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ORIGINAL PAPER

Multiresidue analysis of beta-agonists in bovine and porcine urine, feed and hair using liquid chromatography electrospray ionisation tandem mass spectrometry

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Abstract The use of β-agonists as growth promoters in cattle breeding is forbidden in many countries for reasons of fair trade and consumer protection. In recent years the use of liquid chromatography (LC) tandem mass spectrometry (MS/MS) has been shown to be the method of choice for the control of β-agonists. In this study an LC-MS/MS multiresidue analysis method is presented for trace analysis of 22 β-agonists. A truly generic concept has been designed based on mixed-mode solid-phase extraction and positive electrospray ionisation LC-MS/MS operated in the multiple reaction monitoring mode. This method allows application to a wide variety of sample matrices such as urine, feed and hair, following minor modifications to the analysis procedure only. The method features fit-for-purpose sensitivity in urine as shown by CCα and CCβ values of less than 0.2 and less than 0.5 μg/l respectively, for all β-agonists studied (terbutaline and reproterol, less than 0.3 and less than 1.0 respectively). Similar but semiquantitative application to feed and hair showed CCβ values of less than 10.0 and less than 5.0 μg/kg, respectively. A further simplification and improvement is demonstrated using Ultra Performance LC (UPLC™) and fast-switching MS/MS. The successful validation of this method following the latest EU requirements and its application to real samples demonstrate that a new versatile tool has been achieved for veterinary control of β-agonists.

Keywords Liquid chromatography · Mass spectrometry · Beta-agonists · Feed · Urine · Hair

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Introduction

The use of growth promoters for fattening purposes in cattle has been banned in the European Union since 1988 [1]. Residue analysis on target compounds is carried out by several control laboratories in order to protect the consumer, guarantee fair trade and enforce the ban [2]. Apart from steroids having androgenic, estrogenic or progestagenic activity, thyreostatics and β-agonists are banned as well [3, 4]. Especially in the case of β-agonists, health protection is an issue: several clinical cases of acute food intoxication following the consumption of liver or meat are documented, one of the clinical symptoms being heart disorders which can be fatal [5, 6]. According to a feed producers' magazine more than 300 people at a time were poisoned recently in China following the consumption of pork produced by pigs fed with a β-agonist as a feed additive [7]. Obviously there is a need for a multi β-agonist residue analysis method which is applicable to a wide variety of sample matrices such as feed, urine, hair and tissue. Radio and enzyme immunoassay screening tools were developed in the past and are still being used in many

countries for the control of β-agonists [8, 9]. A more sophisticated immunological approach based on a surface plasmon resonance (SPR) biosensor was presented by Traynor et al. [10]. However, both suffer from the limited degree of cross-reactivity of the antibodies used, covering a maximum of eight to 13 β-agonists only. Many more β-agonists are known from human medicine and moreover "black-market" chemists are highly active in designing structure analogues of β-agonists which cannot be traced by classic routine methods [11–13]. A more generic functional test based on the detection of smooth muscle relaxation of isolated trachea strips from guinea pigs might be considered but is rather laborious, relatively slow and not very sensitive [14]. Meenagh et al. [15] developed a competition binding assay for β-agonists based on solubilised human β_2-adrenoreceptors isolated from transfected Chinese hamster ovary cells and the tritiated antagonist dihydroalprenolol as a radioactivity label. Such an assay is able to detect novel derivatives which exhibit the same pharmacological action as β-agonists but have modified chemical structures. Unfortunately this receptor binding assay is not sensitive enough for the low levels encountered in urine and tissue samples. As an alternative gas chromatography–mass spectrometry (MS) and liquid chromatography (LC) tandem MS (MS/MS) methods are being used to monitor selected ions or multiple reaction monitoring (MRM) MS/MS transitions [16–22]. Huang et al. [23] even omitted the LC separation step by incorporating the selectivities of a polymeric monolith microextraction column and quadrupole time-of-flight MS in the analysis of three β-agonists in pork. The LC-MS/MS methods developed so far include either a smaller number of β-agonists [16–21] than the SPR biosensor assay [10], and/or show a limited scope of either calf or human urine and/or tissue applicability. In this study we developed a multimatrix multianalyte LC-MS/MS method for the residue analysis of β-agonists in feed, in bovine and porcine urine, and in hair. The method described comprises 22 β-agonists and three, isotope-labelled internal standards and was validated using the latest EU requirements [24]. According to these requirements the CCβ and CCα values must be established amongst other parameters. The detection capability CCβ is defined as "the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. 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 - β" [24]. The decision limit CCα is defined as "the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant" [24], or in other words, at which level are you sure that the sample really contains the substance.

Recently, Churchwell et al. [25] showed increased LC-MS/MS performance for a standard solution containing nine β-agonists using Ultra Performance LC (UPLC™) and a fast-switching triple-quadrupole MS/MS instrument. Following purchase of that instrument, we managed to further simplify and speed up our multi method and to present data on the applicability of Ultra Performance LC-MS/MS in real sample matrices.

Experimental

Chemicals

The chemicals and solutions used were of analytical-reagent grade. Water was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Brombuterol, clencyclohexerol, clenpenterol and clenproperol were obtained from the Community Reference Laboratory BVL (Berlin, Germany). Clenbuterol chloride, fenoterol bromide, procaterol chloride, isoxsuprine and salbutamol sulphate were obtained from Sigma (St. Louis, MO, USA). Carbuterol chloride was obtained from Warner-Lambert (Belgium). Mabuterol chloride and mapenterol chloride were from Rephartox (Lelystad, The Netherlands). Terbutaline sulphate was obtained from Bufa (Uitgeest, The Netherlands). Cimbuterol, cimaterol, ractopamine and tulobuterol were a gift from F. André (ENVN, Nantes, France). Salmeterol was a gift from A. Bast (Free University, Amsterdam, The Netherlands). Clenhexyl chloride was a gift from D. Courtheyn (FAVV, Ghent, Belgium). Zilpaterol was a gift from E. van der Vlis (TNO, Zeist, The Netherlands). Reproterol chloride was from laboratory stock. The designer β-agonist clenbuterol-R was obtained from a purified feed extract [12]. The isotope-labelled internal standards clenbuterol-d_6 and salbutamol-d_6 were custom-synthesised at Wageningen University and Research Centre (Wageningen, The Netherlands); ractopamine-d_5 chloride was obtained from the Community Reference Laboratory BVL. Stock solutions of the β-agonists were prepared in methanol at 1 mg/ml. β-Glucuronidase/arylsulphatase (from *suc Helix pomatia*) was from Merck (Darmstadt, Germany). Bondelut Certify mixed-mode solid-phase extraction (SPE) columns (300 mg) were from Varian (Harbor City, CA, USA).

