reaction products

The penicillins all react in the same way with piperidine as was previously reported [53,71]. A reaction product is produced by hydrolysis of the β -lactam ring under formation of the substituted amide (figure 5.23, appendix 5.1). The reaction product is, in contrast to the penicillin itself [59,82], a stable molecule and therefore the hydrolysis procedure is beneficial with regard to method ruggedness.

The cephalosporin hydrolysis is more complicated and results in multiple reaction products. The proposed reaction scheme is presented in figure 5.24 and the proposed reaction products in appendix 5.2. A first nucleophilic substitution occurs in which the β -lactam ring is hydrolysed and the leaving group at the C3' position is removed resulting in the exo-methylene compound as reported previously [53,83]. This intermediate was detected in the continuous infusion full scan mass spectra for all cephalosporins, except for cefalexin. Cefalexin showed the intermediate at two mass units higher, which is explained by the absence of a leaving group on position 3: only the hydrolysis of the β -lactam ring occurs and no double bond can be produced at this position.

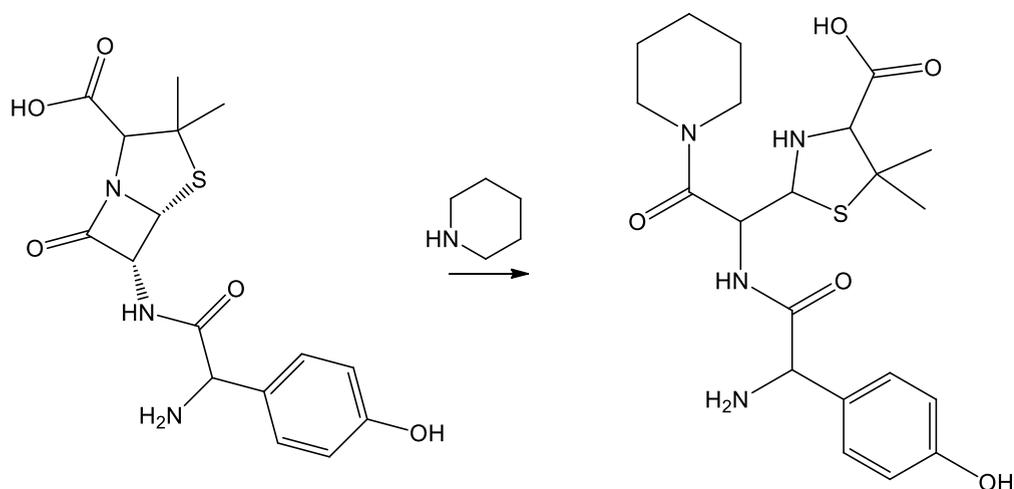


Figure 5.23. Hydrolysis of the penicillin amoxicillin after addition of piperidine.

Two additional reaction pathways are proposed. First, because the exo-methylene group is highly reactive, a second nucleophilic substitution occurs at the 3' position (figure 5.24, pathway 1) [84,85]. This results in a reaction product containing two piperidine moieties (cefazolin, cefalonium, cefacetile and cefoperazone), which was confirmed by a mass increase of 20 Da when carrying out the reaction using piperidine- d_{11} . A peak at the m/z corresponding with this reaction product was observed for all cephalosporins in the continuous infusion mass spectra. The cefalexin intermediate does not contain an exo-methylene group and thus it cannot undergo a second nucleophilic attack at position 3'. Because a reaction product is observed at the corresponding m/z , another reaction must occur that results in an isomeric reaction product. It is proposed that, instead of a nucleophilic attack at position 3', this could also occur at position 4, although this seems less likely at forehand. This would result in a second reaction product containing two piperidine moieties. The formation of multiple isomeric reaction products is confirmed by the observation of several peaks in the chromatogram when monitoring the SRM mass transitions of these reaction products that contain two piperidine moieties.

Second, it is proposed that the nucleophilic attack at position 3', after electron rearrangement, can result in a piperidine amide compound, which was only observed for ceftiofur, cefquinome, cefalexin, cefapirin and cefalonium (figure 5.24, pathway 2). The presence of one piperidine moiety and the side chain on position 7 in this reaction product was confirmed by carrying out the reaction

using piperidine- d_{11} and, if available, cephalosporins containing an isotopically labeled side chain at position 7.

For each cephalosporin a single reaction product was selected for effectively monitoring the presence of β -lactam residues in poultry muscle (appendix 5.2). For ceftiofur, cefquinome, cefalexin and cefapirin the final reaction product containing one piperidine moiety was the most abundant and therefore those were selected as the marker compounds. For cefalonium and cefazolin the reaction product containing two piperidine moieties were the most abundant products and therefore, those were selected as the marker compounds. For cefacetile and cefoperazone reaction products containing two piperidine moieties were observed, but additionally hydrolysis of the nitrile group in the side chain of cefacetile and hydrolysis of the amide in the side chain of cefoperazone occurred [86-88].

Also the carbapenems and faropenem show several reaction products, but it is observed that, as for the penicillins, the hydrolysis of the β -lactam ring was the major reaction occurring (figure 5.25, appendix 5.3). Additional hydrolysis reactions in the side chain were observed for imipenem (comparable to the hydrolysis of amitraz [89]) and biapenem. For all compounds, the proposed reaction products were supported by the exact mass data (error < 1 ppm, table 5.10) and the fragmentation pattern in MS/MS using collision induced dissociation.

According to the MRL of ceftiofur [6] all metabolites containing the intact β -lactam ring should be taken into account. As was previously demonstrated [76] (section 5.3) by using an alkaline hydrolysis also DFC, DCCD and protein bound ceftiofur are taken into account. Another advantage of the presented method is that the hydrolysis reaction products are more stable molecules compared to the intact β -lactams. Therefore, a more robust method is obtained.

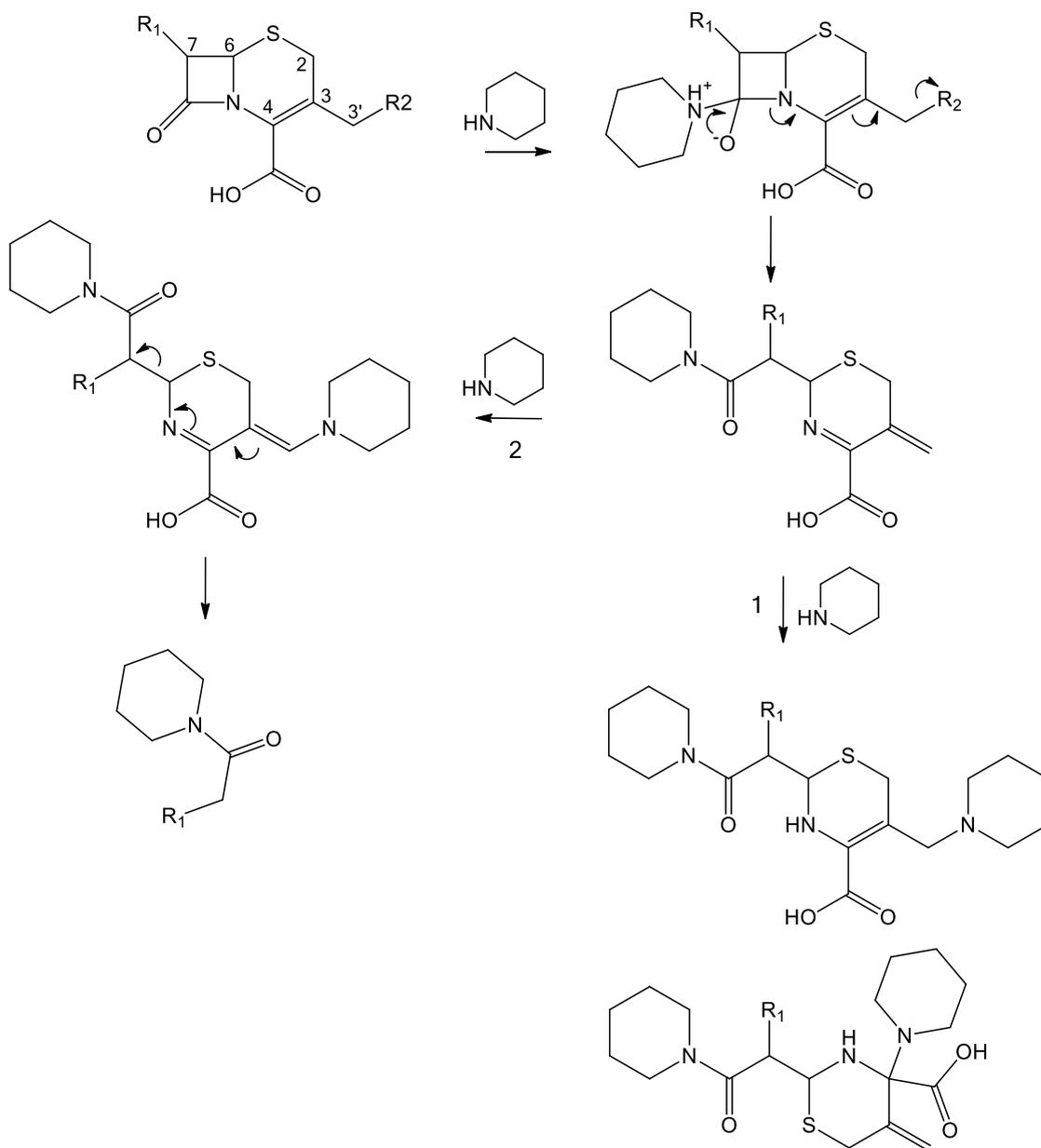


Figure 5.24. Proposed hydrolysis reaction of the cephalosporins in the presence of piperidine.

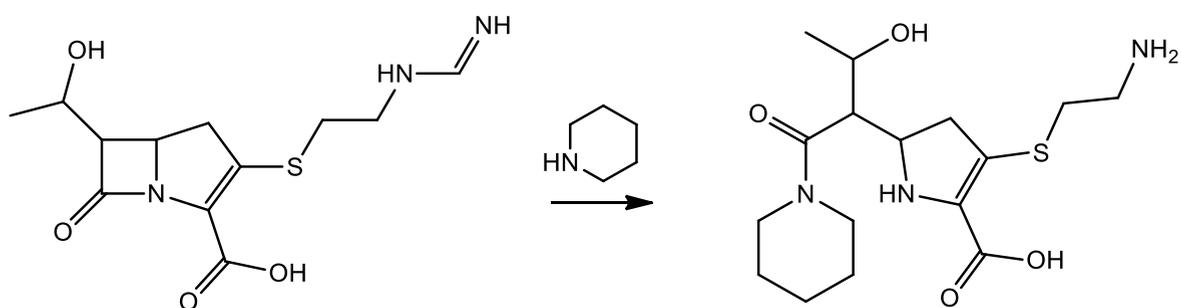


Figure 5.25. Proposed hydrolysis reaction of the carbapenem imipenem in the presence of piperidine.

Table 5.10. Exact mass data for the cephalosporin and carbapenem hydrolysis reaction products.

Product of	Determined exact mass [M+H] ⁺	Molecular formula	Exact mass [M+H] ⁺	Error (ppm)
Amoxicillin	451.2012	C ₂₁ H ₃₁ N ₄ O ₅ S ⁺	451.2010	0.3
Ampicillin	435.2063	C ₂₁ H ₃₁ N ₄ O ₄ S ⁺	435.2061	0.3
Penicillin G	420.1948	C ₂₁ H ₃₀ N ₃ O ₄ S ⁺	420.1952	-0.4
Penicillin V	436.1906	C ₂₁ H ₃₀ N ₃ O ₅ S ⁺	436.1901	0.5
Cloxacillin	521.1618	C ₂₄ H ₃₀ ClN ₄ O ₅ S ⁺	521.1620	-0.2
Dicloxacillin	555.1234	C ₂₄ H ₂₉ Cl ₂ N ₄ O ₅ S ⁺	555.1230	0.4
Oxacillin	487.2013	C ₂₄ H ₃₁ N ₄ O ₅ S ⁺	487.2010	0.3
Nafcillin	500.2214	C ₂₆ H ₃₄ N ₃ O ₅ S ⁺	500.2214	0.1
Ceftiofur	326.1288	C ₁₃ H ₂₀ N ₅ O ₃ S ⁺	326.1281	0.7
Cefquinome	326.1285	C ₁₃ H ₂₀ N ₅ O ₃ S ⁺	326.1281	0.3
Cefapirine	294.1273	C ₁₄ H ₂₀ N ₃ O ₂ S ⁺	294.1271	0.2
Cefalexin	276.1711	C ₁₅ H ₂₂ N ₃ O ₂ ⁺	276.1707	0.5
Cefalonium	507.2104	C ₂₄ H ₃₅ N ₄ O ₄ S ₂ ⁺	507.2094	1.0
Cefazolin	493.2338	C ₂₁ H ₃₃ N ₈ O ₄ S ⁺	493.2340	-0.2
Cefacetrole	468.2280	C ₂₁ H ₃₄ N ₅ O ₂ S ⁺	468.2275	0.5
Cefoperazone	718.3237	C ₃₃ H ₄₈ N ₇ O ₉ S ⁺	718.3229	-0.4
Biapenem	399.2066	C ₁₈ H ₃₁ N ₄ O ₄ S ⁺	399.2061	0.6
Doripenem	506.2109	C ₂₀ H ₃₆ N ₅ O ₆ S ₂ ⁺	506.2102	0.7
Ertapenem	561.2385	C ₂₇ H ₃₇ N ₄ O ₇ S ⁺	561.2377	0.7
Faropenem	371.1643	C ₁₇ H ₂₇ N ₂ O ₅ S ⁺	371.1635	0.8
Imipenem	358.1801	C ₁₆ H ₂₈ N ₃ O ₄ S ⁺	358.1795	0.6
Meropenem	469.2484	C ₂₂ H ₃₇ N ₄ O ₅ S ⁺	469.2479	0.5

Ceftiofur and cefquinome have the same side chain at position 7 and thus they result in the same hydrolysis reaction product. As a consequence, based on this reaction product only, ceftiofur and cefquinome cannot be distinguished. However, a by-product of the cefquinome hydrolysis is the 5,6,7,8-tetrahydroquinoline moiety (the ring structure in the position 3' side chain, appendix 5.2), showing a clear peak in the continuous infusion mass spectrum at m/z 134. By additionally monitoring this reaction product the presence of cefquinome can be distinguished from ceftiofur (figure 5.26). Because the two compounds interfere in each other's quantitation process they can only be quantitated by using separate calibration standards. A molecular structure search

in SciFinder [90] shows 53 substances besides ceftiofur and cefquinome having a comparable molecular structure with variation in the position 3' side chain only. From a theoretical perspective these might all result in the same reaction product. Because a complete side chain group is removed in the hydrolysis of the cephalosporins, the presented method cannot be considered an unequivocal confirmation of the presence of the cephalosporins. However, in the analysis of 21 blank samples no interferences were observed and in practice it is unlikely to find the 53 theoretically interfering compounds in poultry muscle.

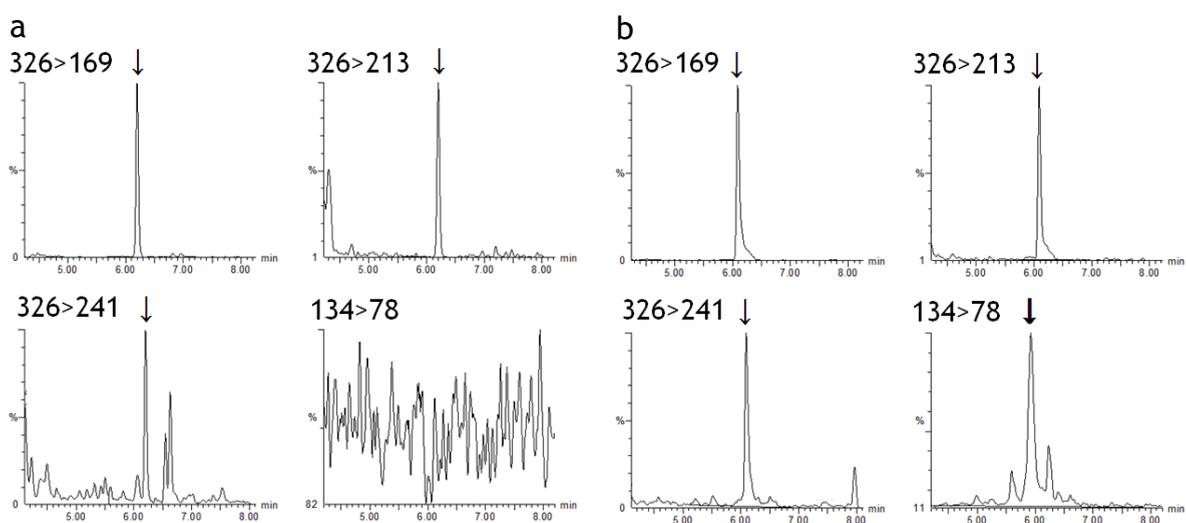


Figure 5.26. SRM chromatograms for three ion transitions of ceftiofur and cefquinome ($m/z=326$) and one ion transition of the cefquinome by-product ($m/z=134$) for a blank poultry muscle sample spiked at $10 \mu\text{g kg}^{-1}$ with (a) ceftiofur and (b) cefquinome.

Optimization of the hydrolysis kinetics

The main factors that possibly influence the hydrolysis reaction were temperature, incubation time and piperidine concentration. From the experimental design ANOVA, it was observed that none of the selected factors affected the hydrolysis of the penicillins and the carbapenems. These reactions were all instantaneous and no significant further degradation of the selected reaction products was observed during the incubation. For the cephalosporins, all three factors and the interaction of temperature with time proved to have a significant effect ($\alpha < 0.05$) on the formation of the selected reaction products. A high piperidine concentration proved to be beneficial for especially the hydrolysis of cephalexin (+ 100 %). At $80 \text{ }^\circ\text{C}$ the cephalexin reaction product

showed an optimal response after 20 min followed by a decrease, which is most likely caused by degradation of the reaction product at this temperature. Overall, the highest response of the reaction products was observed after 1 h incubation at 60 °C using 5 % piperidine.

Optimization of sample extraction

The piperidine has to be added to the raw aqueous extract, in the presence of the muscle matrix in order to hydrolyse protein bound metabolites of ceftiofur. Because a relatively high concentration of piperidine is necessary for the hydrolysis of the cephalosporins, severe gelation caused by the hydrolysis of extracted collagen occurs when the extraction was carried out in pure water. The use of a saturated solution of disodium tetraborate and sodium chloride proved successful in the prevention of gelation. This is most likely caused by inhibiting excessive hydrogen bridge formation by the extracted collagen.

