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DEVELOPMENT AND VALIDATION OF A NEW METHOD FOR DETERMINING NITROFURAN METABOLITES IN BOVINE URINE USING LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

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ABSTRACT

Background. The use of nitrofurans as veterinary drugs in food-producing animals is banned throughout the European Union. Nevertheless, nitrofuran metabolites have been detected not only in animal products, but also in bovine urine. At present there are no methods yet published for the simultaneous detection of nitrofuran metabolites in bovine urine.
Objectives. To develop and validate a method for determination of four key nitrofuran metabolites in bovine urine.
Material and methods. The four nitrofuran metabolites (nitrofurantoin, furazolidone, nitrofurazone and furaltadone), were determined in bovine urine using LC-ESI-MS/MS. The procedure required an acid-catalysed release of protein-bound metabolites, followed by their in situ conversion into 2-nitrobenzaldehyde (NBA) derivatives. The sample clean-up was performed using a polymer extraction cartridge before hydrolysis. Nitrofuran metabolites were then determined using electrospray ionization in the positive mode, that had previously been separated on a Phenomenex Luna C-18 column.
Results. The method was validated in accordance with the procedure outlined in the Commission Decision No. 2002/657/EC. Urine samples were spiked with nitrofuran metabolite solutions at levels of 0.5, 1.0, 1.5 and 2.0 μg/kg. Recoveries ranged between 90 – 108% (inter standard-corrected), with a repeatability precision (RSD) of less than 19% for all four analytes. The decision limit (CC) and detection capability (DC) were obtained from a calibration curve and lay respectively within the following ranges: 0.11 – 0.34 μg/kg and 0.13 – 0.43 μg/kg.
Conclusions. The developed and validated LC-ESI-MS/MS method allows four nitrofuran metabolites to be identified and quantitated in bovine urine. This analytical procedure meets the criteria defined in the Commission Decision No. 2002/657/EC.

Key words: *bovine urine, nitrofuran metabolites, residues, LC-ESI-MS/MS*

STRESZCZENIE

Wprowadzenie. Unia Europejska zabroniła stosowania nitrofuranów jako leków weterynaryjnych u zwierząt, których tkanki przeznaczone są do spożycia. Metabolity nitrofuranów są wykrywane zarówno w produktach pochodzenia zwierzęcego jak i moczu. Brak opublikowanej metody pozwalającej na jednoczesne oznaczanie metabolitów nitrofuranów w moczu bydlęcym.
Cel. Celem badań było opracowanie i zwalidowanie metody analitycznej pozwalającej na oznaczanie czterech metabolitów nitrofuranów w moczu bydlęcym.
Materiał i metoda. Metatabolity nitrofuranów (nitrafurantoiny, furazolidonu, nitrofurazonu i furaltadonu) były oznaczane w moczu bydlęcym metodą LC-ESI-MS/MS. Procedura wymaga stosowania hydrolizy w środowisku kwaśnym w celu rozerwania wiązania białko-metabolit, a następnie przeprowadzenia metabolitów w pochodne z 2-nitrobenzaldehydem. Oczyszczanie próbek prowadzono przed hydrolizą na kolumienkach ze złożem polimerycznym. Metabolity nitrofuranów oznaczane były przy zastosowaniu jonizacji poprzez elektrorozpraszanie w polaryzacji dodatniej, po wcześniejszym rozdzielaniu na kolumnie Phenomenex Luna C-18.
Wyniki. Metoda została zwalidowana zgodnie z decyzją Komisji nr 2002/657/WE. Próbki moczu były wzbogacone roztworem metabolitów nitrofuranów na poziomach odpowiadających 0,5, 1,0, 1,5, i 2,0 μg/kg. Odzysk mieścił się w zakresie 90 – 108%, współczynnik powtarzalności (RSD) był mniejszy niż 19% dla wszystkich czterech związków. Decyzyjną wartość graniczną (CCα) oraz zdolność wykrycia (CCβ) wyznaczono przy użyciu krzywej wzorcowej. Mieszczą się one w zakresie 0,11 – 0,34 μg/kg oraz 0,13 – 0,43 μg/kg.
Wnioski. Opracowana i zwalidowana metoda LC-ESI-MS/MS daje możliwość jakościowego i ilościowego oznaczania czterech metabolitów nitrofuranów w moczu bydlęcym. Procedura analityczna spełnia wymagania decyzji Komisji nr 2002/657/WE.