Sample materials

In the validation studies 25 independent blank bovine and porcine urine samples and 20 blank bovine hair samples from different animals were used, as well as 20 blank feed samples. Incurred bovine urine and hair samples were obtained from a veterinary control programme.

Sample preparation

Urine

Urine sample, blanks and controls (2 ml) were spiked with the internal standard mixture at 2 ng/ml. The control urine samples were prepared by spiking blank urine samples with the β-agonist mixture at 0.125, 0.25, 0.50, 1.0 and 2.0 ng/ml, except for clenbuterol, which was spiked at two-fold lower levels. Following the addition of 3 ml of 0.25 M sodium acetate buffer pH 4.8 and 10 μl of β-glucuronidase/arylsulphatase, enzymatic deconjugation was carried out in a water bath at 55 °C for 2 h. Next, the hydrolysed sample was subjected to SPE on a mixed-mode column, previously conditioned by methanol and sodium acetate buffer. The vacuum-dried SPE column was washed subsequently with 1 ml of 1 M acetic acid, 6 ml of methanol and 2 ml of acetone/chloroform (1:1). Finally the contents of the dried SPE column were eluted with 7.5 ml of 3% ammonia in ethyl acetate and the eluate was evaporated to dryness under a stream of nitrogen gas at 40 °C. The residue obtained was redissolved in 50 μl of acetonitrile and following the addition of 250 μl of 0.24% formic acid 50 μl was injected into the LC-MS/MS system.

Feed

Two 2.5-g portions of homogenised feed sample, blanks and controls were spiked with the internal standards at 24 ng/g. In addition, one of these portions was spiked with the β-agonist standard mixture at 10 ng/g (clenbuterol at 5 ng/g). Following a 15 min equilibration, to both portions 25 ml of 0.2 M phosphoric acid/methanol (1:1) was added. Next, the mixtures were shaken for 30 min in a head-over-head apparatus and centrifuged for 10 min at 2,800 *g*. Finally 1.6 ml of the supernatants was transferred to individual test tubes, 5 ml of 0.25 M sodium acetate buffer pH 4.8 was added and the extracts were further subjected to SPE as described for urine samples.

Hair

Two 500-mg portions of hair sample previously cut to less than 2 cm, blanks and controls were spiked with the internal standard ractopamine-d_5 at 10 ng/g. In addition, one of these portions was spiked with the β-agonist standard mixture at 5 ng/g. The control hair samples were prepared by spiking blank hair with the β-agonist standard mixture at 0, 2.5, 5.0, 10, 20 and 50 ng/g. Following the addition of 4 ml NaOH (1.0 M) solution, digestion was carried out in a water bath at 65 °C for 2 h. Next, 3.5 ml of HCl (1.0 M) and 5 ml of 0.25 M sodium acetate buffer pH 4.8 were added and the test tubes were centrifuged for 10 min at 2,000 g. The supernatants were transferred to a clean test tube, adjusted to pH 4.8 if necessary, and centrifuged again for 10 min at 2,000 g. The supernatants thus obtained were further subjected to SPE as described for urine samples.

Liquid chromatography–mass spectrometry

Conventional LC-MS/MS

The LC-MS/MS system consisted of a Waters (Milford, MA, USA) model Alliance 2690 LC system equipped with a Micromass (Manchester, UK) model Quattro Ultima triple-quadrupole mass spectrometer. The mass spectrometer was operated in the positive electrospray ionisation (ESI) mode at a capillary voltage of 2.7 kV, a desolvation temperature of 300 °C, a source temperature of 120 °C and a cone voltage of 35 V. The desolvation gas was nitrogen and the collision-induced dissociation (CID) gas was argon. For each analyte, the most abundant MRM transition was acquired using the conditions given in Table 1. Because of

Table 1 Positive ion tandem mass spectrometry (MS/MS) conditions for the multiple reaction monitoring (MRM) acquisition of β-agonists

Component	Precursor ion mass (*m*/*z*)	Product ion mass (*m*/*z*)	Collision energy (eV)
Cimaterol	220.1	160.1	15
Terbutaline	226.1	107.0	25
Cimbuterol	234.2	160.1	15
Salbutamol	240.2	148.1	20
Salbutamol-d_6	246.2	148.1	20
Clenproperol	263.1	203.0	20
Clenbuterol	277.1	203.0	15
Clenbuterol-d_6	283.1	204.0	15
Clenbuterol-R	471.2	415.1	20
Clenpenterol	291.1	203.0	17
Mabuterol	311.1	237.0	15
Mapenterol	325.1	237.0	15
Brombuterol	365.0	290.9	20
Zilpaterol	262.2	185.1	22
Carbuterol	268.2	134.1	25
Procaterol	291.2	231.1	20
Fenoterol	304.2	135.1	20
Reproterol	390.2	221.2	22
Tulobuterol	228.1	154.0	15
Isoxsuprine	302.2	150.1	20
Ractopamine	302.2	164.1	15
Ractopamine-d_5	307.2	167.1	17
Clenhexyl	303.1	203.0	20
Clencyclohexerol	319.1	203.0	20
Salmeterol	416.3	232.2	20

the limitation in the number of concurrent MRM transitions with this instrument and because of the coelution tendency of β-agonists in general, two separate injections from each sample extract had to be performed in order to cover all 22 analytes and three internal standards. The analytical column was a 150 mm×3.5-mm internal diameter 5-μm C18 Alltima (Alltech, Breda, The Netherlands) column, kept in a column oven at 30 °C. The two mobile phases used consisted of 100:0.2 water/formic acid (solvent A) and 100:0.2 acetonitrile/formic acid (solvent B) and the flow rate was 0.4 ml/min. Following a 5-min isocratic period at 0% solvent B, a linear gradient was started towards 100% solvent B at 20 min, and was kept at that composition until 20.5 min. The liquid chromatograph was connected to the ESI MS/MS instrument using a 1:2 flow split.