LC-MS/MS analysis

The LC separation of especially the carbapenem hydrolysis products is challenging. Under acidic LC conditions extreme peak tailing is observed for especially imipenem, meropenem and doripenem. Only at a mobile phase pH > 7, the peak tailing decreased but still remained sub-optimal. The addition of 1 % piperidine to the aqueous purified sample extract improved the peak width and shape and thus chromatographic resolution of the isomeric reaction products. A chromatogram of a blank sample spiked at target level with all β -lactams is presented in figure 5.27. As a result of the hydrolysis reaction, the chromatograms of most compounds show multiple peaks. The chromatograms of the penicillins and the carbapenems (except imipenem) show a major and a minor peak whereas imipenem shows two peaks of approximately equal height. It is suggested that stereoisomers are produced during the incubation under the extreme alkaline conditions. For the cephalosporin reaction products that contain two piperidine moieties, up to four peaks are observed. This might be a combination of stereoisomers and structural isomers, due to a variation in the position of the second nucleophilic attack. In most cases, baseline separated peaks are observed, but especially in the case of cefoperazone the resolution of the peaks is limited. In such cases, the combination of peaks is integrated to obtain reproducible peak areas.

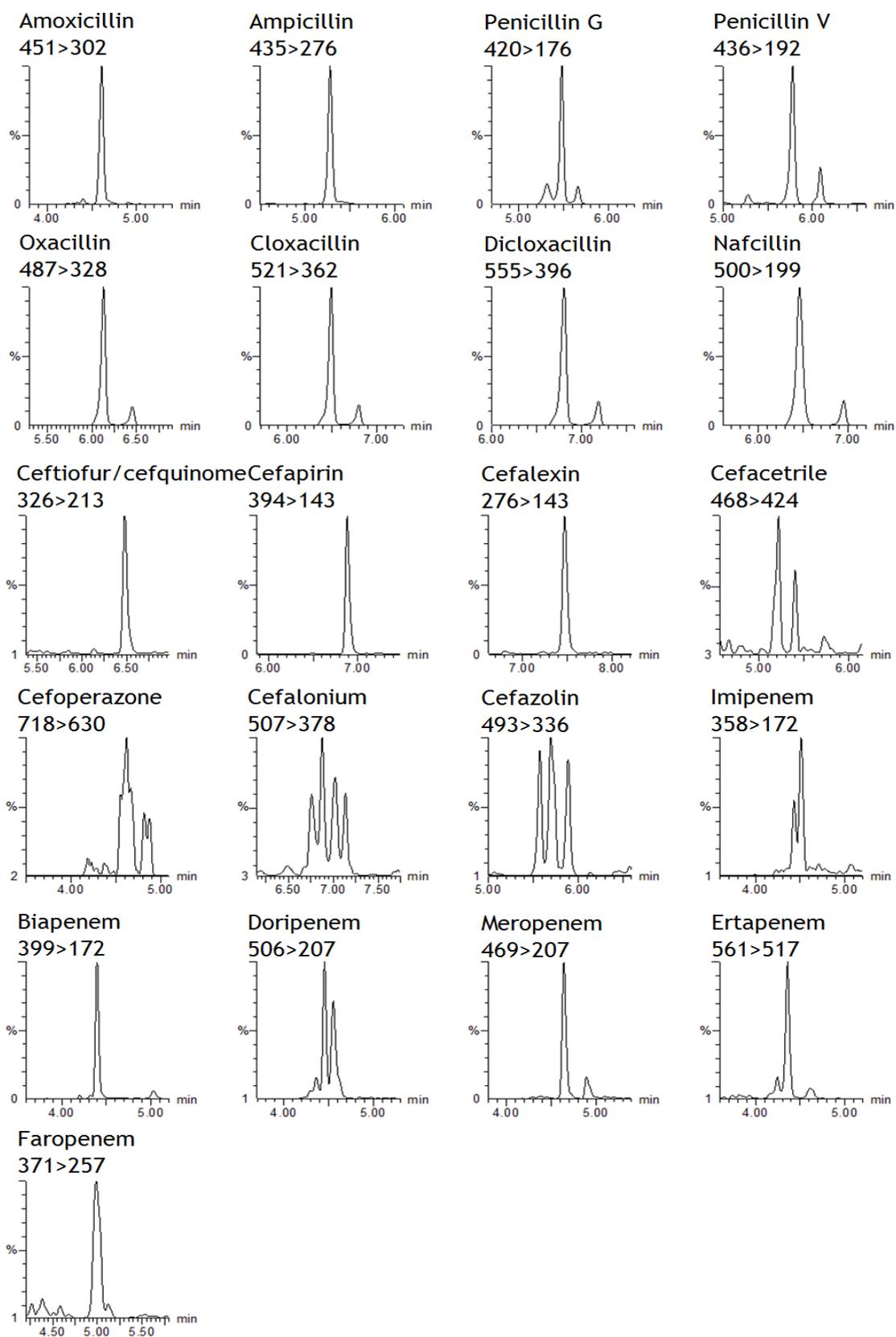


Figure 5.27. Representative reconstructed ion chromatograms of a blank poultry muscle sample spiked at target level with the β -lactams showing the least abundant product ion of each compound's hydrolysis reaction product.

Validation results

An overview of the validation results is presented in table 5.11.

Table 5.11. Summary of the validation study results.

Compound	Validation level ($\mu\text{g kg}^{-1}$)	Trueness (%) n=21	RSD _r (%) n=21	RSD _{RL} (%) n=21	CC α ¹ ($\mu\text{g kg}^{-1}$)	CC β ¹ ($\mu\text{g kg}^{-1}$)	Linearity (r^2)
Amoxicillin	25	97	3.4	4.7	1.1	2.2	> 0.999
	50	98	4.7	4.9	53.9	57.9	
	75	100	4.5	4.5			
Ampicillin	25	106	7.4	8.8	1.7	3.5	> 0.997
	50	102	7.2	11.4	59.5	69.0	
	75	99	7.9	9.1			
Penicillin G	25	100	4.6	4.8	1.1	2.2	> 0.998
	50	98	3.5	3.5	52.9	55.7	
	75	100	5.2	5.5			
Penicillin V	12.5	96	4.4	5.4	0.7	1.5	> 0.993
	25	97	6.0	6.1	27.4	29.9	
	37.5	98	4.8	5.0			
Cloxacillin	150	100	6.8	11.9	1.7	3.4	> 0.995
	300	106	4.2	10.1	353	405	
	450	103	3.6	6.5			
Dicloxacillin	150	101	3.5	5.4	1.6	3.2	> 0.993
	300	103	2.6	2.8	314	328	
	450	104	2.7	2.7			
Oxacillin	150	98	6.1	7.5	1.4	2.8	> 0.997
	300	101	5.2	5.9	329	358	
	450	100	6.5	6.7			
Nafcillin	150	99	7.7	7.8	2.0	4.1	> 0.996
	300	102	6.1	6.1	331	361	
	450	104	8.8	8.8			
Ceftiofur	5	95	6.5	8.6	1.3	2.7	> 0.998
	10	97	6.9	7.3			
	15	101	4.1	5.0			
Cefquinome	5	92	10.7	11.2	1.9	3.9	> 0.996
	10	98	9.4	9.5			
	15	96	8.8	10.1			

Cefapirin	5	88	10.2	13.3	2.4	4.8	> 0.995
	10	97	9.4	9.5			
	15	94	8.5	8.7			
Cefalexin	5	102	10.9	13.9	2.5	5.0	> 0.993
	10	96	7.8	8.4			
	15	91	7.0	10.4			
Cefalonium	25	101	12.6	12.7	5.5	11	> 0.998
	50	108	12.4	13.0			
	75	115	7.1	8.4			
Cefazolin	12.5	93	9.9	10.0	5.1	10	> 0.998
	25	98	8.4	8.7			
	37.5	96	7.0	7.9			
Cefacetriple	25	98	12.0	12.4	10	20	> 0.995
	50	98	14.5	19.0			
	75	104	6.0	15.2			
Cefoperazone	25	100	8.8	17.3	9.3	19	> 0.996
	50	106	10.0	12.6			
	75	110	6.6	10.5			
Biapenem	50	111	27.1	45.1	-	50	> 0.993
	100	118	29.7	46.5			
	150	91	20.7	33.8			
Doripenem	25	101	11.3	15.9	12	25	> 0.999
	50	106	11.1	11.9			
	75	109	7.0	9.2			
Ertapenem	25	91	7.3	7.6	12	24	> 0.990
	50	94	11.0	12.9			
	75	95	7.7	10.7			
Imipenem	50	102	11.2	14.6	20	41	> 0.995
	100	107	9.7	14.9			
	150	103	5.4	9.3			
Meropenem	12.5	86	8.9	12.8	3.6	7.2	> 0.994
	25	97	6.3	7.0			
	37.5	96	5.4	5.6			
Faropenem	12.5	88	12.3	14.8	5.6	11	> 0.995
	25	91	10.2	10.8			
	37.5	94	6.9	7.1			

¹ C_{Ca} and C_{Cβ} based on MRL are presented in regular font, C_{Ca} and C_{Cβ} based on zero tolerance are presented in italics.

For cloxacillin, dicloxacillin, nafcillin and oxacillin, for which the calibration line ranges from 0 - 1500 $\mu\text{g}/\text{kg}^{-1}$ in some cases a clear signal saturation was observed at the highest calibration level. Therefore, these are best calibrated over a range from 0 - 3 * target level. Taking this into account, the linearity over the calibration range is above the criterion of 0.990 for all compounds and does comply with the established criterion. The trueness is between 91 and 110 % for all compounds except cefalonium at 1.5 * target level and biapenem at all levels. From the linear calibration lines, it is concluded that the within-sample variation is limited and thus it is most likely that the observed deviations are caused by matrix effects and could be resolved if an isotopically labeled internal standard would be available. The RSD_f and RSD_{RL} are all below 13.3 % respectively 17.3 % except for biapenem. Based on these outcomes it is concluded that the presented method is suitable for quantitation of the amount of β -lactams present in poultry muscle at relevant levels except for biapenem. The method is suitable for qualitative analysis of biapenem and thus for monitoring biapenem use in poultry breeding.

The calculated decision limits ($\text{CC}\alpha$) based on zero tolerance, indicating the limit of detection, vary from 0.7 - 20 $\mu\text{g kg}^{-1}$. $\text{CC}\alpha$ was found to be of the same magnitude for both mass spectrometers used. Based on the samples spiked at the lowest level included in the validation, the calculated values of $\text{CC}\alpha$ are realistic. The penicillins have detection limits in the low ppb range demonstrating that the method is suitable for the detection of penicillins at levels well below the MRL enabling the monitoring of extra-label penicillin use. The method also proved suitable for the detection of cephalosporins and carbapenems at relevant levels. The calculated detection capabilities ($\text{CC}\beta$) are between 1.5 and 50 $\mu\text{g kg}^{-1}$ which is approximately at the lowest level included in the validation. Therefore the calculated values could easily be verified using the available data. In over 95 % of the cases, based upon the ion ratio, the β -lactams were found positive according to EU criteria [77] indicating that the calculated levels of $\text{CC}\beta$ are realistic. Because this is an initial in-house validation, only limited data are available. Over time the within-laboratory reproducibility should be determined more exact to obtain a more precise estimation of $\text{CC}\beta$.

Selectivity

A hydrolysis upon addition of piperidine is carried out and as a consequence an excessive number of compounds containing a piperidine moiety can be expected in the hydrolysed sample extract. Therefore, protonated piperidine as the product ion (m/z 86) and a piperidine neutral loss (-85 Da) were considered non-selective and were discarded in the selection of ion transitions for confirmatory LC-MS/MS analysis. For each β -lactam reaction product, two product ions were selected and based on those the probability of an interfering signal ($P(I)$) was calculated [81]. If $P(I)$ was above 2×10^{-7} , being a suitable criterion for selectivity as previously proposed [81], as was the case for penicillin G, penicillin V, oxacillin, cefapirin, ceftiofur, cefquinome, imipenem and faropenem, an additional product ion was selected for confirmatory analysis to assure sufficient selectivity. Only for ceftiofur and cefquinome a neutral loss of piperidine was included as the third transition, not to compromise sensitivity too much. Using these ion transitions, no interferences at the retention times of the β -lactam reaction products were observed in the chromatograms of the blank samples ($n=21$).

Ruggedness

Some slight deviations to the procedure that might occur in practice were tested in triplicate: (1) hydrolysis for an additional 60 min, (2) evaporation of the eluent until dryness and leaving the test tubes in the evaporator for 30 min, and (3 and 4) adjustment of the pH of the extract to pH 6.0 and 8.0. Adjusting the pH of the extract to 6.0 resulted in statistically significant deviating results for penicillin V (-12 %), cephalexin (-85 %), cefoperazone (-49 %), biapenem (-92 %), nafcillin (+75 %) and cefalonium (+40 %). Adjusting the pH to 8.0 only showed a statistically significant change in the results of cefoperazone (-16 %) and ertapenem (+34 %). The deviating results can be explained by the break-through occurring during SPE due to ionisation of the compounds and it was concluded that adjustment of the pH of the extracts to pH = 6.8 - 7.3 should be carried out with care. For some penicillins (e.g. nafcillin +60 %) and some cephalosporins (e.g. cefapirin +40 %) prolonged incubation results in an increase in peak response. For faropenem and ertapenem a decrease in peak response is observed of approximately 30 %. An incubation time of 60 min was found to be a good

compromise and because internal standards are used for quantitation, slight deviations of the incubation time are acceptable. No degradation of the compounds was observed during prolonged evaporation, except for cefacetriple which showed a drop in the signal of 15 % when left in the evaporator unit 30 min. after dryness. It is concluded that for this specific compound, the centrifuges tubes should be removed from the evaporator unit directly after dryness.

Stability

Stability data on penicillin solutions were previously reported [82]. It was found that penicillin solutions in methanol are stable for at least 3 months at $< -18\text{ }^{\circ}\text{C}$ with the exception of ampicillin and penicillin G, which are stable for 2 months under these storage conditions.

The stability results of 10 mg L^{-1} academic solutions cephalosporins and carbapenems are presented in table 5.12. As the penicillins, the the cephalosporins and carbapenems are unstable in methanolic solutions, stored at $4\text{ }^{\circ}\text{C}$ during 2 months, with exception of ceftiofur. When storing the solutions at $-18\text{ }^{\circ}\text{C}$ the cephalosporins and faropenem remain stable during at least two months. The carbapenems seem to drop in response for 10 - 30 % and remain stable afterwards. To prevent degradation of the stock solutions it was decided to store them at $< -70\text{ }^{\circ}\text{C}$.

The stability of poultry muscle extracts showed that even if extracts are stored at $-18\text{ }^{\circ}\text{C}$ for 7 days, results similar to those for fresh extracts are obtained. It is concluded that muscle extracts obtained with the described method are stable for at least 7 days when stored at $-18\text{ }^{\circ}\text{C}$.

Table 5.12. Stability of 10 mg L⁻¹ methanolic solutions of cephalosporins and carbapenems during storage at -18 and +4 °C.

Compound	Relative response compared with t=0 (%)			
	+4 °C		-18 °C	
	t = 1 month	t = 2 months	t = 1 month	t = 2 months
Cefalexin	87	65	96	98
Cefapirin	85	66	99	99
Cefquinome	89	68	99	100
Ceftiofur	99	97	100	99
Cefacetrile	81	55	97	93
Cefazolin	83	62	96	94
Cefalonium	44	13	97	95
Cefoperazone	85	63	99	98
Imipenem	31	14	85	82
Biapenem	12	2	72	75
Meropenem	50	25	87	87
Doripenem	43	19	86	89
Ertapenem	59	34	86	91
Faropenem	85	81	99	98

Application to routine samples

Ceftiofur incurred tissue samples, obtained from chicken that were slaughtered four, eight and 24 hours after intramuscular injection of ceftiofur, were analysed using the presented method (n=3 per group). The results were compared to the results of a previously presented method which was found to give comparable results to a routinely applied method [76]. For the samples obtained four hours after treatment the average ceftiofur concentration of the presented method was 625 µg kg⁻¹ and 587 µg kg⁻¹ for the other method. For the samples taken eight hours after treatment this was 273 respectively 247 µg kg⁻¹, and 24 hours after treatment 42 respectively 52 µg kg⁻¹. In all three cases, no statistically significant differences were observed between the results of both methods. From this it is concluded that the method presented here is suitable for detection of incurred poultry muscle samples. No β-lactam antibiotics were detected in the 25 poultry samples obtained from local supermarkets.

Conclusions

A comprehensive quantitative trace analysis method for the analysis of 22 β -lactam antibiotics including penicillins, cephalosporins and carbapenems was successfully developed and fully validated according to 2002/657/EC [77]. In order to stabilise the penicillins and to be able to carry out an effective monitoring for ceftiofur use by including its protein-bound metabolites, a hydrolysis using piperidine is included. Reaction products, which are used as markers for the β -lactams are proposed. The validation showed good trueness, repeatability and within-lab reproducibility for all compounds except for cefalonium at $75 \mu\text{g kg}^{-1}$ and biapenem, for which the method is considered qualitative. The ruggedness and stability proved to be sufficient to apply the presented method in routine analyses. The method is the most appropriate method available today for detection and quantitation of a broad range of β -lactam antibiotics in poultry muscle.

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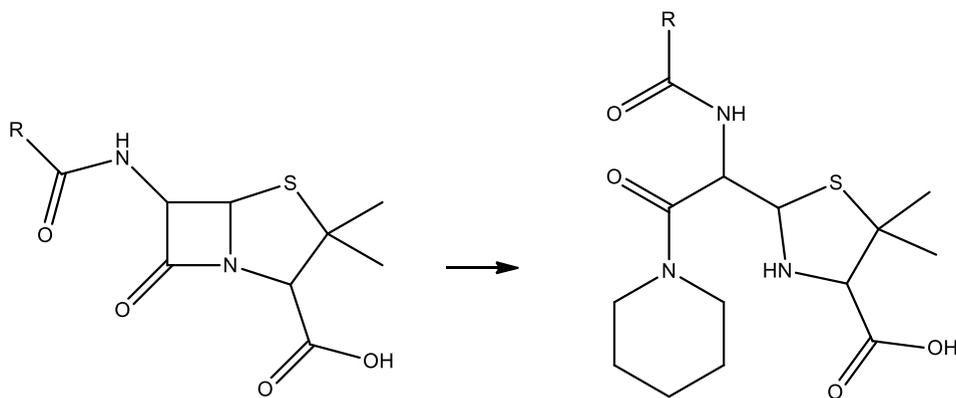
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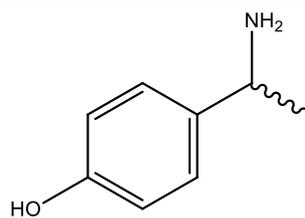
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Appendix 5.1

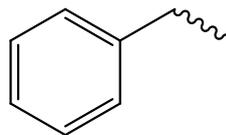
Molecular structure of 6-aminopenicillanic acid nucleus, the piperidine hydrolysis product, and penicillins registered for use in animal practice.