Słowa kluczowe: *mocz wołowy, metabolity nitrofuranów, pozostałości, LC-ESI-MS/MS*

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INTRODUCTION

Nitrofurans (NFs) are widely used as antibacterial agents in feed additives for treating gastrointestinal infections such as bacterial enteritis caused by *Escherichia coli* and *Salmonella* in cattle, pigs, farm reared poultry, fish and shrimps. The most common NFs are nitrofurantoin, furazolidone, nitrofurazone and furaltadone. After administration to animals, these nitrofurans are rapidly metabolized *in vivo* to more stable products. Thus, the analysis of NFs in biological samples is based on the determination of their metabolites. The marker residues for the nitrofurantoin, furazolidone, nitrofurazone and furaltadone are correspondingly; 1-aminohydantoin (AHD), 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM) and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) [12].

The EU has however banned NFs from being used in veterinary drugs in animals intended for human consumption because of their proven carcinogenic and genotoxic effects. According to Commission Decision No. 2003/181/EC [3], the minimum required performance level (MRPL) has been set at 1 µg/kg for each of these NF metabolites in poultry meat and aquaculture products, but not up till now in urine. In Poland, NF metabolites are monitored by the authorities as part of the official control on veterinary drug residues.

NFs and their metabolites are excreted mainly by the animal liver and kidneys. Indeed, AOZ concentrations highly correlated (r>0.97) between the liver and kidneys of pigs after the administration of furazolidone at 400 mg/kg in feed for 7 days. The urinary AOZ half-life was 14.7 days [7]. These results thereby demonstrated that urinary AOZ can provide the basis for monitoring the illegal use of furazolidone in pigs without slaughter, i.e. by non-invasive means.

To date, several LC-ESI-MS/MS methods have been reported for determining the four NF metabolites in various biological matrices (e.g. muscles, eggs, honey, milk and plasma) [2, 8, 9, 10, 11], and all involve simultaneous acid hydrolysis and derivatisation of the NF residues with 2-nitrobenzaldehyde (NBA). The differences between the methods are in the sample clean-up. So far however, there are no LC-ESI-MS/MS methods for determining all four metabolites in animals' urine. Currently, only single, evaluated analytical methods are available allowing measurement of single NF metabolites in animal urine. Two of them, high performance liquid chromatography (HPLC) with UV detection [1] and an immuno-chromatographic method have estimated AHD [13], with a limit of detection (LOD) >10 µg/l. The sensitivity of these methods was nevertheless insufficient to reach the MRPL of 1 µg/kg. A third method using enzyme-linked immunoassay (ELISA) was also developed for the determination of AOZ in urine which had an LOD of approximately 1.0 µg/l.

The aim of this study was to therefore develop and validate a method for simultaneously determining the aforementioned metabolites, i.e. – AHD, AOZ, SEM and AMOZ in bovine urine using a LC-ESI-MS/MS technique. The method involved a preliminary clean-up by solid phase extraction (SPE), followed by acid hydrolysis/derivatisation and liquid-liquid extraction. High sensitivity was achieved with tandem mass spectrometry using API 5500 equipment (AB SCIEX Q TRAP 5500, Canada), providing analyte identification at low concentrations. The method may be used for the detection of all four residues down to 0.5 µg/kg.

MATERIAL AND METHODS

Chemicals and reagents

The AHD, AOZ, SEM and AMOZ metabolites were obtained from Witega (Berlin, Germany) and from Sigma-Aldrich (St. Louis, MO, USA). Internal standards AOZ-d4, AMOZ-d5 and 2-nitrobenzaldehyde (2-NBA) and the 2-NBA derivatives of nitrofuran metabolites NPAHD, NPAOZ, NPSEM and NPAMOZ were supplied by Sigma-Aldrich. Strata X (500 mg/3 ml) cartridges were purchased from Phenomenex (Torrance, USA). Methanol LC-MS grade, methanol LC grade, ethyl acetate LC grade and hydrochloric acid were provided by Baker (Deventer, The Netherlands). Trisodium phosphate dodecahydrate p.a., sodium hydroxide p.a. and ammonium acetate p.a. were received from Merck (Darmstadt, Germany). Ultrapure water was supplied by Milli-Q system Millipore (Bedford, MA, USA). The filters for the extract filtration were from Millipore (Millex GV, 0.45 µm).