The concentrations of β-agonists in urine samples were calculated using the isotope dilution method and a linear calibration graph was constructed from peak areas relative to that of the internal standard obtained with blank and spiked control samples prepared in urine (matrix-matched standards, MMS). The response of the clenbuterol-d_6 internal standard was used, except for the concentrations of salbutamol and ractopamine, which were calculated using the internal standards salbutamol-d_6 and ractopamine-d_5, respectively. The concentrations of β-agonists in feed samples were estimated using the isotope dilution method and standard addition of 10 ng/g of each β-agonist (clenbuterol at 5 ng/g) to each individual sample. The concentrations of β-agonists in hair samples were estimated using the isotope dilution method and standard addition of 5 ng/g of each β-agonist to each individual sample.

Ultra performance LC-MS/MS

The Ultra Performance LC-MS/MS system consisted of a Waters model Acquity UPLC™ system equipped with a Quattro Premier XE triple quadrupole mass spectrometer. The mass spectrometer was operated in the positive ESI mode at a capillary voltage of 2.0 kV, a desolvation temperature of 350 °C, a source temperature of 120 °C and a cone voltage of 20 V. The desolvation gas was nitrogen and the CID gas was argon. The injection volume was 40 μl and the chromatographic separation was performed at 40 °C using an Acquity (Waters) BEH-C18 column (100 mm×2.1-mm internal diameter,1.7-μm particle size). The two mobile phases used consisted of 100:0.1 water/formic acid (solvent A) and 100:0.1 acetonitrile/formic acid (solvent B) and the flow rate was 0.4 ml/min. Following a 1-min isocratic period at 0% solvent B, a linear gradient was started towards 35% solvent B at 8.0 min (or 9.5 min in the case shown in Fig. 4) and 100% solvent B at 8.5 min (or 9.8 min in the case shown in Fig. 4), and was kept at that composition until 12.8 min. The liquid chromatograph was connected to the ESI MS/MS instrument without a flow split.

Quality assurance and validation

In all cases, standards, reagent blanks, matrix blanks and spiked matrix control samples were analysed in the same series. Run acceptance criteria for system stability, minimum sensitivity, recovery of internal standards, recovery of the standard addition or the linearity of the matrix-matched control standards (where applicable) must be fulfilled for each series. Accuracy results from the urine samples spiked with 0.25 ng/ml clenbuterol and 0.5 ng/ml clenproperol and salbutamol were plotted on a long-term quality control chart. The validation study for the quantitative screening of β-agonists in urine was carried out at three concentration levels by the analysis of bovine and porcine urine samples spiked with 1.0, 1.5 and 2.0 ng/ml of each β-agonist, except for clenbuterol, which was spiked at twofold lower levels. To each urine sample 2.0 ng/ml of the internal standard mixture was added, and six replicates of each sample were analysed on three different days. From the data thus obtained, within-day repeatability, within-laboratory reproducibility and accuracy were calculated. By doing so, we included robustness data in the reproducibility results. Furthermore it should be underlined that the ruggedness was monitored in each individual urine analysis by monitoring the signal intensity of the deuterated internal standards. The recovery of the overall sample preparation procedure was determined on two different days by the analysis of bovine and porcine urine samples spiked at 1.0 ng/ml just before the sample preparation (clenbuterol at 0.5 ng/ml) and addition of the internal standard just before injection into the LC-MS/MS system, i.e. internal standard correction for injection volume and ion suppression only. The linearity was determined for a limited concentration range since the analytes of interest are banned substances for which no safe maximum residue limits have been established. To this end, urine samples spiked at 0.0, 0.125, 0.25, 0.50, 1.0 and 2.0 ng/ml, except for clenbuterol, which was spiked at twofold lower levels, were analysed on three different days.

The validation study for the qualitative screening of β-agonists in feed was carried out by the analysis of 20 different feed samples with and without adding each β-agonist at a level of 10 μg/kg (except for clenbuterol, which was added at a level of 5 μg/kg). From the data thus obtained, CCβ and the specificity were determined. Because the analysis of β-agonists in feed is very similar to the analysis of β-agonists in urine, it was not necessary to determine the robustness.

The validation study for the qualitative confirmation of β-agonists in hair was carried out by the analysis of 20

different bovine (calf) hair samples with and without adding each β-agonist at a level of 5.0 μg/kg. To each hair sample, 10 μg/kg of the internal standard mixture was added, and the total set of samples was analysed on three different days. From the data thus obtained, CCα, CCβ and the specificity were determined. Robustness was determined by analysing one hair sample with the addition of each β-agonist and preparing this sample in 18-fold with minor changes in the sample preparation. The linearity was determined by the analysis of hair samples spiked at 0.0, 2.5, 5.0, 10, 20 and 50 μg/kg, which were analysed on four different days.