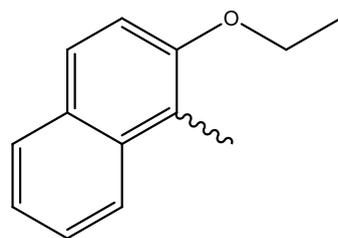
Amoxicillin



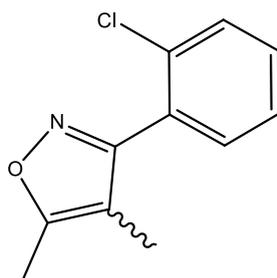
Penicillin G



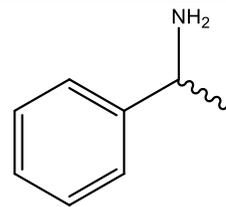
Nafcillin



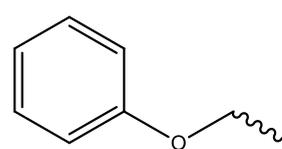
Cloxacillin



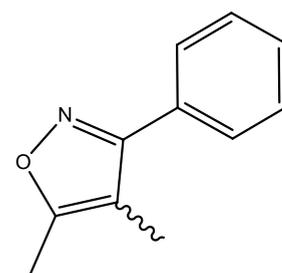
Ampicillin



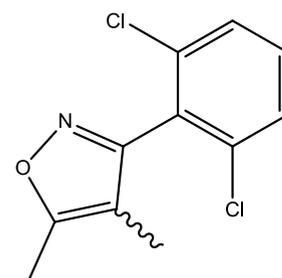
Penicillin V



Oxacillin

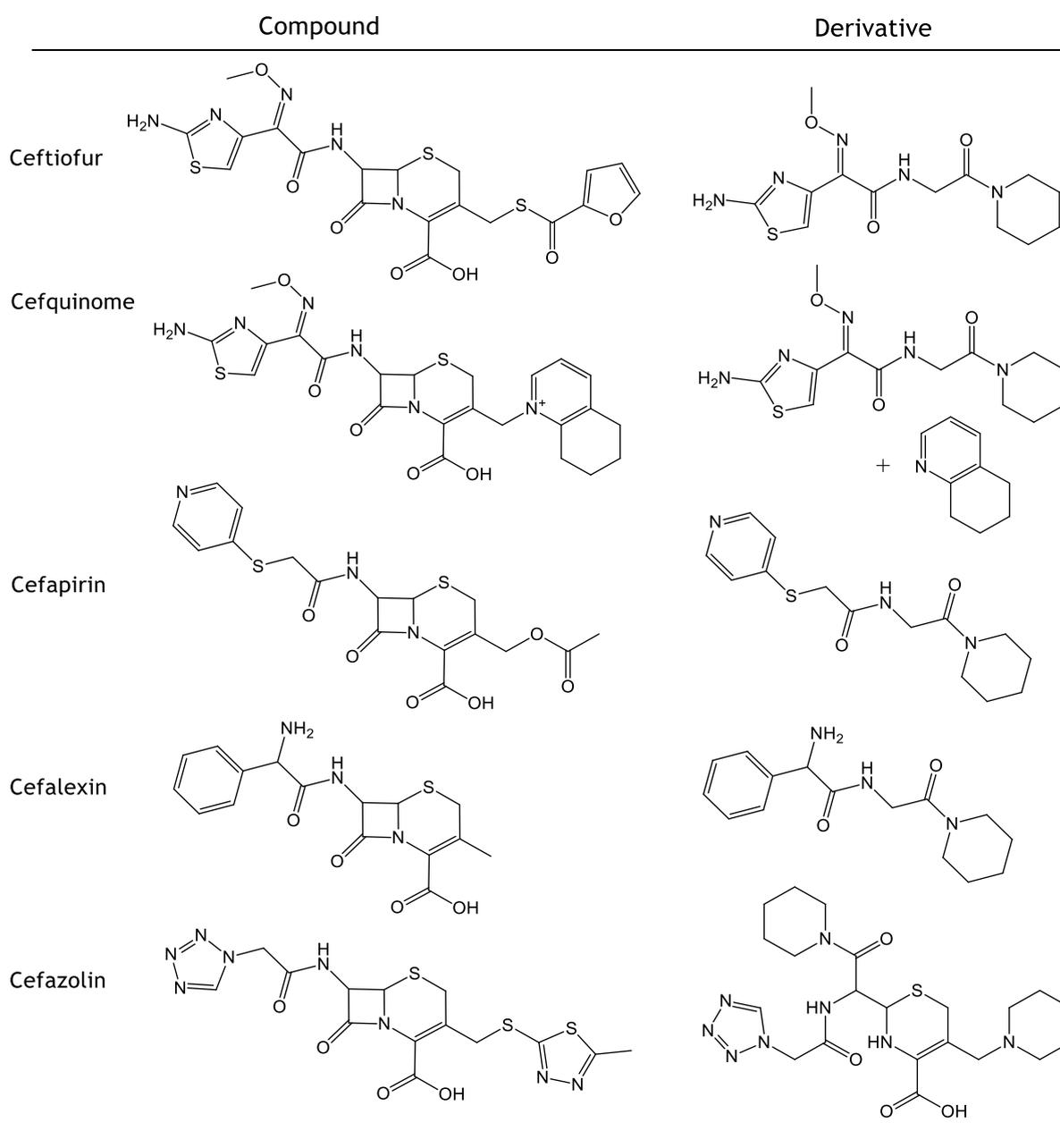


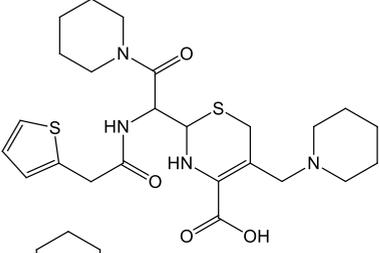
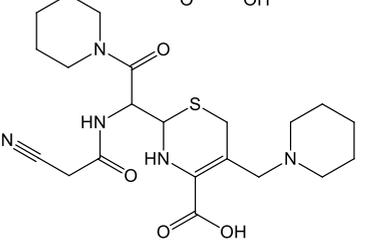
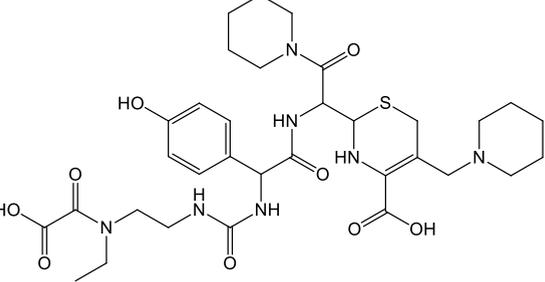
Dicloxacillin



Appendix 5.2

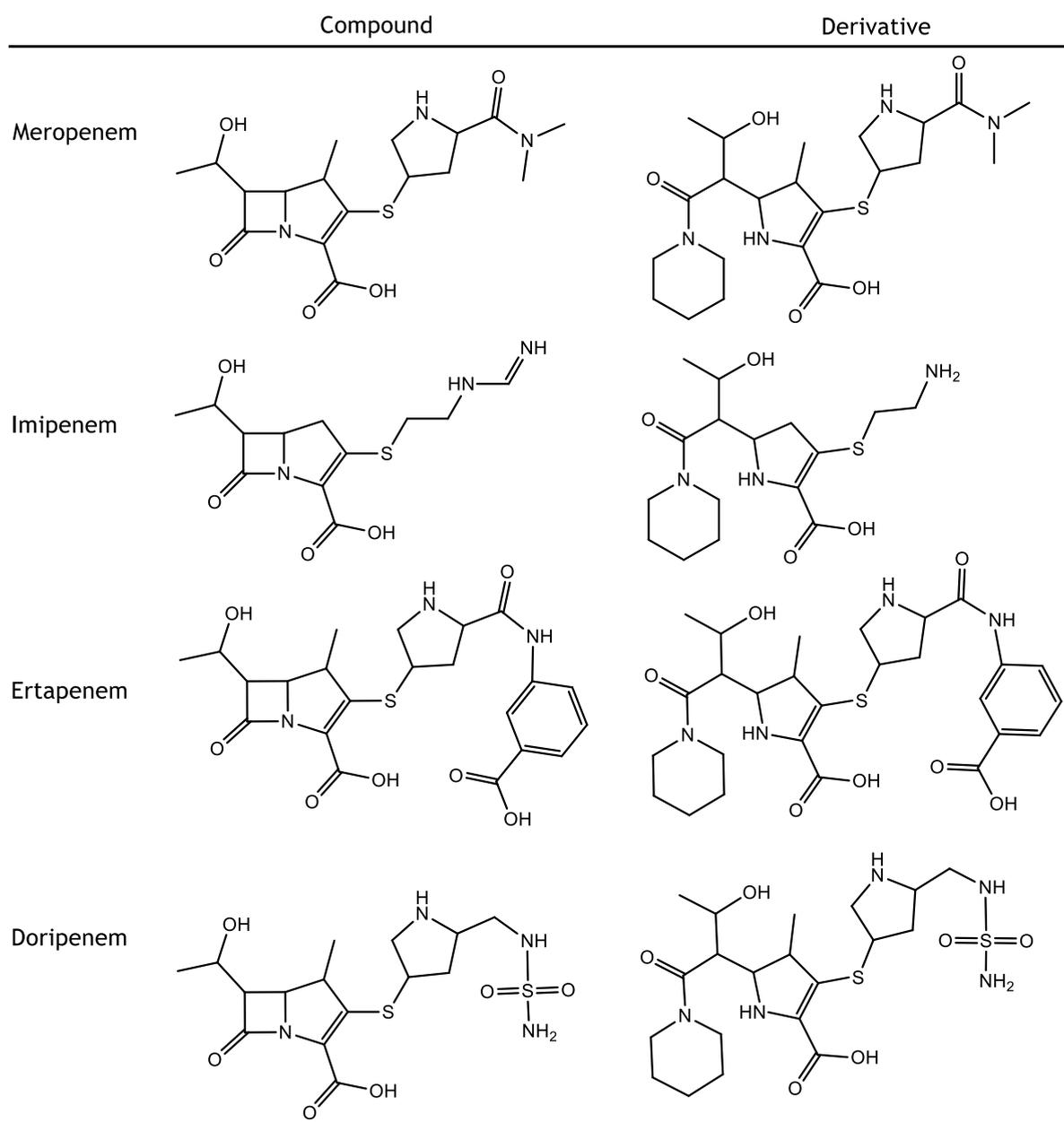
Molecular structure of the cephalosporins registered for use in animal practice including their hydrolysis product.

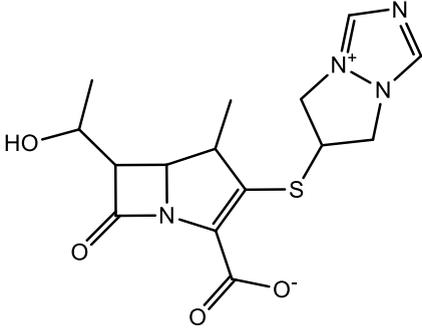
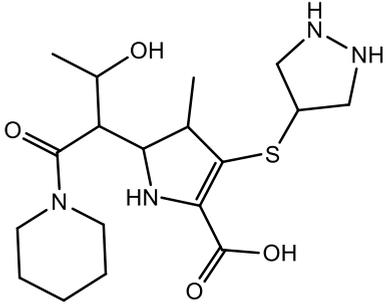
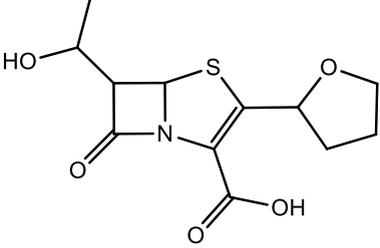
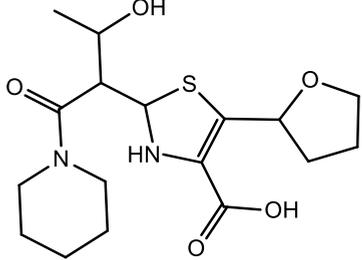


Compound	Derivative
Cefalonium	 <p>The derivative of Cefalonium is a cephemycin core with a piperidine ring attached to the 6-position of the dihydrothiazine ring. The 3-position is substituted with a thienylmethyl group, and the 4-position has a carboxylic acid group. The 7-position is substituted with a piperidine ring via a methylene group. The 2-position is substituted with a piperidine ring via a carbonyl group.</p>
Cefacetile	 <p>The derivative of Cefacetile is a cephemycin core with a piperidine ring attached to the 6-position of the dihydrothiazine ring. The 3-position is substituted with a cyanoethyl group, and the 4-position has a carboxylic acid group. The 5-position is substituted with an acetoxy group. The 2-position is substituted with a piperidine ring via a carbonyl group.</p>
Cefoperazone	 <p>The derivative of Cefoperazone is a cephemycin core with a piperidine ring attached to the 6-position of the dihydrothiazine ring. The 3-position is substituted with a 4-hydroxyphenyl group, and the 4-position has a carboxylic acid group. The 5-position is substituted with a 1,2,4-triazol-5-ylmethyl group. The 2-position is substituted with a piperidine ring via a carbonyl group. The 7-position is substituted with a complex side chain containing a piperidine ring, a hydroxyphenyl group, and a carboxylic acid group.</p>

Appendix 5.3

Molecular structure of the carbapenems and their hydrolysis product.



Compound	Derivative
Biapenem	
 <p>The structure of Biapenem consists of a penam nucleus (a fused 4-membered and 5-membered ring system with a nitrogen atom and a carbonyl group) substituted with a methyl group, a hydroxyethyl group, and a carboxylate group. The 5-position of the penam nucleus is linked via a sulfur atom to a 5-membered ring containing two nitrogen atoms, one of which is positively charged.</p>	 <p>This derivative of Biapenem features the same penam nucleus. The 5-position is linked via a sulfur atom to a 5-membered ring containing two nitrogen atoms. The penam nucleus is also substituted with a methyl group, a hydroxyethyl group, a piperidine ring, and a carboxylic acid group.</p>
Faropenem	
 <p>The structure of Faropenem consists of a penam nucleus substituted with a methyl group, a hydroxyethyl group, and a carboxylic acid group. The 5-position of the penam nucleus is linked via a sulfur atom to a 5-membered ring containing one oxygen atom.</p>	 <p>This derivative of Faropenem features the same penam nucleus. The 5-position is linked via a sulfur atom to a 5-membered ring containing one oxygen atom. The penam nucleus is also substituted with a methyl group, a hydroxyethyl group, a piperidine ring, and a carboxylic acid group.</p>

Chapter 6

General conclusions and future perspectives



Introduction

What selectivity is adequate in residue analysis of antibiotics and other drugs? There is no general answer to this question, because this highly depends on the purpose of the method in question.

In chapter 2 it was elaborated that selectivity is related to the sample clean-up and that usually a tradeoff between selectivity and the number of compounds included in the method occurs.

In chapter 3 it was demonstrated that the selectivity obtained by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) can be graded based upon modeled empirical data. The result can be compared to a threshold value but no sharp boundary between 'selective' and 'non-selective' exists. As a starting point, according to European Union Commission Decision 2002/657/EC [1] the selectivity for detection of banned substances (group A) should be higher than for registered substances (group B). Because consequences of a non-compliant result for banned substances are higher than for a non-compliant finding of a registered substance, this is an acceptable concept.

A second consideration in the selectivity needed is the kind of interferences that can be expected. A clear example thereof is the analysis of chloramphenicol (CAP) as presented in chapter 4. A non-compliant finding of CAP has huge consequences that might include farmers' bankruptcy, bad reputation of certain branches, destruction of shipments of perfect quality food products, and affecting the economy and international relations. Beforehand, it is known that CAP has two chiral centers and that its nitro-group can be substituted at the para- and meta-position. Because 'unequivocal identification' is mandatory, a method for the analysis of CAP should be able to discriminate CAP from its stereo-isomers. Therefore, to prevent false positive results to occur, a method for the analysis of CAP should be extremely selective. The same accounts for other banned compounds having enantiomers, like β -agonists [2,3] and to less extend for regulated compounds like some fluoroquinolone antibiotics [4], some non-steroidal anti-inflammatory drugs (NSAIDs) [5] and albendazole sulphoxide, an anthelmintic drug [6,7]. Enantiomeric separations have usually been carried out using chiral liquid chromatography (LC) [4-6] as also demonstrated for CAP

and/or capillary electrophoresis (CE) [2-4,7], but also new techniques to enhance selectivity have become available. These are further discussed from page 290.

A third consideration in the selectivity needed is the purpose of the analysis. In monitoring programs, usually a broad screening is carried out first, followed by a confirmatory analysis of suspect samples. Ideally, a screening analysis focusses on a broad range of compounds, which compromises selectivity as was discussed in chapter 2. A confirmatory method should by definition result in an unequivocal identification and thus a high selectivity is mandatory. Future perspectives on monitoring programs and their effects on selectivity are further discussed in this chapter.

An example of a method in which selectivity is intentionally compromised is the analysis of β -lactams as presented in chapter 5. Because, ceftiofur metabolises rapidly, off-label use is best detected if protein-bound residues are included. To facilitate this, a hydrolysis of the protein-bound residues is mandatory. By definition, when applying a derivatization, selectivity is compromised: instead of the drug focused at, a derivative is detected and thus the method is not able to differentiate the parent drug from other drugs that result in the same derivative.

It is concluded that selectivity should be fit for purpose and therefore remains a matter of experts' judgment. Nevertheless, it is important to be able to grade selectivity, e.g. in terms of bad, good or extreme, which is facilitated by the method presented in chapter 3. The obtained result is the basis for determining if selectivity is adequate and thus the described procedure is highly valuable when the selectivity of the confirmatory method is challenged in a court case. When selectivity is considered inadequate, additional methods or techniques can be applied to increase method selectivity.