Standard solutions

Stock standard solutions of 1.0 mg/ml were prepared in calibration flasks by initially dissolving 50 mg of AOZ, SEM, AHD and AMOZ in 50 ml of methanol. These solutions were then diluted 100 times in methanol giving an intermediate standard solution at 10 µg/ml. A working standard solution of 10 ng/ml was then made by appropriate dilution of intermediate standard with methanol. Internal standards of AOZ-d4 and AMOZ-d5 were prepared by dissolving 10 mg of each in methanol, which were accordingly diluted until a working solution of 10 ng/ml was achieved. The stock standards solutions (1.0 mg/ml) kept at -20ºC were stable for 1 year. The intermediate standard solutions (10 µg/ml) stored at -20ºC were stable for one month. The working standard solutions (10 ng/ml) stored at temperatures in the range of 2 – 8ºC were stable for 1 week.

The stock solutions of NPAOZ, NPSEM, NPAHD and NPAMOZ were prepared in methanol at concentrations of 20 µg/ml. Standard solutions were prepared from these stock solutions in acetonitrile : water (1:2; v/v) to give concentrations of 0.5, 1, 1.5 and 2 µg/kg.

Sample preparation

Urine samples were first centrifuged at 3000 rpm for 10 min, then filtered with a 0.45 µm PVDF membrane and stored at -20°C until analysis.

A 1 ml urine sample was then weighed and an internal standard mixture was added (10 ng/ml of AOZ--d4 and AMOZ-d5, 100 µl). The sample was passed through a Strata X cartridge (500 mg/3 ml), which had been previously pre-conditioned with 3 ml methanol, followed by 3 ml water prior to use. The cartridge was then rinsed with 3 ml water and the rinsed eluates containing the analytes were collected in suitable glass vials. Subsequently, 9 ml hydrochloric acid (0.1 mol/l) and 100 µl 2-NBA (50 mmol/l) were added to the test eluates followed by vortex mixing.

Vials were then capped tightly and thermostatically incubated overnight at 37 ±1ºC after which samples were then cooled to room temperature and neutralised by adding 1.0 ml trisodium phosphate (0.3 mol/l) with the pH adjusted at 7.00 ±0.5 using sodium hydroxide (2 mol/l) as appropriate. From this mixture, 7 ml of supernatant were transferred into suitable glass tubes, placed into a heating block maintained at 40 ±5°C and evaporated to dryness under a stream of nitrogen. The residues were then resuspended in 500 µl of methanol--water (10:90, v/v) followed by vortex mixing for 30 s. The resulting aliquots were transferred into micro centrifuge tubes and spun at 14000 rpm for 5 min followed by filtration through a 0.45 µm nylon filter directly into the LC vial.

LC-ESI-MS/MS analysis

The LC-ESI-MS/MS system consisted of an Agilent 1200 HPLC (Agilent Technologies, Germany) connected to a Applied Biosystems/AB SCIEX Q TRAP 5500 linear ion trap mass spectrometer (Concord, Ontario, Canada), coupled to a TurboIonSpray (TISP).

LC analyses were performed on a Luna C18(2) column (150 × 2 mm, I.D 3 µm particle size; Phenomenex, Torrance, USA). The column was thermostatted at 40ºC, the flow rate was 200 µl/min, the injection volume was 10 µl and the following programme of mobile phases were used consisting of: 0.5 mmol/l ammonium acetate in 20% methanol in water (A) and methanol (B). To achieve the required separation, the linear gradient run comprised; 0.0 – 0.1 min 10% A, 0.1 – 15 min from 10 to 90% A, 15 – 16 min from 90% to 10% A and 16 – 25 min 10% A.

The MS was operated in Multiple Reaction Monitoring mode (MRM). Two MRM transitions were monitored for each compound. The most intense was used for quantification and the minor one for confirming the analyte identity. Both quantification and identification procedures were optimised. MS tuning was performed in positive ESI mode by infusing solutions of analytes (100 µg/l in methanol) at a flow rate of 10 µl/min mixed with an LC flow comprising solvents A and B (v/v; 0.2 ml/min) using a T-connector. MRM transitions and optimal values for the declustering potential (DP) collision energy (CE) and collision cell exit potential (CXP) are shown in Table 1.