Results and discussion

General considerations

The molecular structures of the β-agonists studied include aniline and phenol derivatives and more complex structures

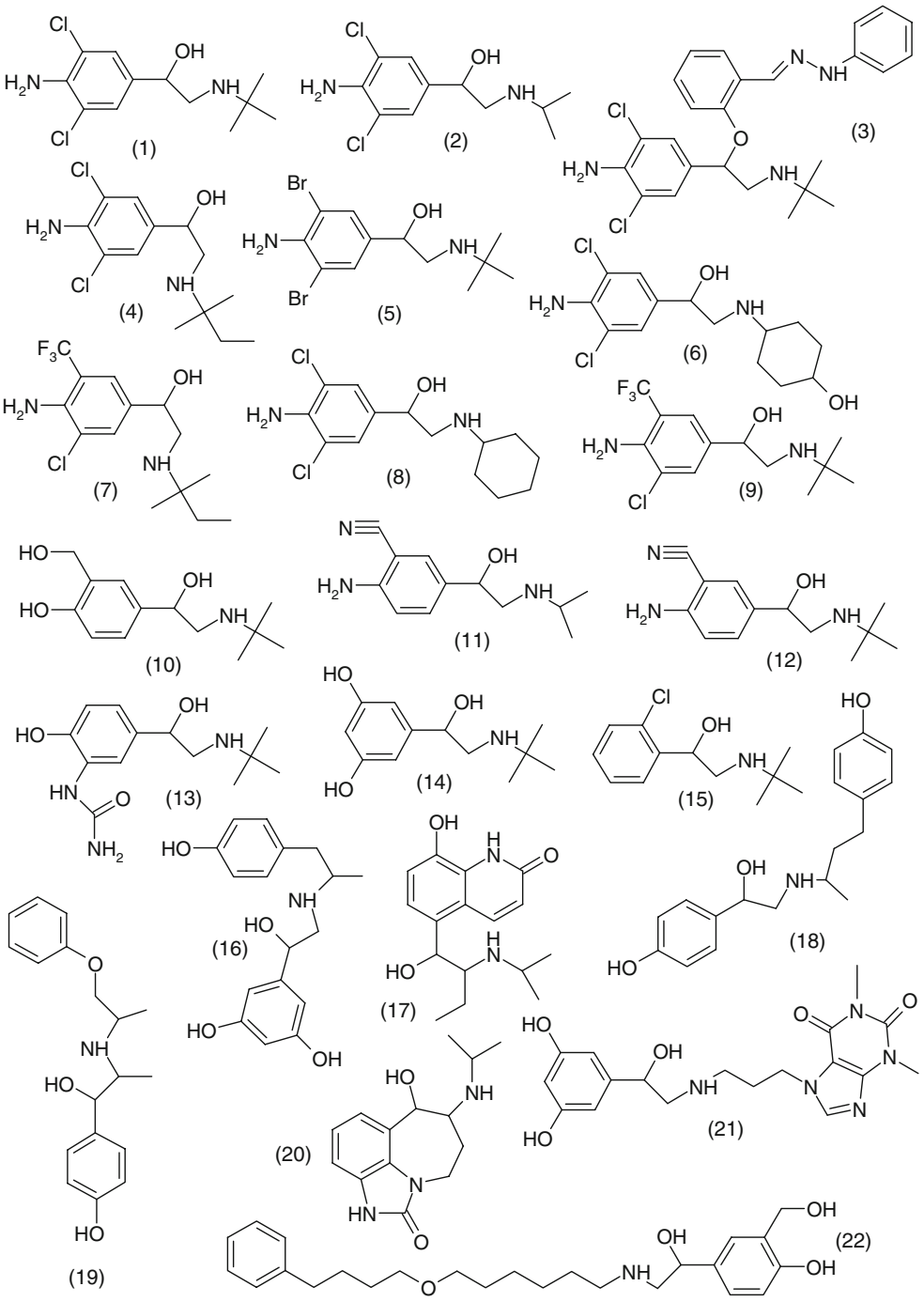

Fig. 1 Structures of the β-agonists studied: *1* clenbuterol, *2* clenproperol, *3* clenbuterol-R [12], *4* clenpenterol, *5* brombuterol, *6* clencyclohexerol, *7* mapenterol, *8* clenhexyl, *9* mabuterol, *10* salbutamol, *11* cimaterol, *12* cimbuterol, *13* carbuterol, *14* terbutaline, *15* tulobuterol, *16* fenoterol, *17* procaterol, *18* ractopamine, *19* isoxsuprine, *20* zilpaterol, *21* reproterol, *22* salmeterol

such as zilpaterol (Fig. 1). From these diverse structures it is obvious that a single antibody can cover only a limited number of them; possibly the development of a multiplex immunological screening assay might become an alternative for LC-MS/MS multiresidue analysis in the future. Classic triple-quadrupole MS/MS instruments are limited in the number of concurrent MRM acquisitions. For the present number of β-agonists this problem was solved by performing two injections of each sample extract using the same LC gradient but acquiring a different set of ion transitions. The ion transitions given in Table 1 provide adequate sensitivity and yield 2.5 identification points according to [24]. A common fragmentation mechanism for β-agonists is the loss of the alkyl group from the secondary amine and the loss of water, yielding a stable unsaturated primary amine ion and a neutral loss of *m*/*z* 116, 100, 88, 74 or 60 for the cyclohexerol, hexyl, pentyl, butyl or propyl derivatives, respectively. Salbutamol and salmeterol show a second loss of water and carbuterol shows an additional loss of urea. In the case of reproterol the substituent at the secondary amine keeps the positive charge, while ractopamine and isoxsuprine show a neutral loss consisting of the phenolic part of the molecule. Once a specific β-agonist is suspected in this initial screening procedure, the same method can be simply adjusted to a confirmatory analysis method by replacing one or more of the MRM transitions of apparently irrelevant β-agonist(s) by a second or more MRM transitions for the suspected β-agonist of interest. By doing so, one can easily achieve the minimum number of 4.0 identification points for confirmation of identity.