In this chapter 6, future perspectives are discussed. First techniques that in my view are highly valuable to further increase selectivity are presented and discussed. Next, future perspectives on monitoring strategies for regulatory control are elaborated upon and finally additional options to further investigate the natural occurrence of CAP are presented.

Enhancing selectivity

As described in chapter 2, during the last decade many developments in state-of-the-art instruments for the analysis of antibiotics in products of animal origin occurred. As a result, instead of aiming for the detection of just one compound or compounds from a single antibiotic group, multi-compound methods are being developed that include different antibiotic groups. As a result extraction and sample-clean-up procedures have become more generic and selectivity is compromised. To obtain a trustworthy confirmatory result additional ion transitions can be monitored in LC-MS/MS to improve the information on the compounds' functional groups and structure. However, in my opinion some other techniques have become available to further increase selectivity, being high-resolution mass spectrometry (HRMS), ion mobility spectrometry (IMS), two-dimensional LC and supercritical fluid chromatography (SFC).

High resolution mass spectrometry

In HRMS, co-eluting compounds are much better separated compared to low resolution MS and, provided target ions are fully separated from background ions, the exact mass is determined more accurate [8]. Quadrupole instruments operate at a resolution of approximately 5000 full width at half maximum (FWHM) [9] which results in nominal mass data. As was presented in chapter 3 (figure 3.2) at nominal mass, the probability of a compound having the same mass ($P(M_{pc})$) is at most 0.0052. Nowadays, Fourier transform-ion cyclotron resonance (FTICR) [10] and orbitrap [11] MS instruments are commercially available operating at a resolution of 100,000 FWHM with a mass accuracy below 3 ppm [10,12-16]. Using specific software tools and applying an additional internal calibration, mass accuracy is enhanced to even sub-ppm errors [12,17-19].

Selectivity in low resolution MS (LRMS) and HRMS has been assessed previously [20-22]. Kaufmann *et al.* [20] reported that single stage MS analysis at a resolution of 100,000 FWHM permits selectivity which exceeds that provided by currently used unit resolving MS/MS instruments. Quenzer *et al.* [23] demonstrated that the number of possible elemental compositions that fit the accurate mass result increases with increasing m/z . For instance, only a few elemental compositions are possible for a compound at $m/z = 100.0000 \pm 2$ ppm,

whereas much more options are available for compounds with $m/z = 900.0000 \pm 2$ ppm. It was concluded that at a higher mass range, a better mass accuracy is required for unequivocal identification compared to the lower mass range. However, this conclusion was based upon the theoretical mathematical number of elemental compositions possible, instead of upon the number of existing molecules or chemically possible elemental compositions. Furthermore, the seven golden rules for filtering molecular formulas obtained from HRMS measurements were not applied [24]. These rules include (1) restriction of element numbers, (2) LEWIS and SENIOR check, (3) isotopic pattern filtering, (4) a hydrogen/carbon element ratio check, (5) a heteroatom ratio check, (6) an element probability check and (7) a TMS check (only if derivatised using N-methyltrimethylsilyltrifluoro-acetamide (MSTFA)). When applying these rules, the number of optional molecular formulas can be severely limited.

Another theoretical assessment of the selectivity in LRMS and HRMS was recently reported by Stoev *et al.* [22]. In this the maximum number of m/z peaks fitting a mass spectrum was calculated. Logically the number of peaks fitting in a spectrum is proportional to the mass resolution. As a result a 10,000-fold increase in selectivity was reported going from nominal mass resolution to HRMS at 100,000 FWHM resolution. It was concluded that confirmatory analysis using LC-MS/MS according to Commission Decision 2002/657/EC [1], monitoring one precursor ion and two product ions thereof, was more selective than full scan exact mass measurement of the precursor ion only at 100,000 FWHM resolution. The reported approach is highly theoretical and some important issues are overlooked. First, the chance of the occurrence of precursor masses is unequally distributed over the mass range as demonstrated in chapter 3. In HRMS these differences can be very extreme, because in the low mass range, the majority of exact masses has a zero probability of occurrence. Second, as was also presented in chapter 3, not all product ions are as likely, as a matter of fact some product ion masses are impossible. Third, it is suggested that selectivity is related to the selected scan range, which is not the case. A more practical approach for the assessment of LRMS versus HRMS is needed.

To assess the selectivity in full scan HRMS analysis, the database containing over 5,000,000 entries of commercially available compounds [25], as introduced in chapter 3, can be used. This information is highly valuable to assess the

selectivity of confirmatory methods in which additionally to LC-MS/MS analysis, a HRMS measurement is used to determine the exact mass of the compound present to obtain additional selectivity. The probability distribution of the precursor ion m/z at 2 ppm mass accuracy is presented in figure 6.1a showing that with high resolving power a precursor m/z of 351.1592 is the most probable, having a probability of $3.8 \cdot 10^{-4}$. This is over a factor 10 improved compared to nominal mass resolution. Looking at the details of the distribution (figure 6.1b) it is clear that the distribution, in contrary to nominal mass resolution, is not continuous and thus the selectivity highly depends on the elemental composition of the compound: e.g. at $m/z = 351.1592$ the probability is $3.8 \cdot 10^{-4}$ and at $m/z = 351.2421$ the probability is only $8.9 \cdot 10^{-6}$.

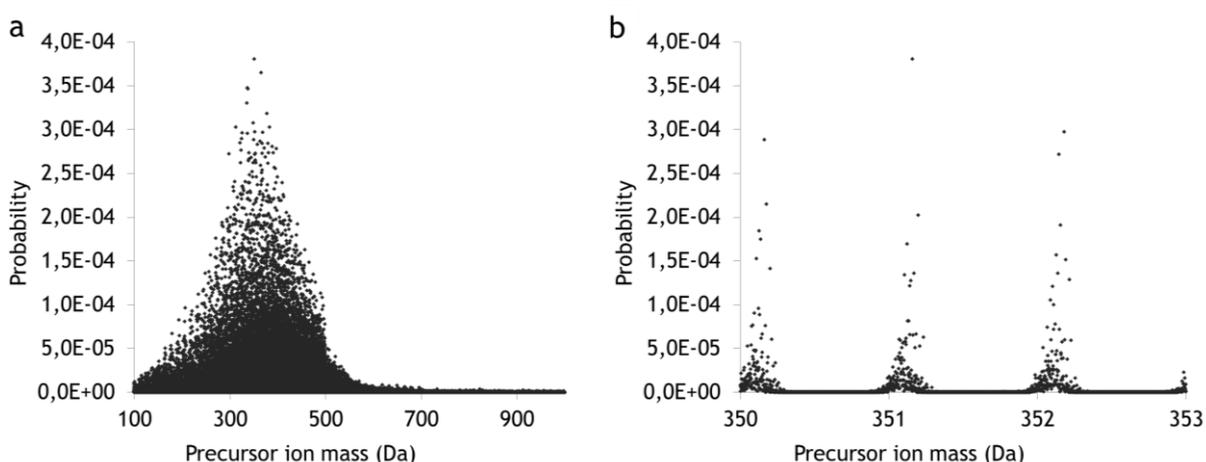


Figure 6.1. Probability distribution of the precursor ion m/z at 2 ppm mass accuracy over a m/z range of (a) 100 - 1000 and (b) 350 - 353.

This approach is valuable when the precursor ion is detected at high resolution, but it does not cover the LC-MS/MS analysis using currently available hybrid instruments like Q-TOF and linear ion-trap-Orbitrap instruments [26] in which the precursor is determined at nominal mass resolution and the product ion(s) at high resolution. To assess the selectivity of these LC-MS/MS measurements, exact mass data on product ions is needed for a high number of compounds. Then, as was done for LRMS, the selectivity in high resolution LC-MS/MS can be calculated and compared to low resolution LC-MS/MS. Because HRMS is nowadays used more frequently for confirmatory analysis, in my opinion, this work should be carried out urgently, so that statistically supported criteria for confirmatory analysis in HRMS can be established.

Ion mobility

Ion mobility spectrometry (IMS) was developed to separate volatile and semi-volatile compounds in the gas phase. The separation is based on different drift velocities (mobility) of compounds in low and high electric fields and enables differentiation by mass, charge and collision cross section (derived from structural parameters of size, shape, and the charge location or distribution) [27]. Therefore it is a separation mechanism that is orthogonal to liquid chromatography and mass spectrometry [28,29].

In conventional IMS, after ionisation of the sample compounds, a cloud of sample ions is introduced into a drift region via an electronic shutter. The ions in the drift region move toward a detector down a voltage gradient through a gas atmosphere. Velocities in electric fields of 300 V cm^{-1} are often 2 m s^{-1} , so a spectrum can be generated every 5 to 25 ms. The velocity of the ions (drift velocity, v_d) is proportional to the strength of the electric field (E) with the mobility (K) of the ions being constant: $K = \frac{v_d}{E}$ [27,30-33].

In addition to conventional IMS, the desire for further miniaturization has prompted the introduction of other methods for ion separation. Two IMS devices are commercially available for coupling to LC-MS: traveling-wave (T-wave) ion mobility spectroscopy (TWIMS) [29,31] as applied in the Waters SYNAPT, and differential mobility spectrometers (DMS), also called field asymmetric waveform ion mobility spectrometers (FAIMS) [27] as applied in the AB Sciex SelexION™.

In TWIMS (figure 6.2a) the drift cell is placed in the vacuum of the instrument and is made of a series of ring electrodes. In conventional IMS a low electric field is uniformly applied on the cell, whereas in TWIMS a high electrical field is swept sequentially from one electrode to the next in the direction of ion migration [27,34,35]. As for conventional IMS, a pulsed method is used for ion injection with the duty cycle time depending on the speed of separation. While waiting for previously injected ions to separate, many ions are being discarded. This can be prevented by including a trap device in front of the drift tube [34].

In DMS/FAIMS (figure 6.2b) the ion mobility device is placed in the ion source region and ions are separated under ambient conditions. DMS/FAIMS differentiates ions by their difference in ion mobilities in high and low electric

fields, induced asymmetrically and orthogonal to the ion path. The different ion mobility during the application of high and low electric fields causes the ions to drift toward one of two electrodes. A compensation voltage is applied to correct the trajectory of targeted ions along the radial axis and thus to avoid ion discharge. DMS and FAIMS instruments are based on the same principle, but their instrumental design differs: two plate electrodes are used in DMS, whereas cylindrical electrodes are used in FAIMS [27,32].

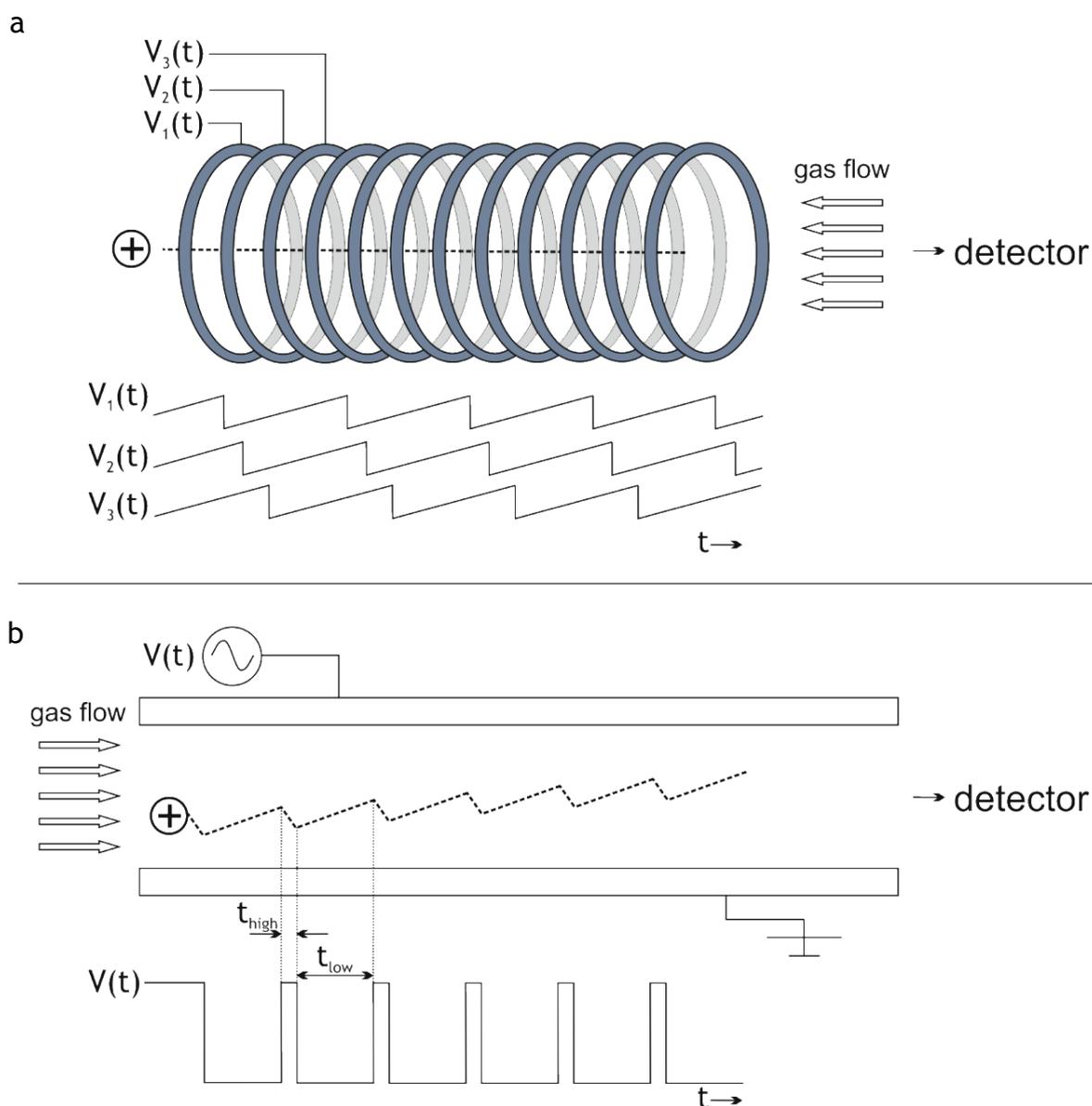


Figure 6.2. Principles of ion mobility separation of ions in (a) traveling-wave ion mobility spectrometry (TWIMS, per example only $V_1 - V_3$ are depicted) and (b) field-asymmetric waveform ion mobility spectrometry (FAIMS).

A clear example of additional selectivity obtained using IMS is the ability to separate closely related compounds. Direct injection electrospray-IMS has been applied for the analysis of different antibiotics, including CAP and some penicillins for the analysis of aquatic environmental samples [36]. Corona discharge IMS has been applied for the analysis of three antibiotics, including CAP, in poultry muscle [37]. IMS in combination with MS detection was recently reviewed [38] and has been applied for the analysis of pharmaceuticals [39-41] and was found extremely useful for the discrimination of positional isomers [41]. LC-IMS-MS has recently been applied for antibiotic analysis showing increased overall resolving power as a result of IMS [42].

Diastereomeric separation of ephedrine, pseudoephedrine and their metabolites using FAIMS without addition of a chiral selector was reported [43]. The separation of carotenoids diastereomers [44] and small pharmaceutical diastereomeric compounds [45] using TWIMS was also reported. Chiral separations are more complicated: IMS is not capable of carrying out chiral separation by itself, because m/z , shape and the collision cross section are identical. Using IMS, chiral molecules in the gas phase can be separated as a result of differences in their stereospecific interaction with a chiral gas (e.g. (S)-(+)-2-butanol) that is brought into the drift tube [46,47]. As a drawback, the addition of the chiral modifier to the drift gas reduced the mobility of both enantiomers and thus cycle times severely increase [34]. Another option to obtain a chiral separation is to add a chiral selector to the injected sample solution to produce diastereomeric complexes that can be separated by IMS [48] or to carry out a complexation reaction prior to injection as has been reported for terbutaline enantiomers analysed using electrospray-IMS-MS [40].

In my opinion, IMS is a valuable addition to residue analysis, because it is simple to apply, it is commercially available and it increases selectivity. In the analysis of CAP isomers (chapter 4) and β -lactam antibiotics (chapter 5) IMS may have a clear added value. In the analysis of CAP isomers, the chromatographic resolution of the isomers is critical. As was presented in chapter 4.3, using reversed phase LC SS-*p*-CAP was not chromatographically separated from RR/SS-*m*-CAP which excludes the use of the product ions that origin from the RR/SS-isomers. Most likely, the different pairs of stereoisomers show different drift times in IMS, resulting in better (or even completely) resolved peaks. As a result the selection

of product ions used for quantitation and confirmation becomes less critical and more abundant product ions can be selected for monitoring. Furthermore, if the isomers are separated by IMS, another LC separation may be used speeding up the chromatographic separation and thus sample throughput.

In the analysis of β -lactams (section 5.4) it was found that for some cephalosporin and carbapenem drugs several isomers were formed during derivatization. In some cases these are not baseline separated, which negatively influences the automatic peak integration performance. As a result, the integration should be checked carefully and additional manual integration is usually mandatory. Most likely, IMS will discriminate among the isomers, facilitating baseline separation of the isomeric compounds. As a result, besides the gain in selectivity, the quantitation process is simplified and more robust. Furthermore, by using IMS, information on the type of isomers present is obtained (e.g. stereoisomers versus enantiomers, cf. above).

Two-dimensional LC

Two-dimensional (2D) LC, either off-line or on-line, is a useful tool to improve the peak capacity and is especially useful for the separation of compounds in complex mixtures [29]. In the off-line mode two chromatographic systems with orthogonal separation principles are used of which the first chromatogram is fractionated using a fraction collector and each fraction is subsequently injected in the second chromatographic system. In this way superior selectivity is obtained. Examples of off-line 2D LC are presented in table 6.1.

Table 6.1. Applications reported for off-line 2D-LC.