Table 1. Mass spectrometry detection conditions using MRM mode

Analytes	MRM transition (m/z)	Retention time (min)	DP (V)	CE (V)	CXP (V)
NPAHD	249 → 134	10.1	46	21	8
	249 → 178		48	21	8
NPAOZ	236 → 134	10.5	46	14	10
	236 → 104		46	26	10
NPSEM	209 → 192	10.8	36	13	8
	209 → 166		36	10	8
NPAMOZ	335 → 291	13.0	42	21	10
	335 → 262		42	19	10
NPAOZ-d4 (IS)	240 → 134	10.4	48	20	12
NPAMOZ-d5 (IS)	340 → 296	12.9	46	24	12

Other ESI parameters used were as follows; ion spray voltage (IS) at 4500 V: source temperature (TEM) at 400ºC: curtain gas (CUR) at 30 psi: CAD gas was medium: ion source gas at 1 (GS1), 40 psi: and gas 2 (GS2) at 70 psi. Nitrogen served both as turbo gas and collision gas. The dwell time was set to 100 ms. Data collection and subsequent processing were performed with Analyst 1.5.2 Software.

Confirmation

In accordance with the Commission Decision 20002/657/EC, a sample can only be determined positive when the following criteria are met; the signal-to--noise ratio (S/N) of diagnostic ions have to be greater than three, the relative ratio of analyte retention time to that of the IS corresponds to that of the calibration solution within a ±2.5% tolerance for liquid chromatography as well as for the relative peak area ratios of the various transition reactions within the tolerances set by the EU criteria [4].

RESULTS AND DISCUSSION

Overview of approach

A common approach to analysing NF metabolites in biological samples involves an acid hydrolysis and derivatisation with 2-nitrobenzaldehyde (NBA), where the latter is necessary for the determination of NF

metabolites. All NF metabolites have low molecular masses, from 75 m/z (SEM) to 201 m/z (AMOZ). The LC-ESI-MS/MS analysis of low molecular mass substances displays relatively poor sensitivity due to the high abundance of mass spectrometric background noise within this mass range, as well as non-specific fragmentation behaviour. The derivatisation of the NF metabolites with NBA however usually significantly increases the sensitivity of the MS analysis, as has been described in the literature. In the presented paper, derivatisation was performed according to the procedure published by *Leitner* et al. [6], although with some modifications. Unfortunately, derivatisation may increase matrix side-reactions which can interfere with subsequent LC-ESI-MS/MS analysis. Removing any drugs from the matrix has been found to be one of the most difficult steps in the analysis of NF metabolites in urine samples. The current study describes such a method for determining NF metabolites in urine where solid-phase extraction (SPE) was used as a clean-up step prior to hydrolysis and derivatisation. The three following polymeric columns for SPE were tested: Strata X (Phenomenex, 500 mg/3 ml), Strata SDB-L (Phenomenex, 200 mg/3ml) and Oasis HLB (Waters Co, 30 mg/3 ml). The clean-up was assessed through recoveries, that were calculated by comparing peak areas of spiked samples (at 1 µg/kg) with corresponding derivatised standards. In these instances, no internal standard was used and quantification was carried out using the external absolute response. The samples were prepared as described in the 'Sample preparation' section. Results so obtained, showed that all three sorbents provided similar recoveries, but that the Strata X gave the greatest reductions of interfering compounds and hence allowed NF metabolites to be determined at low levels. Therefore, the Strata X was selected as the SPE cartridge for the main study. The absolute recoveries for the Strata X cartridge were 66%, 37%, 18% and 25% respectively for NPAHD, NPAOZ, NPSEM, and NPAMOZ. These low recoveries were due to extensive matrix effects; a common problem of the ESI technique. However, improved and satisfactory recoveries were in fact obtained by quantification using calibration curves based on matrix matched standards and also due to the use of internal standards which compensate for the signal loss resulting from matrix effects as well as the analyte loss during sample preparation; the internal standard being added before sample preparation. In the present study, the relative recoveries were thereby higher than 90% and relative standard deviations were below 19%.

Validation

The developed method was validated on the basis of procedures described in Commission Decision No. 2002/657/EC [4]. The following performance characteristics were checked: specificity, linearity, authenticity (i.e. through recovery), precision (repeatability and within-laboratory reproducibility), decision limit (CC) and detection capability (DC). The method was validated for the simultaneous analysis of AHD, AOZ, SEM and AMOZ by their derivatised NF counterparts; NPAHD, NPAOZ, NPSEM and NPAMOA.