With the aim of a multianalyte multimatrix method, the issue of matrix-matched standards (MMS) for validation and quantification should be discussed. Urine is a homogeneous sample matrix and has a relatively constant background composition. By selecting an appropriate set of blank calf, bovine and porcine urine samples, one can easily validate the method using MMS according to the EU requirements [24] and it is justified to screen samples quantitatively using an external multipoint MMS calibration curve. Still it should be kept in mind that ideally each individual β-agonist would have its own isotope-labelled analogue as an internal standard; the three internal standards in the present method are a compromise in that respect and will not correct completely for recovery loss, ionisation suppression or ionisation enhancement. For feed the situation is quite different since the actual background composition of a specific feed sample might differ

Table 2 Validation data for the quantitative screening of clenbuterol and 20 other β-agonists in urine by liquid chromatography (LC)–MS/MS

Analyte	**Clenbuterol**			**20 other β-agonists**		
Spike level (μg/l)	0.5	1.0	0.75	1.0	1.5	2.0
Repeatability (RSD,%)	6	4	3	5 – 24	3 – 26	3 – 16
Within-lab reproducibility (RSD,%)	14	11	14	6 – 30	5 – 32	5 – 25
Accuracy (%)	101	98	101	91 – 110	88 – 111	85 – 108
Recovery (%) at 1.0 μg/l, 0.5 μg/l for clenbuterol	76			54 – 85		
Linearity (r^2, 0.15 – 2.0 μg/l, 0.0625 – 1.0 μg/l for clenbuterol)	≥0.990					
CCα (μg/l)	0.02			0.01 – 0.28		
CCβ (μg/l)	0.04			0.09 – 0.99		
Specificity	Passed					
Ruggedness	Passed					

Validation parameters according to [24]; other conditions, see the text
RSD relative standard deviation

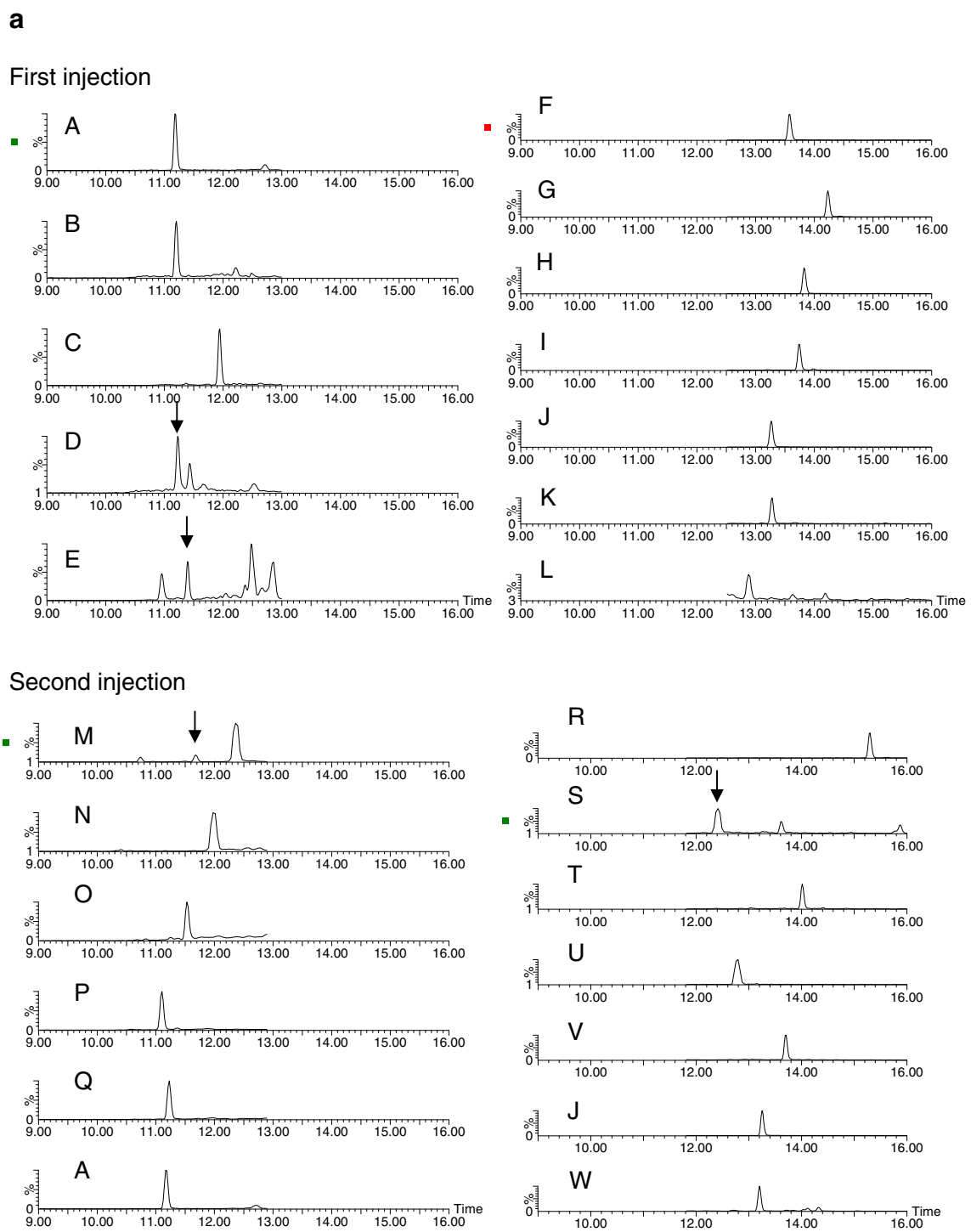

Fig. 2 Reconstructed multiple reaction monitoring (MRM) chromatograms for the screening of β-agonists in a control bovine urine sample spiked at 1.0 μg/l and analysed by **a** two injections in a conventional liquid chromatography (LC)–tandem mass spectrometry (MS/MS) system and **b** one injection in an Ultra Performance LC (UPLC™) MS/MS system represented by reconstructed MRM overlays. The MRM ion transitions are given in Table 1. *A* salbutamol-d*6*, *B* salbutamol, *C* cimbuterol, *D* terbutaline, *E* cimaterol, *F* brombuterol *G* mapenterol, *H* mabuterol, *I* clenpenterol, *J* clenbuterol-d_6; *K* clenbuterol, *L* clenproperol, *M* reproterol, *N* fenoterol, *O* procaterol, *P* carbuterol, *Q* zilpaterol, *R* salmeterol, *S* clencyclohexerol, *T* clenhexyl, *U* ractopamine, *V* isoxsuprine, *W* tulobuterol