Analysis	Dimension		Detection	Ref.
	First	Second		
Triacylglycerol samples of plant oils	Non-aqueous reversed-phase	Silver-ion	APCI-MS	[55]
Pharmaceutical samples	Cyano	Reversed-phase	ESI-MS	[56]
Lipidomic profiling of biological tissues	HILIC	Reversed-phase	ESI- and APCI-MS	[57]

HILIC: Hydrophilic lipophilic interaction chromatography

APCI: Atmospheric pressure chemical ionisation

ESI: Electrospray ionisation

In the on-line mode two chromatographic systems with orthogonal separation principles are coupled by one or more multi-port valves. Two fundamental challenges to realise the full potential of 2D-LC are (1) coupling two orthogonal separation mechanisms and (2) retaining the first dimension separation [49]. The first challenge mainly relates to solvent incompatibility, which is usually caused by solvent immiscibility or because a strong solvent in one mode is a weak solvent in the other [49]. The second challenge relates to interfacing the two dimensions. A fraction from the first dimension (e.g. of 30 sec) is trapped (usually in a loop or trap column) and subsequently injected on the second dimension. While the second dimension separation takes place, a new fraction is collected in the first dimension. In theory, peak capacity of the 2D-LC system would be the product of the peak capacities of the individual dimensions. However, this is not the case due to inefficient transfer of separated compounds from one dimension to the other resulting in remixing of the compounds [50]. Therefore, a prerequisite of on-line 2D-LC is a fast separation in the second dimension, so the trapping cycles in the first dimension remain limited in time to obtain sufficient chromatographic resolution in the first dimension [51]. Two parallel columns can be used for the second dimension to limit the fractions collected in the first dimension [52]. Clearly, the separation mechanisms in both dimensions should be selected with care and interface dead volume should be limited to benefit most from the advanced peak capacity that can be achieved with 2D-LC. Another consideration is that total analysis time severely increases using 2D-LC which is disadvantageous in a routine situation. Comprehensive 2D-LC (LCxLC) was reviewed [50,53,54] and examples, relevant to residue analysis of antibiotics, are presented in table 6.2.

In my opinion, because LCxLC can further enhance the selectivity of a chromatographic separation, it should be the method of choice for confirmatory analysis. It may be possible to develop a single system that is able to analyse all major antibiotic groups (including the highly polar aminoglycosides) within a single run. Furthermore, the increased selectivity of LCxLC would tremendously add to the identification of unknowns and to profiling strategies. The application of LCxLC as a confirmatory method and as a tool in profiling are discussed in more detail in the paragraph 'monitoring strategies'.

Table 6.2. Applications reported for on-line 2D-LC.

Analysis	interface	Dimension		Detection	Ref.
		First	Second		
Sulfonamide and steroid mixture	2 10-port valves	Diol	Reversed-phase	APCI-MS	[55]
Antiviral compounds in poultry muscle	2 6-port valves	Hypercarbon	Reversed-phase	ESI-MS	[58]
Drug mixture	12-port valve	Reversed-phase	Phenyl	ESI-MS	[52]
Chinese medicines	8-port valve	Cyano	Reversed-phase	APCI-MS	[51]
Corn metabolites	2 6-port valves	Reversed-phase	Reversed-phase	UV	[59]
Triacylglycerols in vegetables oil	10-port valve	Silver ion	Reversed-phase	UV	[60]
Oxygen heterocyclic components of a lemon essential oil	10-port valve	Silica normal-phase	Reversed-phase monolithic	Diode array	[61]

Supercritical fluid chromatography

SFC was first introduced in 1962 [62]. In packed column SFC regular LC columns can be used, but instead of a liquid mobile phase, supercritical fluids are used. The supercritical state occurs when pressure and temperature are both above the thermodynamic critical point. A supercritical fluid has a higher density compared to the gas state and a lower viscosity and higher diffusivity relative to the liquid state. These unique characteristics result in a higher solvation power and lower backpressures across a packed column [63-65].

CO₂ is the most commonly applied eluent because its critical pressure and temperature are relatively low (respectively 73 atm and 31 °C) and thus the instrumental requirements are limited and thermo-labile compounds can be analysed. Furthermore, when the pressure is decreased to atmospheric pressure, the CO₂ converts to the gaseous state and is easily removed [65]. As a result SFC is a technique with an extremely low waste production and a low environmental impact [66-68]. Note that often organic modifiers like alcohols, acetonitrile or hexane are added to the mobile phase to change the polarity of the mobile phase to allow tuning of the mobile phase polarity [69,70]. Besides the advantage of

less organic waste production, the sample-throughput [71] and chromatographic resolution [71-73] (all using 5 μm particle size) in SFC were reported to be increased relative to LC. The advantage of the high throughput in SFC is clearly observed from the increased linear velocity, u , at optimal (low) plate height, H , in the Van Deemter curves of SFC compared to LC (figure 6.3) [74]. In this specific study, the separation efficiency, expressed by a low value of H , in SFC when using 3.5 μm particles was slightly better than in LC using the same particle size. However, when using particles of 1.7 μm internal diameter, LC shows slightly better separation efficiency compared to SFC using the same particle dimension. This is explained by the occurrence of an inverted radial temperature gradient (the wall region of the column is warmer than the center) inducing an endothermic mobile phase decompression in SFC [74].

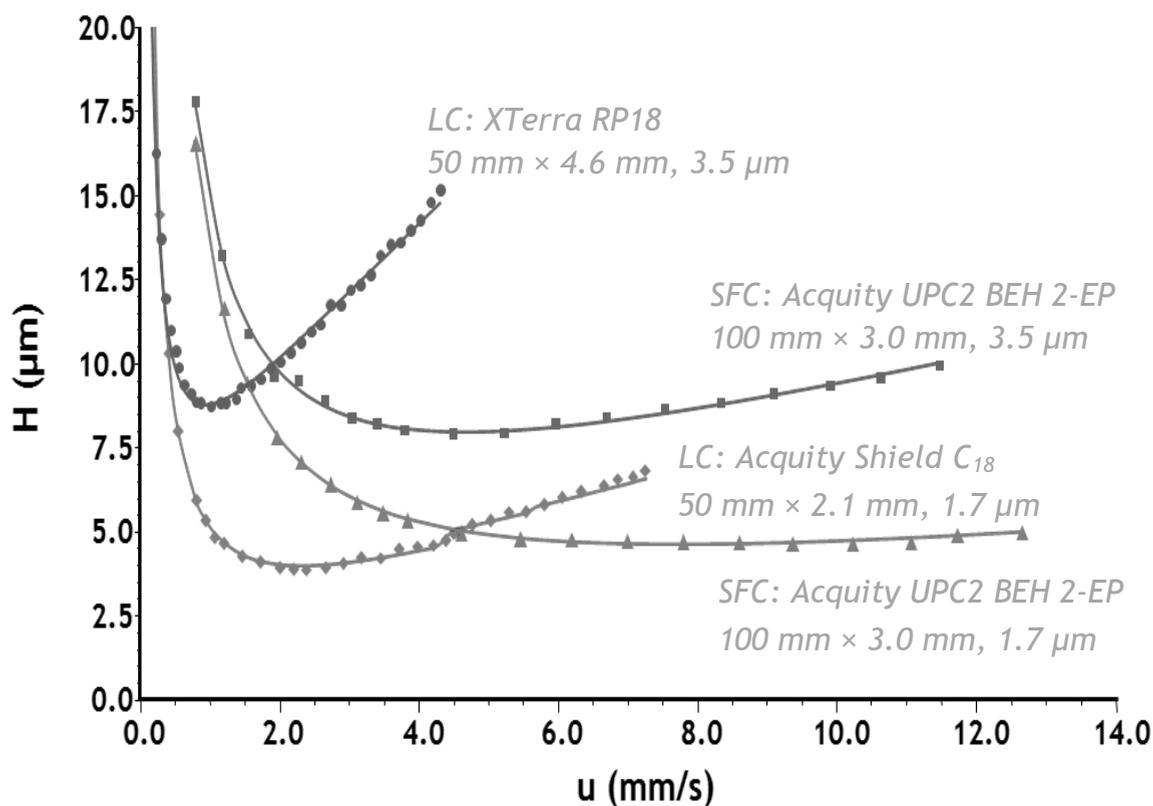


Figure 6.3. Plate height (H) as a function of linear velocity for 1.7 and 3.5 μm particles in HRLC and HRSFC. Van Deemter curves for butylparaben on 2 systems equipped with 1.7 or 3.5 μm particles columns. LC conditions: H₂O/ACN (60/40, v/v), 30 $^{\circ}\text{C}$, 1 μL injected. SFC conditions: CO₂/MeOH (96/4, v/v), 40 $^{\circ}\text{C}$, 150 bar backpressure, 1 μL injected. Reproduced with permission of the author [74].

Due to the high efficient separations when using $> 3 \mu\text{m}$ particles, SFC is becoming more popular for chiral separations [70,75,76]. Note that, as for chiral LC, in chiral SFC a chiral selector is used, either bonded to the stationary phase (e.g. α -acid glycoprotein or polysaccharide) or in the mobile phase (e.g. cyclodextrins) [70]. Several chiral applications of SFC were previously reported [70] of which the majority aim for impurity separation in pharmaceuticals. Applications of SFC in veterinary drugs analysis include the separation of benzimidazole sulphoxide enantiomers in academic solutions [77-79], ketoprofen enantiomers in human plasma [80], ibuprofen [81], naproxen [82] and β -agonist [83] enantiomers in academic solutions.

In my opinion, now SFC bench-top equipment, compatible to MS instruments, has become commercially available, it will become more popular for the analysis of small-molecules. When using $> 3 \mu\text{m}$ particle sizes, chromatographic resolution is increased by SFC and thus this technique is especially beneficial for chiral separations for which no sub- $2 \mu\text{m}$ particle size materials are commercially available. Furthermore, the low environmental impact and high sample throughput serve well in a routine situation. Also because of its flexibility in separation mechanisms, solvent compatibility, high efficiency and speed, SFC would be an excellent option for SFCxSFC [84] (comparable to LCxLC), SFCxLC or LCxSFC.

Concluding remarks on selectivity

Several new techniques are available on the market today to further enhance the selectivity in residue analysis. The use of HRMS is becoming increasingly popular in a routine situation, especially for screening analysis but it is also increasingly applied for confirmatory analysis. In my opinion HRMS has added value in the screening of antibiotics in products of animal origin (e.g. the low detection limits) but the benefits of this technique are not fully exploited yet. Because databases are used for data evaluation this technique remains a targeted approach. New software tools may improve the process of data evaluation making HRMS applicable for fast non-targeted screening analysis. Until then, the use of microbial inhibition tests [85,86] should be maintained (in parallel to HRMS), because this is a fully orthogonal technique and is able to detect all compounds showing antimicrobial activity.

IMS, LCxLC and SFC all contribute to a certain extent to an increased selectivity in the analysis of veterinary drugs in product of animal origin. I expect that many MS instruments will be equipped with IMS during the upcoming years, because it is easy to implement. IMS will be used in a research environment as well as in routine analyses and it will be applied in combination with both nominal mass MS and HRMS. The implementation of SFC is less obvious. For many applications the increase in chromatographic power compared to HRLC is expected to be limited and thus SFC is primarily beneficial for decreasing the environmental impact and to increase the sample-throughput which is primarily of interest for routine analyses. Furthermore, unless an MS system is exclusively used in combination with SFC, flexibility is needed. Many currently applied methods use LC-MS/MS, and if the same MS equipment is used (as is likely, especially during the SFC implementation phase) the SFC equipment should be easily disconnected from (and later reconnected to) the MS. This practical issue might hamper the implementation of SFC-MS. LCxLC can easily be implemented in the laboratory by purchasing a complete LCxLC system or by constructing a system using a couple of multi-port valves and LC pumps, which are usually already present in the laboratory. However, before this technique can be fully operational, research is needed to develop an easy to use LCxLC system that is widely applicable. Also the use of SFC in multi-dimensional applications should be considered.

Monitoring strategies

Regulatory monitoring of antibiotic residues has focused on the analysis of products of animal origin based upon 96/23/EC [87]. Due to the economic situation a cut down on budgets is a realistic scenario, and thus there is a need for a more efficient design of the monitoring strategy. In my opinion this will result in changes in the regulatory control of antibiotics in food products:

- Due to the advances in biochemical analysis techniques, parts of the regulatory monitoring will move from the laboratory to the farms, fields and slaughter houses and become part of the self-control policy.
- More generic methods will be applied for the screening of regulatory control samples to increase sample-throughput. Because in this selectivity is compromised, highly selective confirmatory methods are needed as a

follow up. For sake of efficiency the development of a single highly selective confirmatory method would be of interest.

- The monitoring programs will become more risk based, thereby limiting the number of regular monitoring samples and increasing the number of irregular samples being other matrices and/or containing compounds not routinely included in the monitoring plan. Methods should be available to detect antimicrobial activity at relevant levels and procedures should be developed to determine the identity of the 'unknown' compound present.

Monitoring plans aim for detection of banned substances at levels as low as reasonably possible and at least at the minimum required performance limit (MRPL). For registered substances, screening and confirmatory methods are implemented aiming for detection and quantitation of antibiotic residues at and around the maximum residue limit (MRL). It is important to realise that “in order to protect public health, MRLs should be established in accordance with generally recognised principles of safety assessment, taking into account toxicological risks, environmental contamination, as well as the microbiological and pharmacological effects of residues” [88]. More specifically, MRLs are established based on pharmacological and toxicological data, immunotoxicity, microbiological properties and their effects on the human gut flora [89,90]. The possible occurrence of bacterial resistance as a result of antibiotic usage is not specifically mentioned in the establishment of MRLs. Because of emerging antibiotic resistance the government policy focuses on the decrease of antibiotic treatment and therefore additional requirements for the registration of antibiotics usage in animal breeding were established [91]. To enforce this requirement, I expect that the focus of monitoring plans will broaden:

- Instead of only aiming at MRL level, monitoring programs will focus on antibiotic usage in general and thus on levels as low as reasonably possible.
- The focus will extend from monitoring the levels of antibiotics in food products to other matrices, like manure, feathers, hair and swap samples. By analysing these noninvasive samples antibiotic usage can possibly be monitored effectively. Monitoring programs should also include environmental samples to study the role of antibiotic residues in the dissemination of bacterial resistance.

Efficient monitoring strategies

For the regulatory control of products of animal origin aiming at MRL level, the current trend observed is towards the use of more generic methods as presented in chapter 2. This compromises selectivity and therefore, most of these methods are considered screening methods. I expect that, in the near future a few screening methods, be it microbiological, biochemical or instrumental, that include a broad range of compounds will be applied to monitor the complete range of antibiotic, anthelmintic and coccidiostat drugs. In current practice, suspect samples are re-analysed using confirmatory methods that focus on a single compound group and if instrumental screening techniques are used it is common to re-inject samples on the same LC system after adjusting the MS settings to focus on a single compound using two or more ion transitions. These confirmatory methods run within the quality system's scope and as a result, operability of all of these methods has to be demonstrated. Because only a relatively low number of samples are found suspect, these methods are applied sparingly and thus additional effort is needed to demonstrate operability to keep accreditation for these methods. This is extremely inefficient.

In my opinion, a solution to this problem would be to use a single, highly selective confirmatory method that replaces all individual confirmatory methods. To facilitate this, a detection system should be developed that is able to sufficiently retain and separate antibiotics from all relevant antibiotic classes including the tetracyclines, sulfonamides, (fluoro)quinolones, macrolides, β -lactams and aminoglycosides. Especially including the latter compound group is challenging because these antibiotics are highly polar and are not retained in reversed phase chromatography.

A promising technique to fulfill this challenging task is 2D chromatography like LCxLC, SFCxSFC or the combination of LC with SFC. Such a system yields superior chromatographic resolution and thus is high selectivity. Because these systems are more complicated than regular LC systems, for the upcoming years, these will not function within a routine situation. Until then, I advise to carry out the screening analysis using generic screening methods at the official laboratories and that all suspect samples are transferred to the national reference laboratory (NRL) in order to carry out confirmatory analysis using a comprehensive two-dimensional technique. A huge advantage of this approach is that samples are

analysed by two different laboratories and using two different systems, which enhances the selectivity and trustworthiness of the result. Furthermore, this approach is more efficient for the official laboratories because they can focus on broad screening methods under a fixed scope whereas the NRL can assure accreditation under a flexible scope which is an additional strain on the laboratory's quality system. For this approach to be successful, a high degree of collaboration between the official laboratories and the NRL is mandatory. A disadvantage of this approach is that the NRL cannot carry out the counter-analysis of the non-compliant samples, because of a conflict of interest. Therefore, also collaboration with other laboratories, e.g. the European Reference Laboratories (EURL) is mandatory.

Risk based monitoring

Currently, the implemented monitoring programs are relatively fixed and only slight changes occur from one year to the next. Therefore, also the scope of methods of analysis needed for regulatory control is relatively fixed. In my opinion monitoring programs should become more risk based. The prescribed part in the monitoring program should become more limited and each member state should carry out a risk assessment regularly on basis of which the national monitoring programs are adjusted. This will result in a focus on different antibiotic compounds, different matrices and different levels and as a result, additional compounds and matrices will have to be analysed. As an example of a risk based approach, national legislation will be enforced stating that, to prevent further dissemination of antimicrobial resistance, third and fourth generation cephalosporins and (fluoro)quinolones are only to be used in veterinary practice after it has been demonstrated that the aimed bacteria are resistant towards other, more common drugs like tetracyclines [91]. A more stringent monitoring on the use of third and fourth generation cephalosporins and (fluoro)quinolones is then inevitable. This should not focus on the detection of these compounds at MRL level, but at the use of these compounds in general. As a result, instead of determining the concentration of these compounds in animal tissue, the control should focus on the use of these compounds in general and thus be able to detect these antibiotics at levels as low as reasonably possible. Furthermore, the focus should be extended from species for which a certain antibiotic is registered, to species for which the use of these compounds is to be expected. Cephalosporins

are registered for use in pork and cattle, but because the use of these compounds is likely in poultry breeding as a result of penicillin resistance, monitoring should include different matrices.

This development demands a highly flexible approach from official laboratories and NRLs. Furthermore, fast development of robust methods and efficient validation and method implementation is of importance. Also a more flexible quality control system is needed to be able to report results under accreditation.