The specificity of the method was demonstrated by analysing 20 different blank and spiked urine samples at 0.5 µg/kg in order to investigate any possible interferences that had similar retention times with the analytes. For each of the transitions, chromatograms demonstrated a significant increase in peak area and intensity at their specific retention time compared to the blanks, taking into account a signal to noise ratio of at least 3. Figure 1 shows the LC-ESI-MS/MS chromatograms of blank and spiked urine; at a concentration of 0.5 µg/kg. In the blank samples, interfering peaks were observed at the SEM retention time for the 209192 and 209166 ion transitions. However, when comparing chromatograms of blank bovine urine with the bovine urine spiked with 0.5 µg/kg, the SEM clearly shows that the interference is minimal and of little consequence since the interference signal is very low compared to that obtained from the positive sample, even at its low levels. All the chromatograms obtained throughout the validation study demonstrated very stable retention times for the spiked samples, with relative deviation always being better than ±2,5%.

A minimum of four identification points are required for the identification of banned substances (according to Decision No. 2002/657/EC). This is fulfilled using a LC-ESI-MS/MS method with 1 precursor ion (1 IP) and at least 2 transition product ions (2×1.5 IP). Precursor (1 IP) and product ions (1.5 IP) of each analyte are presented in Table 1.

The linearity of the developed method was evaluated for each of the NF metabolites by preparing calibration curve blank matrices, which consisted of six

Table 2. Recovery, repeatability and reproducibility in spiked bovine urine samples (n=6)

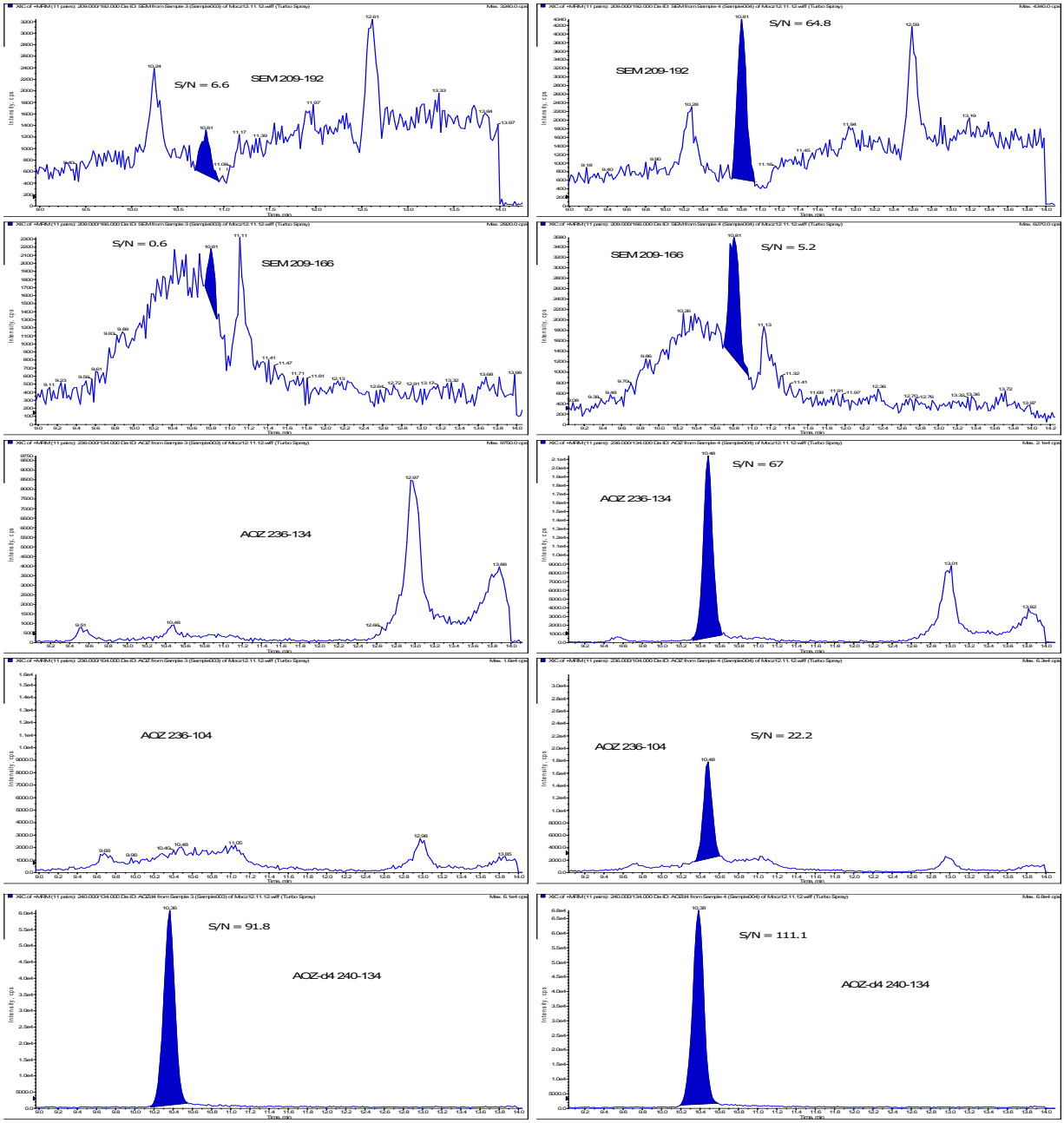
Analytes	Parameter	Level of spiking (µg/kg)			
		0.5	1.0	1.5	2.0
AHD	Recovery (µg/kg)	101.7	99.2	103.4	102.5
	Repeatability (RSD,%)	10.8	8.6	7.5	7.8
	Reproducibility (RSD,%)	13.9	10.1	10.7	8.2
AOZ	Recovery (µg/kg)	102.3	102	92.8	94.1
	Repeatability (RSD,%)	9.7	7.6	6.8	6.1
	Reproducibility (RSD,%)	11.3	9.3	8.1	7.3
SEM	Recovery (µg/kg)	101.9	99.4	102.5	107.5
	Repeatability (RSD,%)	18.8	15.8	15.8	14.5
	Reproducibility (RSD,%)	23.6	21.1	20.9	18.7
AMOZ	Recovery (µg/kg)	90.4	94.1	97.5	103.2
	Repeatability (RSD,%)	8.9	6.5	6.3	5.3
	Reproducibility (RSD)	9.7	9.4	8.7	6.1