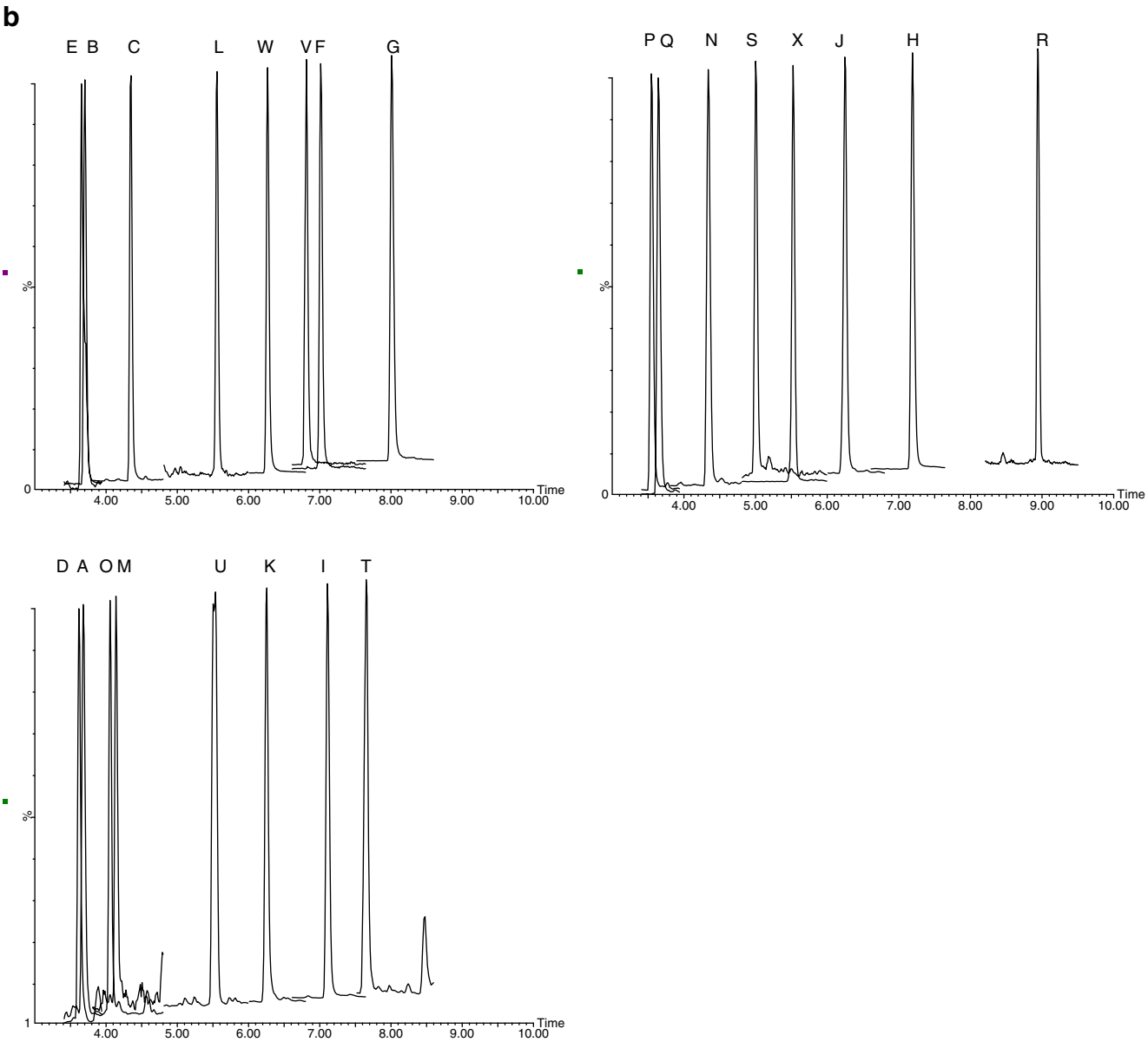

Fig. 2 (continued)

significantly from that of the feed samples used during validation and from that of the MMS used for quantification. Incomplete recovery and ionisation suppression are likely to occur and only three internal standards are available to indicate the potential occurrence of false-compliant screening results. Therefore, we do not recommend using the external multipoint MMS calibration approach for feed. Alternatively we propose a one-point standard addition procedure in which each sample under investigation is split into two portions, one being analysed straight away and the second being analysed following the addition of the standard mixture of β-agonists. In this way, each specific feed sample under investigation allows a realistic check for false compliants due to incomplete recovery and/or ionisation suppression. Moreover, a semi-quantitative screening result is obtained. According to Gratacós-Cubarsí et al. [26] hair samples should be also considered as a complex matrix differing significantly in composition. Again, because of the increased risk of incomplete recovery and ionisation suppression and the limited number of labelled internal standards we also prefer the standard addition approach in the case of hair.

Validation studies

According to [24] the following performance characteristics must be determined for a quantitative screening method: detection capability CCβ, precision, selectivity/specificity and ruggedness/stability. However, following an initial multi β-agonists screening, confirmatory analysis of the

suspect sample will be required. In that case also the decision limit CCα and the trueness/recovery validation parameters must be assessed. At the beginning of the validation study the specificity was checked by the analysis of 23 urine samples from different bovines and porcines. No interference on the β-agonist screening was found owing to the highly specific MRM acquisition method and the use of an appropriate deuterated internal standard. The validation results for the quantitative analysis in urine are summarised in Table 2. Despite the fact that only three isotope-labelled internal standards were available the accuracy is 85–111% in all cases. All β-agonists show CCβ values less than 0.5 ng/ml, except terbutaline and reproterol (less than 1.0 ng/ml), and all MMS calibration curves show a correlation coefficient higher than 0.990. From Table 2 it can be concluded that the method developed shows relevant CCα and CCβ values, good precision, accuracy and linearity, and fulfils the validation criteria set for quantitative residue analysis methods [24]. Note that no quantitative validation data are presented for the designer β-agonist clenbuterol-R because of the lack of a sufficient amount of a high-purity standard. However, it