Another effect of a more risk based monitoring strategy is that methods are applied to monitor if new 'unknown' antibiotics are being used in veterinary practices. Therefore methods focusing on the detection of microbial activity are needed and as a follow up, highly selective methods are needed to be able to identify such compounds. A method for the identification of antimicrobially active compounds in feed is currently being developed within RIKILT. Samples showing antimicrobial activity are extracted and fractionated on an LC system with a subsequent microbial inhibition test to determine the fraction that contains the antimicrobially active compound. This fraction is further analysed by HRMS and the data were evaluated using a large compound database. This procedure is highly successful if the antimicrobially active compound is present at relatively high concentration, but especially when the antimicrobially active compound is present at low level, this procedure results in a long list of possible molecular formulas, each having several structural isomers. The possible candidates might not be false positive finding but could be other compounds present in the sample, like vitamins, flavones and plant hormones. So these can be eliminated because they do not show antimicrobial activity, but still it remains difficult to assign the identity of the antimicrobially active compound present. There are several options to further improve this procedure and limit the number of possible candidates: (1) using a database of compounds that all show antimicrobial activity, (2) modeling the LC system to eliminate false positive results based on retention time, (3) the use of a second fractionation system to limit the number of candidates, (4) the application of fragmentation or neutral loss trees for identification and (5) the application of IMS-HRMS.

The first option is to only include antimicrobially activity compounds in a database. This would be highly beneficial, even though it might be possible that not all biological data on the possibly relevant compounds are available in

literature. Furthermore, it is an immense task to create a database that includes all antimicrobially active compounds and some natural (herbal) antibiotics might be missed.

Second, the analytical LC system can be modeled according to the procedure described in chapter 3. With this, for each possible candidate reported, the retention time found can be related to the chemical characteristics of the compound. If these are not in agreement, the suggested compound can be eliminated from the list of possible candidates. Because for each possible candidate a number of physical and chemical properties have to be determined, this procedure can be quite laborious when the number of possible candidate compounds is high. Furthermore, other compounds that are truly present but are not responsible for the antimicrobial inhibition are not eliminated using this procedure. Therefore, other procedures to limit the number of possible candidates might be preferred.

A third option is the use of an additional, and preferably orthogonal, fractionation system, e.g. using alkaline conditions, that is used in parallel to the acidic LC fractionation system. By correlating the list of determined possible candidates found by HRMS in the antimicrobially active fraction of both fractionation systems, the number of possible candidates can be limited severely. Furthermore, by comparing the retention time of the fraction containing the antimicrobially active compounds for both systems, information is obtained on the chemical properties of the compound (acidic, neutral, basic) that may not always be obtained by comparing the data of positive and negative ionisation in MS.

A fourth option is to identify unknown compounds based upon multi stage (MS^n) fragmentation and/or neutral loss trees. Related compounds result in similar fragmentation patterns in tandem MS (MS/MS) showing similar product ions or neutral losses. As an example, for all penicillins included in the method as presented in section 5.4, a product ion at m/z 160 is found (not in all cases among the two most abundant ions). The observation of a product ion of m/z 160 for an unknown compound could indicate it is related to the penicillins. The use of fragmentation and neutral loss trees has been reported in literature as a tool to elucidate sub-structures of unknown compounds [92-95]. Problems related to this technique are the complexity of the data and therefore the need of

expertise [92], the reproducibility of the data from one instrument to the other and the limitation of this approach to the use of multi-stage MS^n mass spectrometric equipment (ion trap).

Last, the use of IMS-HRMS can aid in the structure elucidation of the antimicrobially active compound. Besides the higher separation power obtained, using IMS, the collision cross section of the candidate compounds can be determined, which is valuable information for structural characterization of the compound [45,96].

Sub-MRL detection

It is recognised only recently that antibiotic usage in veterinary practice and the presence of low levels of antibiotics in food products and the environment contribute to the emergence of antibiotic resistance [97]. Therefore, instead of only monitoring the level of antibiotic residues present in food products, the use of antibiotics in animal breeding in general should be monitored. Due to increasing antibiotic resistance, the Dutch government emphasised on the development of new antibiotics and on lowering antibiotic usage in humans [98] and veterinary practice [99]. In 2011, a reduction of 32 % of antibiotic usage compared to 2009 was reported. However, these figures are based on official sales data rather than usage data and might thus not fully reflect the use of antibiotics. In my opinion, to be able to determine the true antibiotic usage in veterinary practice, on-farm antibiotic usage registration data should be used that are enforced by a monitoring program.

This program should not focus on monitoring antibiotic residues in food products aiming at MRL levels as is current practice, but instead on the control of the on-farm registration data itself. For this purpose non-traditional matrices should be sampled including manure, hair, feathers and surfaces (e.g. by methanolic swaps). These samples are non-invasive and can easily be taken from any farm. Next, methods should be developed for the analysis of these samples aiming at detection limits as low as possible and the results of the analyses using these methods should be correlated to the on-farm registration system. Especially for the analysis of swap samples, if detection limits allow, ambient ionisation mass spectrometry might be a valuable technique as was previously demonstrated for

the analysis of explosives and drugs on fabrics [100,101] and surfaces [102], and pesticides on vegetable and fruit surfaces [103,104].

Because no information is available on the levels of antibiotics that can contribute to the emergence of bacterial resistance, analytical methods should be able to detect antibiotics at levels far below the MRL. Many analytical methods, especially the ones based on LC-MS/MS are already capable of doing this of which the method for analysis of β -lactams as described in section 5.4 is a clear example. However, for routine monitoring, quick, easy, cheap and reliable screening methods are beneficial. Unfortunately, microbial screening tests, as routinely applied in the monitoring of antibiotics in food products, are unsuited because of their relative high detection limits [105]. Therefore, cheap, easy and reliable screening tests, that are able to detect a broad range of antibiotics at levels well below the MRL, are required. Besides generic instrumental methods, immunoassay biosensors based on (imaging) surface plasmon resonance (SPR) [106-113], electrochemical immunosensors using magnetic beads [114] and luminescent bacteria biosensors [115,116] are promising techniques.

Antibiotic-free animal breeding

Especially when outbreaks of resistant bacteria get more common, from a producer's commercial perspective it could be of interest to introduce a quality label indicating antibiotic-free animal breeding practices. But is it possible to distinguish between for instance broilers that were raised without antibiotics and broilers that were treated, even if it was in the first days after hatching? Antibiotics are excreted from the body and a few days or weeks after treatment, residue levels in body tissues and manure are usually too low to be detected (as was also shown in section 5.3) [117]. Therefore, regular testing of food products aiming at the MRL level or well below, are insufficient and thus new approaches are needed.

The first option could be monitoring unconventional matrices, e.g. bones, legs, feathers or hair. Antibiotics may be excreted from tissue, they might accumulate in other matrices. In a study enrofloxacin was administered to broilers and kidney, muscle, liver and feather samples were analysed until nine days after treatment. It was found that nine days after treatment enrofloxacin and its metabolite ciprofloxacin were present at higher levels in feathers compared to

tissues [118]. Furthermore, it was reported that chlortetracycline binds to bones and can be released under acidic conditions [119]. This could be of interest to detect the use of tetracycline antibiotics long after administration.

The second option is the use of an untargeted metabolomics approach [120-124] to distinguish treated animals from untreated animals. A first step would be to study the applicability of this approach based on one antibiotic substance. Samples of a large and highly diverse group (species, age, region, etc.) of treated and non-treated animals (preferably treated at different time points in their lives) should be analysed using a generic sample preparation procedure and a full scan detection technique (e.g. LC-Orbitrap-MS). Using multivariate data analysis (e.g. principal component analysis), the treated and untreated group should be distinguished [125,126]. As a second step, animals treated with a different antibiotic from the same compound group and, later, animals treated with entirely different antibiotics can be added to the data set. The main disadvantage of this approach to be successful is the need of a blank population that fully covers all natural variation and thus an extreme number of animals is needed to set up the method. As an alternative, biomarkers for antibiotic usage may be found.

Concluding remarks on monitoring strategies

Due to the current economic situation and the renewed attention for bacterial resistance, changes will occur in the monitoring strategies for veterinary drugs in products of animal origin. There is a clear need for more efficient methods and new monitoring strategies have to be developed to be able to control farmers' administration. This includes the detection of antibiotics at levels as low as reasonably possible and research to determine what samples are suitable for this purpose, e.g. manure, swaps, feathers (all non-invasive) and bones.

The analysis of chloramphenicol

In this thesis the analysis of CAP, a banned antibiotic compound which is frequently detected in products of animal origin [127], corn silage and straw [128] was studied. Findings of CAP have had a huge impact on farmers' lives and international trade [129,130].

Confirmatory analysis

A first step in the analysis of CAP is to confirm the identity of the compound present. Because CAP occurs in eight different stereoisomers, of which only one is biologically active, the confirmatory method should be able to discriminate among these eight isomers. As described in section 4.4 a method was developed based on chiral LC coupled to MS/MS which is able to confirm the presence of the individual isomers at levels well below the minimum required performance limit (MRPL) of $0.3 \mu\text{g kg}^{-1}$ in urine. This method also proved to be applicable for the analysis of animal feed and plant samples.

The reported method consists of an extensive sample clean-up procedure which limits the sample throughput. Furthermore, relatively large amounts of toxic chemicals are used. Therefore, it would be advisable to study new ways to discriminate the CAP isomers. Possibilities would be to use ion mobility spectrometry in line with (HR)MS detection. A second option would be to use supercritical fluid chromatography (SFC) instead of chiral LC as discussed above.

Natural occurrence

A second important aspect is the study of the possible natural occurrence of CAP residues. CAP was first isolated from the soil bacteria *Streptomyces venezuelae* [131,132] and can therefore occur naturally. In section 4.2 it is shown that CAP was detected in plant material, mainly obtained from Mongolian pastures. Later the detection of CAP in straw samples from different origins within Europe was reported [128], indicating the natural occurrence of CAP. In the meantime, also in other European countries unexpected findings of CAP have been reported. The possible natural occurrence of CAP was studied by (1) comparing isomeric profiles in positive samples and CAP formulations, (2) studying the possibility of CAP production when exposing corn silage to air and (3) investigating the possibility of crops to take up CAP from the soil.

In section 4.3 it was shown that no differences in isomeric profiles of CAP were observed in positive samples and CAP formulations: in all cases only the active isomer was detected. It was concluded that naturally occurring CAP and chemically synthesised CAP cannot be discriminated on basis of the isomeric profiles. Furthermore, it was found (results not presented in this thesis) that

during corn silage preparation and after exposing corn silage to air, conditions are unfavorable for CAP production. Therefore it is unlikely that CAP is naturally produced in the corn silage production process. In section 4.5 it is shown that wheat and corn can take up CAP from the soil with the uptake rate primarily depending on the type of soil cultivated on. It was also demonstrated that CAP can be produced by *Streptomyces venezuelae* in non-sterile soil and, on the other hand, that CAP is biodegraded in soil. This might explain why no or only very low levels of CAP have been detected in soil samples. Nevertheless, it was shown that CAP can be produced by soil bacteria in non-sterile soil in appreciable amounts, allowing the transfer to crops and the subsequent detection of free CAP residues in crops. This is considered evidence of the natural occurrence of CAP in crops and animal feed.

If such crops are consumed by animals, CAP residues in products of animal origin may occur via this route. To support this, transfer studies of CAP in animal feed products (corn and wheat straw) to products of animal origin, including muscle, casings and urine, are needed. A preliminary oral dosing study in veal calves showed that direct oral administration of CAP corresponding to at least $3 \mu\text{g kg}^{-1}$ CAP in feed is needed to obtain non-compliant findings of CAP ($>$ MRPL) in urine and that CAP levels drop well below the MRPL within one day after consumption. Because no contaminated feed was available, this study was carried out, as a worst case scenario, by applying CAP preparations directly into the calves' mouths. In this way the exact administered amount of CAP is known and no cross-contamination among the calves can occur. However, it may not be fully representative with the intake of CAP through contaminated feed or stall bedding. Therefore, a transfer study using contaminated feed is urgently needed to confirm the preliminary results and to study other matrices like muscle and casings.

Another study that is scientifically relevant is the investigation of the fate of CAP in soil and crops. The transfer study presented in section 4.5 solely focused on free CAP and it was observed that over 99 % of the CAP administered to the soil was undetectable. Besides microbial degradation, it may be possible that CAP is conjugated in soil or in crops and that it is reactivated after ingestion by animals. Therefore, the determined transfer of free CAP is the bare minimum of CAP that is transferred. To study the fate of CAP, HRMS could be applied searching for CAP

conjugates and degradation products. Furthermore the mass balance of CAP could be studied in a transfer study in which the biodegradation of CAP in soil is eliminated. This could be done by cultivating crops on sterile soil or in the absence of soil (e.g. on water). If biodegradation in soil was the primary process that lead to the loss of CAP, a severely higher percentage of CAP should be recovered in this experiment. In parallel, the presence of CAP conjugates and metabolites in plant and soil samples should be studied by analysing these samples using HRMS.

Because it is likely that CAP can occur naturally in crops, this affects the enforcement of legislation. Therefore, for CAP, either the status of banned antibiotic should be abandoned and a threshold level should be established based upon low-level toxicological data [130] or methods should be developed that are able to discriminate between naturally produced and chemically synthesised CAP. The latter might be possible in two ways. The first option is to study if intermediates and/or by-products of the naturally occurring CAP biosynthesis are detectable in contaminated samples. Several intermediates and by-products are produced in the biosynthesis of CAP among which N-dichloroacetyl-p-aminophenylserinol, p-amino-phenylserine and chorismic acid (figure 6.4) [133-136]. If one or more of these intermediates or by-products is detectable besides CAP itself, these compounds might be useful markers in the discrimination of biosynthesised and chemically synthesised CAP. Chemical CAP preparations are purified to obtain the highest possible purity. Nevertheless, to prevent the selection of a marker compound that could also be the result of chemical synthesis, the chemical synthesis of CAP should be taken into account as well. The main route for chemical synthesis seems to deviate severely for the biosynthesis, having 2-amino-1-(4-nitrophenyl)-1,3-propanediol as the intermediate [137-139], whereas in the biosynthesis the intermediate products contain an aminophenyl moiety.

Another option is to study the ratio of naturally occurring isotopes (e.g. ^{12}C versus ^{13}C) in CAP using isotope ratio MS (IRMS) [140] in order to determine the origin of the compound. This technique has been used in authenticity control of food products [141,142], the detection of production fraud [143], the differentiation of exogenous from endogenous steroids [144-147] and in the determination of the origin of cyanide in forensic investigations [148].

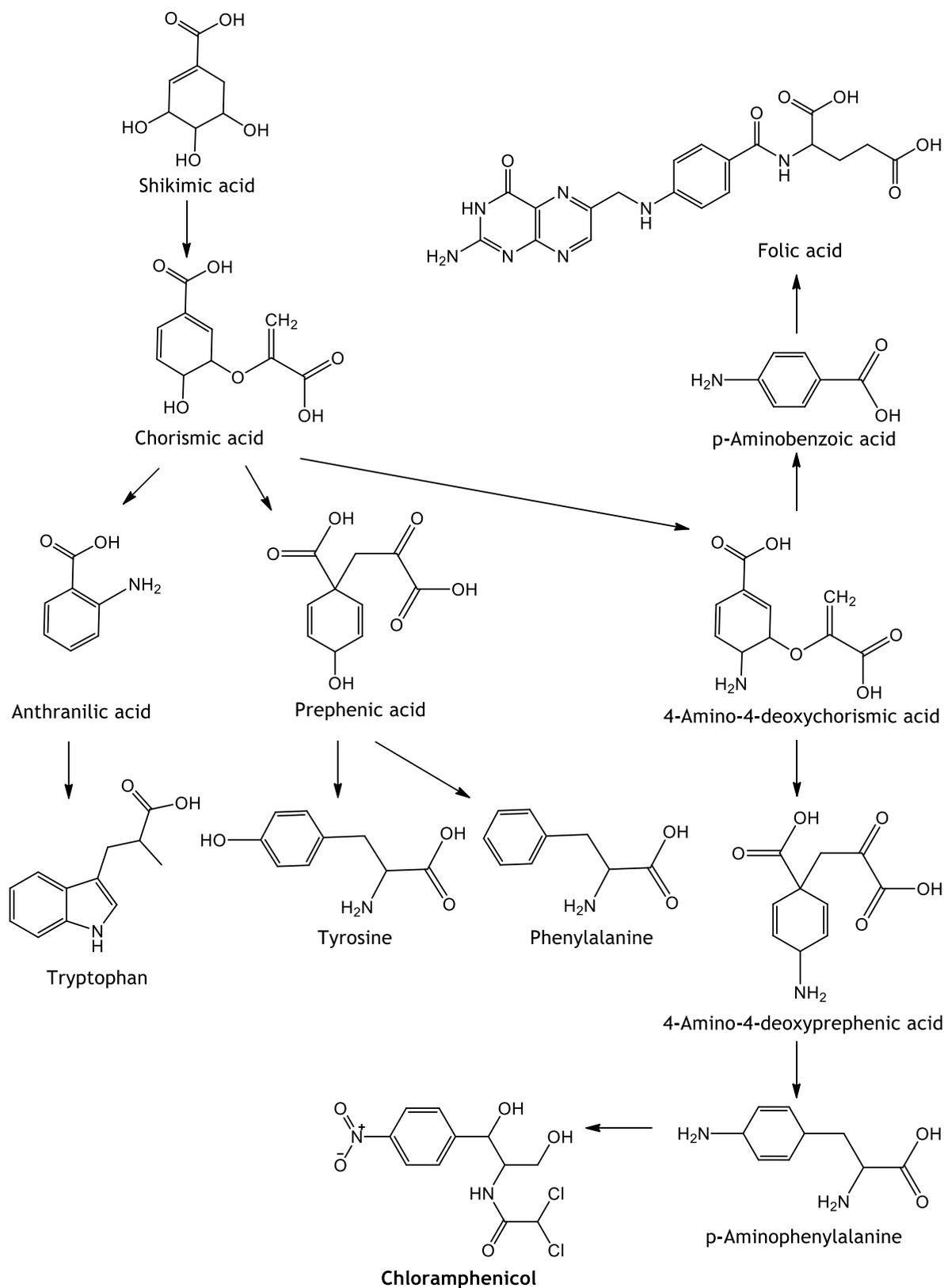


Figure 6.4. Chloramphenicol biosynthesis including other primary and secondary aromatic metabolites in the shikimic acid pathway [134].