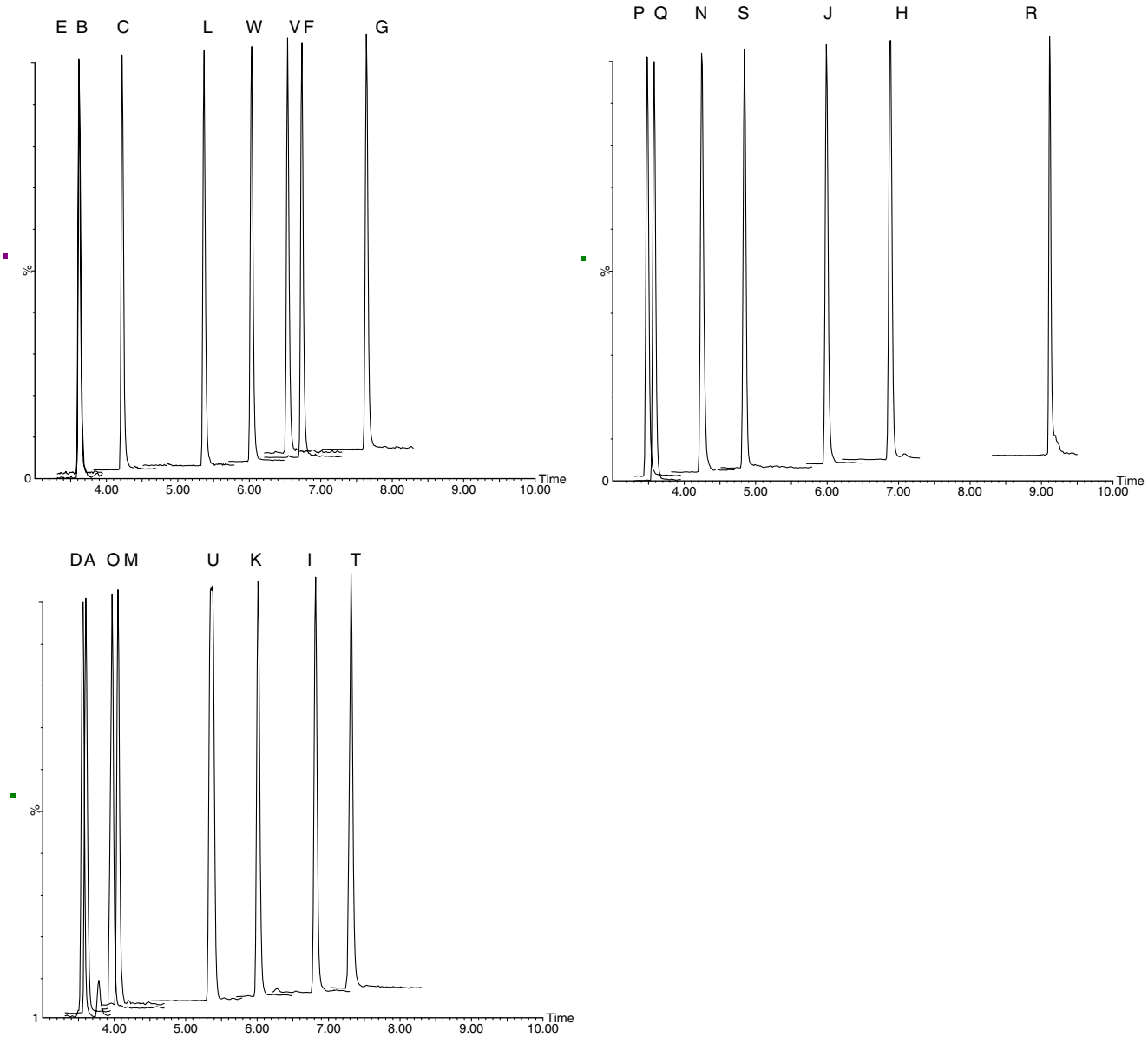

Fig. 3 Reconstructed MRM chromatograms for the screening of β-agonists in a control pig feed sample spiked at 10 μg/kg with the β-agonist mixture from Fig. 2, and analysed by Ultra Performance LC (UPLC™) MS/MS. The MRM ion transitions are given in Table 1. For an explanation of the labelling, see the legend to Fig. 2

Table 3 Confirmation data of a salmeterol suspect in urine and hair analysed by Ultra Performance LC-MS/MS

Matrix/analyte	RRT_{exp} [a]	RRT_{req}	RRT passed	Ion $ratio_{exp}$ [b]	Ion $ratio_{req}$	Ion ratio passed
Urine salmeterol	1.68	1.63–1.72	y	0.71	0.56–0.84	y
Hair salmeterol	1.69	1.65–1.73	y	0.66	0.60–0.89	y

For conditions, see the text
RRT relative retention time
[a] RRT versus clenbuterol-d_6 internal standard
[b] Area ratio of product ions from reconstructed MRM chromatograms

has been shown previously that a urine level of approximately 0.5 ng/ml (expressed as clenbuterol equivalents) was sufficient for successful confirmation of identity [12].

The analysis procedure for the other matrices of interest i.e. feed and hair is very similar (see "Experimental"). Because of the use of the semiquantitative single-point standard addition procedure advocated above, these applications were validated as qualitative screening (feed) and qualitative confirmation (hair) methods. According to [24] the following performance characteristics must be determined for a qualitative screening method: detection capability CCβ, selectivity/specificity and ruggedness/stability. At the beginning of the validation studies for feed and hair, the specificity was checked by the analysis of 20 feed samples and 20 bovine (calf) hair samples. No interference on the β-agonist screening was found owing to the highly specific MRM acquisition method. Interferences are inherently checked in each individual sample owing to the standard addition procedure and the stability is checked in each analysis series. The CCβ in feed was less than 10 μg/kg, except for clenbuterol (less 5 μg/kg). When β-agonists are illegally applied as growth promoters the expected level of a β-agonist in feed will be in the milligram per kilogram range, i.e. the sensitivity of the feed method is also adequate for the detection of carryover and/or residues in the feed supply following discontinued application. The CCβ in hair was less than 5 μg/kg. β-Agonists are expected to accumulate in hair owing to a "multiple pool" model which includes (but is not restricted to) passive diffusion from the bloodstream into the growing cells at the base of the hair follicle [26]. For example in a study by Gaillard et al. [27] the levels of clenbuterol found in real samples ranged from 20 to 4,372 μg/kg; consequently the CCβ values obtained in the hair method were considered realistic and fit for purpose.

Ultra performance LC-MS/MS

A serious drawback of multiresidue analysis using a conventional LC-MS/MS instrument is the restricted num-

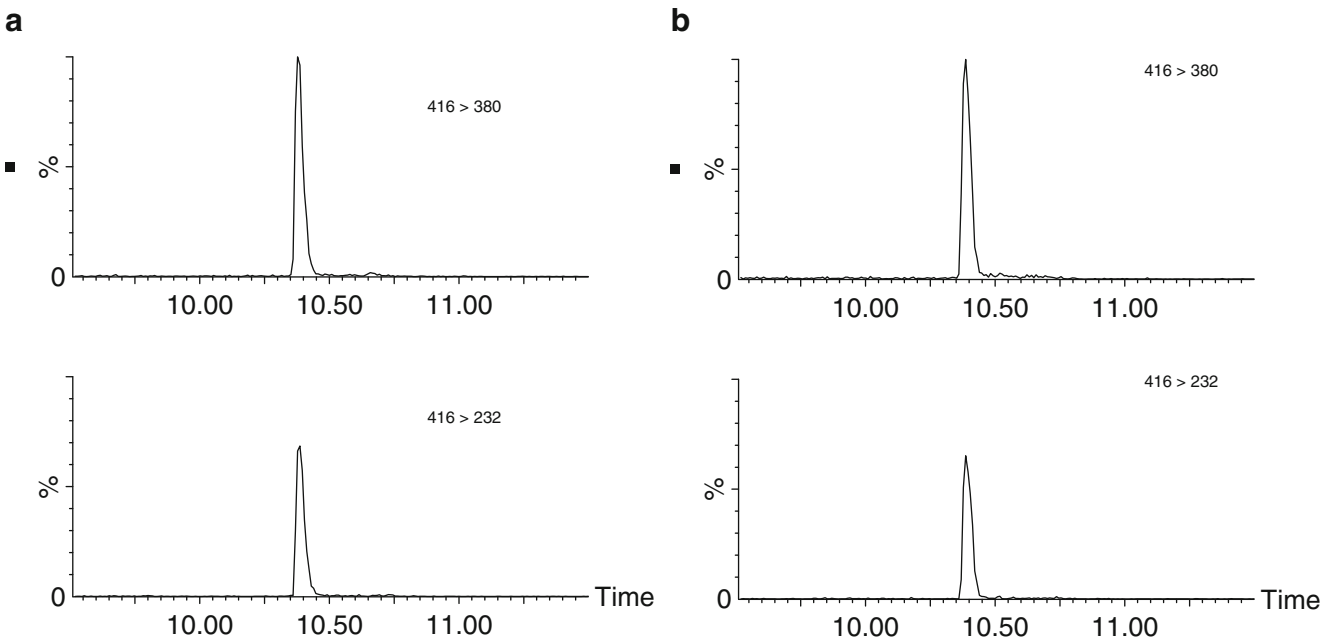

Fig. 4 Reconstructed MRM chromatograms for the confirmatory analysis of the β-agonist salmeterol in **a** a noncompliant urine sample and **b** a noncompliant hair sample, analysed by Ultra Performance LC (UPLC™) MS/MS

ber of MRM transitions which can be concurrently acquired. Consequently in the procedure each sample must be injected twice, thereby doubling the analysis time and no additional MRM transitions can be added, either to extend the scope to more β-agonists, or to allow for second MRM transitions in order to comply with screening and confirmatory analysis requirements in a single run. Recently an Ultra Performance LC system (UPLC™) and a fast-switching triple-quadrupole MS/MS instrument have been installed and indeed many more MRM transitions can be acquired now, allowing multi β-agonist screening by one injection only, without compromising the sensitivity. Moreover, the UPLC™ system provides a much faster LC separation, thereby reducing the runtime to less than 15 min. Figure 2 shows a comparison of a 1 μg/l spiked control bovine urine sample analysed by conventional LC-MS/MS requiring two injections, and by Ultra Performance LC-MS/MS in just one injection. Apart from the overall increase in speed the chromatographic resolution and peak shape improved considerably, yielding an improved signal-to-noise ratio, thanks to the high-resolution column. Figure 3 shows a 10 μg/kg spiked control pig feed sample analysed by Ultra Performance LC-MS/MS. Even in this highly complex sample matrix an excellent signal-to-noise ratio is obtained for all β-agonists in a single injection and at a fast runtime.

Applicability in veterinary control

The multianalyte multimatrix procedure developed has been applied to calf, bovine and porcine urine, and to a wide variety of dry and wet feed samples within the scope of national control and monitoring programmes; more recently the method has been applied to hair analysis as well. So far this β-agonist screening method has been shown to be extremely robust: the percentage of reanalysis caused by noncompliance with the run-acceptance criteria is lower than 5%. In general, findings of incurred samples being noncompliant for one or more β-agonists are rare; two examples are given here for residues of salmeterol. In the screening, the samples were suspect by showing a clear signal in the reconstructed MRM chromatogram at the correct retention time. Next, the confirmation of identity according to [24] was demonstrated by reanalysis of the suspect urine and hair samples. In order to achieve this a second MRM ion transition (m/z 416.4 > m/z 380.3) was added to the MS/MS acquisition method. The precursor ion and two product ions recorded in MRM mode yielded the minimum requirement of four identification points. The results are given in Table 3 and show that all identification criteria were fulfilled, thus yielding noncompliant results [24]. The concentrations in the incurred urine (Fig. 4a) and hair (Fig. 4b) samples were 0.6 μg/l and approximately 4 μg/kg, respectively.

Conclusions

A truly multianalyte multimatrix robust LC-MS/MS residue analysis concept has been developed and validated for β-agonists in urine, feed and hair at relevant levels. The method is suitable for both conventional LC-MS/MS screening as well as the most recent Ultra Performance LC (UPLC™) MS/MS instruments, the latter showing the benefit of having screening results from a single injection, faster chromatography, improved peak shape and signal-to-noise ratio, and the possibility of additional ion transitions for other existing or emerging β-agonists in veterinary control.

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