Concluding remarks on chloramphenicol analysis

As a result of my research a highly selective method for the analysis of CAP is available and evidence was found for the natural occurrence of CAP in crops and animal feed. Because CAP is a banned substance, it is of main importance to discuss the European regulations. Should the banned status of CAP be remained or should a tolerance limit be implemented? In my opinion additional research is needed. First, transfer studies from CAP contaminated feed or crops to products of animal origin are needed to determine if naturally contaminated samples can result in positive findings in such samples. Second, research on the possibility to discriminate biosynthesised from chemically synthesised CAP is needed. Third, toxicity studies based on sub-ppb concentrations of CAP in products of animal origin should be carried out. If naturally contaminated samples are not likely to result in positive findings in products of animal origin or if biosynthesised and chemically synthesised CAP can be discriminated, the banned status of CAP can be maintained. If CAP turns out not to be toxic at residue levels, an MRL could be established. Up until then, I advise to take straw and feed samples when taking regular samples for CAP analysis. If this is not possible, e.g. because samples are taken in the slaughter house, conclusions based on positive CAP findings should be drawn with utmost care.

Concluding recommendations

To summarise, the following recommendations for future research are made:

- The selectivity of HRMS compared to MS operating at nominal mass resolution should be studied. Based on the outcomes, regulations on the use of HRMS in confirmatory analysis should be assessed.
- The use of IMS, LCxLC and SFC to further enhance selectivity in confirmatory analysis should be studied. Also the applicability of these techniques in research studies, e.g. coupled to HRMS should be investigated.
- Efficient screening methods that include a broad range of compounds in combination with highly selective, broadly applicable confirmatory methods should be developed in order to efficiently carry out the (fixed and risk based) national monitoring plan.

- Monitoring strategies should be developed to effectively control farmers' antibiotic usage registration. This includes method development to further decrease detection limits of regulated antibiotics and studying the use of other matrices for antibiotic residue detection, e.g. hair, feathers, swaps, manure (non-invasive) and bones.
- The transfer of CAP from naturally contaminated crops or feed to products of animal origin (e.g. urine, muscle and casings) should be studied to determine if naturally contaminated crops and feed can result in non-compliant finding of CAP in products of animal origin.
- The mass balance of CAP in a transfer study from soil to crops should be studied in which the biodegradation of CAP in soil is eliminated. In this way it is studied if only free CAP is present in crops or if other forms of CAP, e.g. conjugated CAP, is formed in the crop and/or non-sterile soil. In parallel, the presence of CAP conjugates and metabolites in plant and soil samples should be studied by analysing these samples using HRMS.
- It should be studied if the biosynthesis of CAP by soil organisms can be discriminated from chemically synthesised CAP. This may be possible by studying biosynthesis by-products or by applying IRMS.

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Summary - Samenvatting



Summary

In animal breeding the use of antibiotics has become common practice. Antibiotics are used to treat bacterially infected animals but are also administered as a preventive measure. From an animal and human health perspective, responsible use of antibiotics is of importance. Therefore, regulatory monitoring programs are in place. In residue analysis of antibiotics in products of animal origin, quantitative and qualitative aspects are involved in declaring a sample non-compliant (positive). The quantitative aspect regards the determination of the amount of the compound present in the sample. The qualitative aspect regards the confirmation of the identity of the compound present. In this, selectivity is the main parameter of importance which is defined as the ability of a method to distinguish the analyte being measured from other substances. In *chapter 1*, the background on antibiotics usage in animal breeding and the legal framework of antibiotic analysis are discussed.

In *chapter 2*, the observed trends in sample preparation of veterinary drug analysis using liquid chromatography coupled to mass spectrometry (LC-MS/MS) are discussed. Due to recent advances in LC-MS instrumentation the detection of many compounds within one run became possible and methods for the simultaneous analysis of different compound groups were developed. To be able to simultaneously analyse compounds having different physical and chemical properties, generic sample preparation procedures are applied. The most frequently reported generic sample preparation methods are a solvent extraction only, solid phase extraction and a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) approach. These multi-analyte methods - sometimes including over 150 different compounds - are of much interest for analytical laboratories due to the reduction of costs. A clear drawback of generic sample preparation procedures is the occurrence of abundant matrix effects which compromise detection limits, quantitative aspects, maintenance frequency and method selectivity.

In *chapter 3* the concept of selectivity of qualitative LC-MS/MS methods is discussed. Validation procedures are available to determine the uncertainty of the quantitative result, which is taken into account in the decision making process. For the qualitative aspect, criteria for confirmation of the identity of a

compound using LC-MS/MS are laid down in several regulations. These criteria concern the minimum requirements for the instrumental set-up and criteria for retention time and relative product ion abundances in LC-MS/MS systems operated in the multiple reaction monitoring mode. Procedures to determine the uncertainty of the qualitative aspect of a method of analysis are lacking and, as a result, whether or not a method is adequately selective is a matter of experts' judgment. A procedure was developed on basis of which the selectivity of an LC-MS/MS method can be graded. A measure for the (un)certainty of the selectivity is the probability of any compound showing the same precursor ion, product ions and retention time as the compound of interest. In the developed procedure this is calculated based upon empirical models constructed from three large compound databases. Based upon the final probability estimation, additional measures to assure unambiguous identification can be taken, like the selection of different or additional product ions. The reported procedure in combination with criteria for relative ion abundances result in a powerful technique to determine the (un)certainty of the selectivity of any LC-MS/MS analysis and thus to decrease the risk of false positive results.

To demonstrate the importance of selectivity, in this thesis two analytical challenges are presented in which selectivity plays an important role. One in which selectivity is extreme to be able to discriminate between a banned antibiotic and its antimicrobially inactive isomers. Second a method in which selectivity is deliberately compromised to obtain an effective monitoring strategy in which not only the parent drugs are detected, but also their protein bound metabolites.

In *chapter 4*, the analysis of the broad spectrum antibiotic chloramphenicol (CAP) is discussed. Due to its suspected carcinogenetic properties and linkages with the development of aplastic anemia in humans, CAP is banned for use in food producing animals in the EU and many other countries. This topic is introduced in detail in *section 4.1*. In *section 4.2* it is reported that CAP was detected in several herb and grass samples from different geographic origin. The results of this study demonstrate that non-compliant findings in animal-derived food products may in part be due to the natural occurrence of CAP in plant material. If so, this has implications for the application of current legislation and the interpretation of analytical results with respect to the consideration of CAP

as a xenobiotic veterinary drug residue and the regulatory actions taken upon its detection in food and feed.

CAP occurs in the *para*-configuration and in the *meta*-configuration and contains two chiral centers, thus eight different isomeric configurations exist, namely four (RR, SS, RS, SR) *meta*-stereoisomers and four *para*-stereoisomers. It is known that only RR-*p*-CAP has antimicrobial properties. To find out if the CAP detected in the plant material samples is the active configuration, in **section 4.3** a high resolution reversed phase LC-MS/MS system was tested for its ability to separate the different isomers. After a detailed elucidation of the product ions and the fragmentation patterns of all isomers, reversed phase LC-MS/MS proved to be able to discriminate among some isomers, but not between the enantiomeric pairs, including RR-*p*-CAP (the active isomer) and SS-*p*-CAP. A chiral LC separation with MS/MS detection that is able to distinguish all isomers was developed. Using this new method it was demonstrated that the CAP present in the plant materials was indeed the antimicrobially active isomer.

In **section 4.4** a quantitative method for the analysis of ultra-trace levels of eight chloramphenicol isomers in urine by chiral LC-MS/MS is reported. The separation of the isomers on the analytical column, the selectivity of the monitored product ions and the clean-up of urine turned out to be critical parameters. To obtain reproducible retention times, isocratic elution on a chiral α -acid glycoprotein column was applied. For urine samples, matrix compounds present in the final extract caused decreased retention of the isomers on the chiral stationary phase and a lack of chromatographic resolution. Therefore an extensive clean-up procedure that combines solid phase extraction and liquid-liquid extraction had to be developed. The final method was fully validated and showed satisfactory performance for all isomers with decision limits (CC α) ranging from 0.005 to 0.03 $\mu\text{g L}^{-1}$ and within-laboratory reproducibility of all isomers below 20 % at the minimum required performance limit of 0.3 $\mu\text{g L}^{-1}$.

In **section 4.5** the natural occurrence of CAP is studied in more detail. The hypothesis is that CAP can occur in crops and animal feed through the production of the drug by soil bacteria and the subsequent uptake by crops. Two conditions should be fulfilled to accept the posed hypothesis: (1) soil bacteria must be able to produce CAP in soil under natural conditions and (2) crops should be able to take up CAP from the soil. It is shown that the soil bacteria *Streptomyces*

venezuelae can produce CAP in appreciable amounts in non-sterile soil. Even though CAP is biodegraded in non-sterile soil, it is taken up by plants in relatively small amounts, where it might accumulate. Based on the observations it is stated that CAP can occur in crops, and therefore in animal feed, through the natural production of CAP in soil by soil bacteria.

In **chapter 5**, the analysis of β -lactam antibiotics is presented. β -lactam antibiotics include the penicillins, cephalosporins and carbapenems. Especially penicillins are frequently applied in animal breeding and human medicine. Nowadays, antibiotic resistance against the penicillins is extremely high. Also resistance against cephalosporins and even carbapenems has been reported, which is a major threat to human health. This subject is introduced in detail in **section 5.1**. The main challenges in β -lactam analysis are (1) the instability of some of the analytes and (2) the fast metabolism ceftiofur and cefapirin and the protein binding of ceftiofur residues.

In **section 5.2** the research on the degradation of ceftiofur and cefapirin is presented. A slight instability of cefapirin and desfuroylceftiofur was observed at elevated temperatures. Ceftiofur and cefapirin degraded immediately and completely in an alkaline environment, resulting in antimicrobially inactive degradation products. Ceftiofur and cefapirin also degraded immediately and completely in kidney extract resulting in both formerly reported metabolites as well as not previously reported products. It is shown that conditions often occurring during the analysis of ceftiofur or cefapirin can result in rapid degradation of both compounds. From this, on a theoretical basis, it is concluded that underestimation of the determined amounts of ceftiofur and cefapirin is likely to occur when using conventional methods for the quantitative analysis of these compounds in tissue, and that a new approach is needed that takes the metabolism and degradation into account.

To effectively detect off-label ceftiofur usage an analytical method is needed that, besides the native compound, also detects its active metabolites. In **section 5.3** the applicability of three approaches for the quantitative analysis of ceftiofur using LC-MS/MS is assessed, viz. (A) the analysis of ceftiofur, desfuroylceftiofur and/or desfuroylceftiofur cystein disulfide, (B) the hydrolysis of ceftiofur metabolites to desfuroylceftiofur acetamide and (C) a new method based on the chemical hydrolysis of ceftiofur metabolites using ammonia in order

to produce a single marker compound that is representative for the sum of all for ceftiofur metabolites. It was found that approach A is not suited for quantitative analysis of total ceftiofur concentration nor for effectively detecting off-label use of ceftiofur. Approach B resulted in adequate quantitative results, but is considered to be a single compound method. Approach C showed adequate quantitative results as well, but in contrast to approach B, this approach is applicable to a range of cephalosporin antibiotics and therefore applicable as a broad quantitative analysis method for cephalosporin compounds in poultry tissue samples.

In *section 5.4* a comprehensive method is reported based on hydrolysis using piperidine, for the quantitative analysis of a broad range of β -lactam antibiotics including penicillins, cephalosporins and carbapenems. The hydrolysis was optimised using a full experimental design. Using isotopically labeled analogues and high resolution mass spectrometry (HR-MS), molecular structures of the derivatization products were proposed. The method was fully validated showing satisfactory method performance. It was shown that this method is suitable for the quantitative analysis of 21 out of 22 compounds included and that it is effective for the detection of off-label ceftiofur use, because protein bound metabolites are included.

In *chapter 6* future perspectives on antibiotic analysis are discussed. The use of HR-MS is one of the main trends observed. A study comparable to the work described in chapter 3 is needed to assess the selectivity of that technique. Based upon the outcome, regulations on the use of HR-MS for confirmatory analysis should be assessed. Other methods have become available to further enhance the selectivity of a method. In ion mobility spectrometry compounds can be separated based on their size, shape and collision cross section and therefore it is orthogonal to LC-MS/MS. If IMS is coupled to LC-MS/MS the selectivity is enhanced. The use of comprehensive LC (LCxLC) would also severely increase selectivity. Research is needed to determine optimal and compatible conditions for both dimensions and to effectively couple the two chromatographic systems. Next, supercritical fluid chromatography (SFC) is a promising technique. Due to the fast diffusion rate and the limited back pressure, fast separation at high resolution can be obtained. Also combinations of these techniques, e.g. IMS-HR-MS or LCxSFC should be considered.

In *chapter 6* also some expected changes in monitoring strategies are discussed. The national monitoring plan will become more risk based and therefore, a more generic and flexible approach is needed. The most efficient would be to use generic screening methods that include a broad range of compounds in combination with highly selective confirmatory methods. Also, due to the increasing bacterial resistance, the focus of regulatory control will be more on antibiotic usage in general next to the control of food products itself. In this, detection limits should be as low as reasonably possible and the use of other matrices should be investigated, e.g. manure, hair, feathers, swap (all non-invasive) and bone samples.

Finally, future research regarding CAP analysis is discussed. Because it is shown that CAP can occur naturally in crops and feed, transfer studies from contaminated crops and feed to products of animal origin, including urine, meat and casings are needed to determine if the natural levels in crops and feed can result in non-compliant results in animal products. Furthermore, a study on the possibility to discriminate biosynthesised CAP from chemically synthesised CAP is needed. Based on the outcome of these studies, the banned status of the drug should be reconsidered.

Based on the findings in this thesis we can now answer the question: “What selectivity is adequate?” Unfortunately, there is no general answer to this question, because this highly depends on the purpose of the method in question. Usually there is a trade-off between selectivity and the number of compounds that can be included in a method. For a screening method a low selectivity is acceptable (as long as the number of false positives remains limited), whereas selectivity should be high for a confirmatory method. Furthermore, the selectivity needed depends on the interferences that can be expected. In some cases high selectivity is needed as in the case of CAP, in other cases, like in ceftiofur analysis, selectivity has to be compromised to obtain an effective monitoring strategy. To conclude, whenever developing or validating a method, it is of extreme importance to consider the parameter selectivity in detail.

Samenvatting

In de veehouderij is het gebruik van antibiotica gangbaar geworden. Antibiotica worden gebruikt om bacterie-infecties te behandelen, maar worden ook toegepast als preventief middel. Het verantwoordelijk gebruik van antibiotica is van belang vanuit het oogpunt van dierlijk welzijn en humane gezondheid. Om antibioticumgebruik in de veehouderij te controleren bestaan er wettelijke monitoringsprogramma's. In de residuanalyse van antibiotica in producten van dierlijke oorsprong spelen kwantitatieve en kwalitatieve aspecten een rol in het bepalen of een monster conform de regelgeving is. Het kwantitatieve aspect betreft de bepaling van de hoeveelheid van het aanwezig antibioticum en het kwalitatieve aspect betreft het vaststellen van de identiteit van het antibioticum. Hierbij speelt de parameter 'selectiviteit' een belangrijke rol. Selectiviteit wordt gedefinieerd als 'het vermogen van een methode om onderscheid te maken tussen de te analyseren component en andere aanwezige componenten'. In **hoofdstuk 1** wordt de achtergrond van antibioticumgebruik in de veehouderij en de wetgeving omtrent de monitoring hiervan besproken.

In **hoofdstuk 2** worden de trends in de monstervoorbewerking in de analyse van dierbehandelingsmiddelen met behulp van vloeistofchromatografie gekoppeld aan tandem-massaspectrometrie (LC-MS/MS) bediscussieerd. Ten gevolge van de laatste instrumentele ontwikkelingen is het mogelijk geworden een groot aantal verschillende componenten tegelijkertijd te detecteren. Om componenten met verschillende fysische en chemische eigenschappen tegelijkertijd te kunnen analyseren, dient een generieke monstervoorbewerking toegepast te worden. Het meest frequent toegepast zijn vloeistofextractie, vastefase-extractie en QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe). Deze multi-componentmethoden, die soms meer dan 150 componenten bevatten, leiden tot een verlaging van de analysekosten, maar een nadeel is dat de kans op matrixeffecten groot is, waardoor ingeleverd wordt op detectielimieten, kwantitatieve aspecten, de onderhoudsfrequentie en de selectiviteit.

In **hoofdstuk 3** wordt de selectiviteit van kwalitatieve methoden besproken. Validatierichtlijnen voor het bepalen van de onzekerheid van het kwantitatieve resultaat van een methode, dat wordt meegenomen in de besluitvorming, zijn voorhanden. Wat het kwalitatieve aspect betreft zijn criteria voor bevestiging

van de identiteit van een component, gebruikmakend van LC-MS/MS vastgelegd. Deze criteria betreffen de minimaal toe te passen instrumentele techniek, de retentietijd en relatieve ion-intensiteit. Procedures om de onzekerheid van het kwalitatieve resultaat te bepalen zijn niet voorhanden. Daarom is het vaststellen of een methode voldoende specifiek is een kwestie van de inschatting van experts. Er is een methode ontwikkeld op basis waarvan de selectiviteit van een LC-MS/MS meting kan worden beoordeeld. Een maat voor de (on)zekerheid van de selectiviteit is de kans dat een component eenzelfde precursor-ion, product-ionen en retentietijd heeft als de component die van belang is. In de ontwikkelde procedure wordt dit bepaald op basis van empirische modellen, opgesteld aan de hand van drie stofdatabanken. Op basis van de verkregen schatting kunnen aanvullende maatregelen genomen worden om te komen tot een eenduidige bevestiging van de identiteit, zoals de selectie van andere product-ionen of de selectie van een additioneel product-ion. De gerapporteerde procedure in combinatie met de gestelde wettelijke criteria voor relatieve ion-intensiteiten resulteert in een krachtige techniek om de onzekerheid van de bevestigingsanalyse te bepalen, zodat het risico op een vals-positief resultaat beperkt wordt.

Om het belang van selectiviteit te illustreren, zijn in dit proefschrift twee uitdagingen gepresenteerd waarin selectiviteit een belangrijke rol speelt. Ten eerste een methode waarin de selectiviteit extreem hoog moet zijn om verschil te kunnen maken tussen een verboden antibioticum en haar antimicrobiologisch inactieve isomeren. Ten tweede een methode waarbij de selectiviteit bewust wordt gecompromitteerd zodat een effectieve monitoringsstrategie wordt verkregen waarbij niet alleen het antibioticum zelf wordt gedetecteerd, maar ook eiwitgebonden metabolieten daarvan.

In **hoofdstuk 4** wordt de analyse van het breedspectrumantibioticum chlooramfenicol (CAP) bediscussieerd. Doordat dit antibioticum verdacht carcinogeen is en geassocieerd wordt met aplastische anemie bij de mens, is het verboden voor gebruik bij voedselproducerende dieren. Dit onderwerp is in **sectie 4.1** geïntroduceerd. In **sectie 4.2** wordt gerapporteerd dat CAP gedetecteerd is in diverse kruiden en grassen van verschillende geografische oorsprong. Dit resultaat laat zien dan positieve bevindingen van CAP in dierlijke producten mogelijk een natuurlijke oorsprong kunnen hebben. Als dat zo is,

heeft dit mogelijk invloed op de bestaande wetgeving en de interpretatie van analytische resultaten.

CAP komt voor in de para- en in de meta-configuratie en bevat twee chirale centra, waardoor acht verschillende isomeren bestaan, namelijk vier (RR, SS, RS, SR) para- en vier meta-stereo-isomeren. Het is bekend dat alleen RR-*p*-CAP antimicrobieel actief is. Om te onderzoeken of de CAP gedetecteerd in de gras- en kruidenmonsters de actieve isomeer is, is in **sectie 4.3** getest of hogeresolutie-reversed-phase-LC-MS/MS onderscheid kan maken tussen de verschillende isomeren. Na een uitgebreide opheldering van de structuur van de product-ionen en de fragmentatiepaden, blijkt reversed-phase-LC-MS/MS geschikt om enkele isomeren te onderscheiden, maar niet de spiegelbeeldisomeren zoals RR-*p*-CAP en SS-*p*-CAP (dextramycine). Daarom is een chiraal LC-systeem ontwikkeld waarmee alle isomeren wel onderscheiden kunnen worden. Met dit scheidend systeem is aangetoond dat de gras- en kruidenmonsters de antimicrobieel actieve vorm van CAP bevatten.

In **sectie 4.4** is een kwantitatieve methode voor de analyse van de acht CAP-isomeren in urine met behulp van chirale LC-MS/MS gerapporteerd. De isomeerscheiding op de analytische kolom, de selectiviteit van de gemonitorde product-ionen en de monstervoorbewerking zijn kritische parameters. Om reproduceerbare retentietijden te verkrijgen wordt isocratische elutie toegepast op een α -zuurglycoproteïnekolom. In het geval van urinemonsters zijn matrixcomponenten in het eindextract aanwezig die ervoor zorgen dat de retentietijden van alle isomeren afnemen, waardoor onvoldoende chromatografische resolutie wordt verkregen. Om dit te voorkomen is een intensieve opschoning van de monsterextracten ontwikkeld, bestaande uit een vaste fase extractie met daarna een vloeistof-vloeistofextractie. De ontwikkelde methode is volledig gevalideerd en voldoet aan de gestelde criteria voor alle isomeren. De beslisgrens (CC α) varieert van 0.005 tot 0.03 $\mu\text{g L}^{-1}$ en de binnenlaboratoriumreproduceerbaarheid van alle isomeren ligt lager dan 20 % op niveau van de minimaal vereiste prestatielimiet van 0.3 $\mu\text{g L}^{-1}$.

In **sectie 4.5** is het natuurlijk voorkomen van CAP onderzocht. De hypothese is dat CAP van nature voor kan komen in gewassen en diervoeders doordat het in de grond geproduceerd wordt door bodembacteriën en vervolgens wordt opgenomen door gewassen. Om deze hypothese te kunnen accepteren dient aan twee

voorwaarden voldaan te worden: (1) bodembacteriën moeten CAP kunnen produceren in natuurlijke grond en (2) gewassen moeten CAP vanuit de grond kunnen opnemen. Er is gedemonstreerd dat *Streptomyces venezuelae* CAP in voldoende mate kan produceren in onbehandelde grond. Ondanks dat CAP in niet-gesteriliseerde grond degradeert, wordt het in beperkte mate opgenomen door gewassen. Op basis van deze observaties is geconcludeerd dat CAP in gewassen en voeders kan voorkomen ten gevolge van de natuurlijke productie van CAP door bodembacteriën.

In **hoofdstuk 5** wordt de analyse van β -lactamantibiotica besproken. β -lactamantibiotica omvatten de penicillines, cefalosporines en carbapenems. Met name penicillines worden veelvuldig gebruikt in de veehouderij en als humaan medicijn. Dit heeft geleid tot een extreem hoge bacteriële resistentie voor penicillines. Resistentie voor cefalosporines en in minder mate carbapenems komt tevens voor, wat een gevaar vormt voor de volksgezondheid. Dit onderwerp wordt in detail geïntroduceerd in **sectie 5.1**. De uitdagingen voor de analyse van β -lactams zijn (1) de instabiliteit van enkele componenten behorende tot deze groep en (2) het metabolisme van ceftiofur en cefapirine, en eiwitbinding van ceftiofurresiduen.

In **sectie 5.2** is het onderzoek naar de degradatie van ceftiofur en cefapirine gepresenteerd. Een beperkte instabiliteit van cefapirine en desfuroylceftiofur is waargenomen bij verhoogde temperatuur. Ceftiofur en cefapirine degraderen meteen en volledig in basisch milieu, resulterend in antimicrobieel inactieve producten. Ceftiofur en cefapirine degraderen tevens in de aanwezigheid van nierextract resulterend in eerder gerapporteerde en nog niet eerder gerapporteerde producten. Condities die vaak voorkomen bij de analyse van ceftiofur en cefapirine kunnen leiden tot snelle afbraak van deze componenten. Daarom wordt op theoretische gronden geconcludeerd dat het waarschijnlijk is dat een onderschatting van het ceftiofur- en cefapirinegehalte plaatsvindt bij toepassing van de conventionele methoden voor de analyse van deze componenten en dat een nieuwe methode, die rekening houdt met het metabolisme en deze degradatie noodzakelijk is.

Om effectief off-label gebruik van ceftiofur aan te kunnen tonen dient een analysemethode naast ceftiofur zelf ook de actieve metabolieten daarvan te kunnen detecteren. In **sectie 5.3** is de toepasbaarheid van drie kwantitatieve

methoden onderzocht, namelijk (A) de analyse van ceftiofur, desfuroylceftiofur en/of desfuroylceftiofur-cysteïne-disulfide, (B) de derivatisering van ceftiofurmetabolieten naar desfuroylceftiofur-acetamide en (C) een binnen RIKILT ontwikkelde methode gebaseerd op de basische hydrolyse van ceftiofurmetabolieten met ammonia ter verkrijging van een markerresidu dat representatief is voor de totale hoeveelheid ceftiofurmetabolieten. Er is vastgesteld dat methode A niet geschikt is voor de kwantitatieve analyse van de totale hoeveelheid ceftiofurmetabolieten, noch voor de effectieve detectie van off-label gebruik van ceftiofur. Methode B resulteert in goede kwantitatieve resultaten, echter deze methode is uitsluitend geschikt voor de analyse van ceftiofur, niet voor andere cefalosporines. Methode C geeft tevens goede kwantitatieve resultaten en deze methode lijkt geschikt voor toepassing van de analyse van diverse cefalosporines in kippenvlees.

In *sectie 5.4* is de ontwikkeling van een methode gerapporteerd, gebaseerd op de basische hydrolyse met piperidine, voor de kwantitatieve alomvattende analyse van β -lactamantibiotica, inclusief penicillines, cefalosporines en carbapenems. De hydrolyse is geoptimaliseerd met een 'full experimental design'. Gebruikmakend van isotoopgelabelde componenten en hoge resolutie massaspectrometrie (HR-MS) zijn voorstellen gedaan voor de molecuulstructuur van de gevormde reactieproducten. De methode is volledig gevalideerd en de bepaalde methodekarakteristieken zijn acceptabel bevonden voor de kwantitatieve analyse van 21 van de 22 β -lactams. Daarnaast is aangetoond dat de ontwikkelde methode effectief is voor het opsporen van off-label gebruik van ceftiofur, omdat met deze methode ook eiwitgebonden residuen worden gedetecteerd.

In *hoofdstuk 6* zijn toekomstperspectieven betreffende antibioticumanalyse besproken. Het toenemend gebruik van HR-MS is één van de meest opvallende trends over de afgelopen jaren. Een onderzoek, vergelijkbaar aan het werk gepresenteerd in hoofdstuk 3, moet uitgevoerd worden om de selectiviteit van HR-MS-analyses te bepalen. Op basis van de uitkomsten hiervan dient wetgeving op het gebied van het gebruik van HR-MS voor bevestigingsanalyse heroverwogen te worden. Enkele andere methoden voor het verbeteren van de selectiviteit zijn voorhanden. Ion-mobiliteitspectrometrie (IMS) scheidt componenten op basis van grootte, vorm en botsingsdoorsnede, en is daarmee orthogonaal aan LC-MS/MS.

De koppeling van IMS met LC-MS/MS resulteert in een verhoogde selectiviteit. Het gebruik van tweedimensionale chromatografie (LCxLC) verhoogt de selectiviteit aanzienlijk. Er is onderzoek nodig om de optimale condities voor beide dimensies te bepalen en om een goede koppeling te maken tussen beide dimensies. Ook superkritische fasechromatografie (SFC) is een veelbelovende techniek. Door de hoge diffusiesnelheid en de lage tegendruk kunnen zeer snelle scheidingen met hoge resolutie verkregen worden. Als laatste zijn combinaties van deze technieken mogelijk zeer interessant, zoals bijvoorbeeld IMS-HR-MS en LCxSFC.

In hoofdstuk 6 worden ook de verwachte ontwikkelingen in de monitoring van residuen besproken. Het nationaal monitoringsplan zal meer risicogebaseerd worden en daardoor is een flexibelere aanpak vereist. Het meest efficiënt is het gebruik van generieke, veel componenten omvattende, screeningsmethoden in combinatie met enkele zeer selectieve bevestigingsmethodes. Daarnaast zal, door de verregaande ontwikkeling van bacteriële resistentie, monitoring steeds meer gericht worden op het testen van antibioticumgebruik in het algemeen naast de monitoring van antibiotica in voedselproducten. Hierbij moeten detectiegrenzen zo laag mogelijk zijn en is het gebruik van andere matrices mogelijk relevant, zoals mest, haar, veren, veegmonsters (alle niet-invasief) en botten.

Als laatste wordt toekomstig onderzoek gerelateerd aan CAP voorgesteld. Omdat aangetoond is dat CAP van nature kan voorkomen in gewassen en diervoeder, zijn overdrachtsstudies van natuurlijk gecontamineerde gewassen en voeders naar producten van dierlijke oorsprong (urine, vlees, darmen) noodzakelijk om vast te stellen of de niveaus in gewassen en voeders kunnen leiden tot detecteerbare CAP-residuen in producten van dierlijke oorsprong. Daarnaast dient onderzoek gedaan te worden naar de mogelijkheid om gebiosynthetiseerd CAP te onderscheiden van chemisch gesynthetiseerd CAP. Op basis van de uitkomsten van dat onderzoek moet de wettelijk verboden status van CAP opnieuw tegen het licht gehouden worden.

Op basis van het onderzoek gepresenteerd in dit proefschrift, kunnen we de volgende vraag beantwoorden: “Hoeveel selectiviteit is vereist?” Helaas is er geen eenduidig antwoord op deze vraag, omdat dit afhangt van het doel van de betreffende methode. Normaal gesproken bestaat er een wisselwerking tussen

selectiviteit en het aantal componenten dat met één methode geanalyseerd kan worden. Voor screeningsmethoden zou derhalve een lagere selectiviteit volstaan, terwijl de selectiviteit van bevestigingsmethoden hoog moet zijn. Daarnaast hangt de vereiste selectiviteit af van de verwachte interferenties. In het geval van CAP-analyse is hoge selectiviteit nodig, maar in andere gevallen, zoals ceftiofuranalyse, dient de selectiviteit doelbewust verlaagd te worden om een effectieve monitoringsstrategie te verkrijgen. Hieruit wordt geconcludeerd dat bij methodeontwikkeling en -validatie, het van groot belang is de parameter selectiviteit in detail te bestuderen.

Dankwoord



Het is algemeen bekend dat het uitvoeren van een PhD-traject stressvol is. Deadlines, mislukte experimenten, lastige reviewers... Gelukkig heb ik de afgelopen jaren de druk die ik voelde enigszins weten te beperken. Echter, zodra de promotiedatum bekend werd, ontstond toch een licht gevoel van spanning in mijn onderbuik. Het gaat nu echt gebeuren...

Zoals zo vaak de laatste maanden, zit ik weer eens in de voorkamer achter de laptop aan tafel. Het is alweer donker buiten, de gordijnen dicht. De hond ligt snurkend op de bank en op de achtergrond hoor ik zacht het geluid van de televisie. Een documentaire of zoiets... Het grootste deel van het proefschrift ligt gelukkig al lang klaar, de opmaak is gedaan. Alles is geregeld. Op tafel liggen verschillende proefschriften, alle opengeslagen op één van de laatste pagina's. Ik kijk ernaar, de pendule tikt confronterend op de achtergrond. Terwijl ik inspiratie probeer te krijgen herinner ik me ineens de woorden van Ingrid: "Het dankwoord mag best met een beetje humor geschreven worden". Ik voel ineens die enorme last op mijn schouders; meer druk dan ik tijdens het gehele onderzoek en het schrijven van dit proefschrift heb ervaren. Ik kijk op en zie dat de hond echt eerst nog even naar buiten moet en ik loop een rondje dat net iets langer is dan normaal. Eenmaal terug, de hond weer op de bank, tuur ik afwisselend naar het scherm en naar de opengeslagen proefschriften. Ik lees nog eens vluchtig een dankwoord door. Eerst een kop thee. Ik sta op, loop naar de keuken en zet de waterkoker aan. Onderweg zie ik de modderpoten en haren van de hond op de vloer; daar moet toch echt eerst iets aan gebeuren, anders kan ik me niet concentreren. Een kwartier later zit ik weer aan tafel, een kop thee rechts en een handje rozijnen links van de laptop. Inspiratieloos. Het belangrijkste is dat ik niemand vergeet te noemen en iedereen in passende volgorde laat passeren, wat dat dan ook moge zijn. Maar humor... Ingrid bedankt! En ineens is zomaar de kop eraf.

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mij te hebben. Jullie hebben me alle ruimte gegeven mijn onderzoek zelfstandig uit te voeren, maar waren er altijd als ik reflectie nodig had. Michel, ik moet eerlijk zeggen dat ik even moest wennen aan jouw kritische blik en je ‘advocaat-van-de-duivel-aanpak’, maar ik ben er wel veel verder mee gekomen. Het heeft me gedwongen beter na te denken over keuzes die ik maakte en me geleerd mijn standpunten helderder te beargumenteren. Dat is iets wat ik in mijn verdere loopbaan zeker mee zal nemen. Ook wil ik jullie beiden bedanken voor jullie feedback op manuscripten en teksten voor dit proefschrift. Ondanks dat jullie enorm druk zijn, kreeg ik toch altijd onverwacht snel een uitgebreide reactie op mijn concepten. Altijd eerlijk en zonder er doekjes om te winden; althans, dat denk ik.

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Bjorn

About the author



Curriculum Vitae

Bjorn Berendsen was born on the 10th of March 1978 in Bommel, The Netherlands. In 1995 he finished his secondary education at Over-Betuwe College in Bommel. He started his Bachelor study, Higher Laboratory Education, at Hogeschool van Arnhem en Nijmegen. His thesis was on tetracycline analysis in milk and the in-line coupling of capillary electrophoresis with solid phase extraction, which was carried out at RIKILT. After obtaining his BSc degree with specialism Analytical Chemistry *cum laude* in 1999, he immediately got employed at RIKILT. After working as a BSc researcher for about ten years in the section of veterinary drug research, in January 2008 he started a part-time Master education in Chemistry, with specialism Analytical Chemistry at the University of Amsterdam in collaboration with the Free University Amsterdam. He conducted his MSc thesis at RIKILT and studied the degradation of cephalosporins. In April 2010 he obtained his MSc degree *cum laude*. Next to working as an MSc scientist at RIKILT, he started working on his PhD research at Wageningen University and was situated at RIKILT, Wageningen UR, of which this thesis is the result. Currently he works as an MSc scientist in the business unit veterinary drugs of RIKILT, Wageningen UR.

List of publications

Peer-reviewed papers related to this thesis:

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Overview of completed training activities

Discipline specific activities

International Symposium on Hormone and Veterinary Drug Residue Analysis, Ghent, Belgium (2010).

Mini-symposium Nieuwe Voedselveiligheidsrisico's, VLAG, Wageningen, The Netherlands (2010).

Symposium NWO Study Group Analytical Chemistry, Lunteren, The Netherlands (2010).

Course Advanced Food Analysis, VLAG, Wageningen, The Netherlands (2010).

Course Advanced Chromatography, Wageningen University, Organic Chemistry, Wageningen, the Netherlands (2011).

Symposium MS in Food and Feed, KVCV, Merelbeke, Belgium (2011).

Symposium Recent Advances in Food Analysis, Institute of Chemical Technology, Prague, Czech Republic (2011).

Symposium EuroResidue VII, Egmond aan Zee, The Netherlands (2012).

Symposium NWO Study Group Analytical Chemistry, Lunteren, The Netherlands (2012).

General courses

Writing for Academic Publication, L. McPhee (2008).

Schrijven voor beleid, Taalcentrum VU (2008).

Metabolomics in Plant Science, Leiden University (2010).

Praktisch leidinggeven, Boertiengroep (2010).

How to write a world class paper?, Elsevier BV, Wageningen UR (2010).

Training and coaching in quality of reports, Buro Spiff (2011).

Regie over de eigen loopbaan, Wageningen UR (2011).

Toegepaste statistiek: statistiek en modellering, WBS (2012).

Succesvol acquireren voor professionals, Boertiengroep (2012).

Optionals

Preparation of the research proposal (2010).

Seminars Organic Chemistry, Wageningen University, Wageningen (2010-2012).

PhD meetings, RIKILT, Wageningen (2011-2013).

Meetings expertise group veterinary drugs, RIKILT, Wageningen (2010-2013).

Training School for Advanced Residue Analysis: group A6 antibiotics, Nantes. The analysis of banned antibiotics in food (2010). Followed and assisted in practical sessions.

Training School for Advanced Residue Analysis: group A6 antibiotics, Cochin, India. The validation of methods for the analysis of veterinary drugs in food products (2010). Taught.

Training Saudi Arabia: group B antibiotics, RIKILT, Wageningen. The analysis of antibiotics using LC-MS/MS (2011). Taught.

Training School for Advanced Residue Analysis: group A6 antibiotics, Kiev, Ukraine. The analysis of anthelmintics in food products (2011). Taught.

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