different bovine urine matrices. The range of the calibration curves were from 0.0 (blank sample) to 2 µg/kg (i.e. at the following points; 0.0, 0.5, 1.0, 1.5 and 2.0 µg/kg). A fixed amount of internal standard (AOZ-d4 and AMOZ-d5) was added to all the samples. The AOZ-d4 was used for AHD, AOZ and SEM whilst AMOZ-d5 for AMOZ. Calibration curves were constructed using analyte/internal standard peak area ratios versus analyte concentration. Adequate linearity was obtained for each analyte with a correlation coefficient higher than 0.993 and a slightly inferior but still acceptable SEM of 0.985.

The measurement accuracy (i.e. corrected recovery of internal standard), was assessed by spiking six replicates of blank urines for each standard level; 0.5, 1.0, 1.5 and 2.0 µg/kg. The results are listed in Table 2.

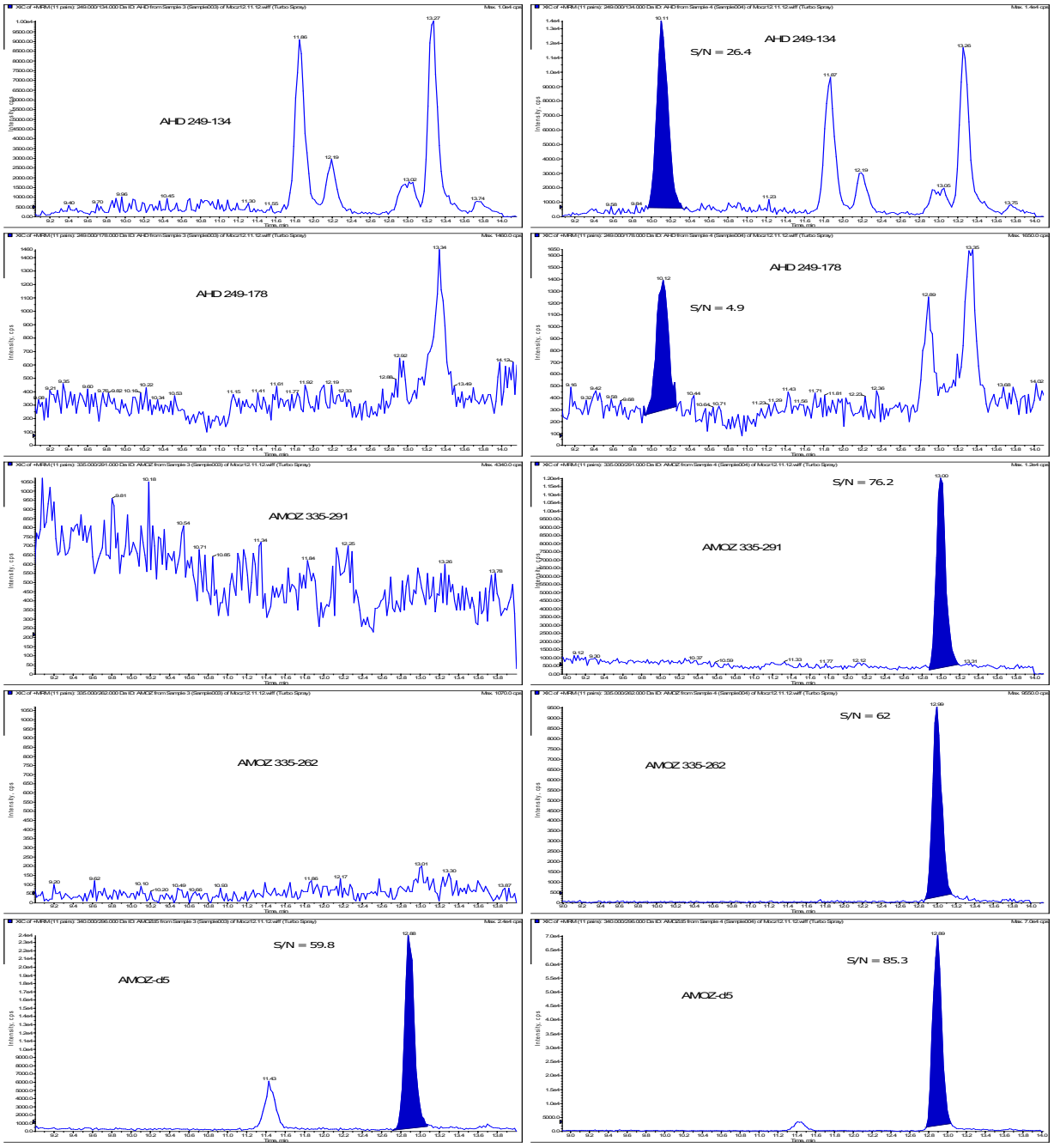
The average recoveries for each compound ranged from 90% to 108% and fulfil the criteria put forward in the 2002/657/EC Decision stating that a mass fraction, which is less than or equal to 1 µg/l should give a mean recovery of 50 – 120%, while 70 – 110% of recovery should be obtained when the mass fraction ranges from 1 to 10 µg/kg. The recoveries obtained by this analytical method were therefore highly satisfactory.

The precision (repeatability and within-laboratory reproducibility) of the assay was determined by the relative standard deviation (RSD). The repeatability of



a b

Figure 1 (a i b). LC-ESI MS/MS chromatograms of blank (a) and spiked (b) urine samples at 0.5 µg/kg



a b

Figure 1 (a i b). (Continued)

the method was evaluated by spiking blank samples of urine in three sets, each consisting of six replicates at levels of 0.5, 1.0, 1.5 and 2.0 µg/kg. These were analysed on different days by the same instrument and the same operator. The within-laboratory reproducibility was calculated identically except that analyses were performed by different operators. The results are shown in Table 2. Satisfactory repeatability and within-laboratory reproducibility were obtained for AHD, AOZ and AMOZ at all levels; RSDs were lover than 14% and 25%, respectively. For SEM, repeatability and within-laboratory reproducibility (RSD) were below 19% and 24%, respectively.

The precision (RSD) outcome was not evaluated by the *Horwitz* equation because too high values would be obtained due to the concentration range used being too low. However, in accordance with Decision 2002/657/EC, the RSD obtained for mass fraction lower than 100 µg/kg were as low as possible.

The CC and DC values were determined by the matrix calibration curve procedure according to case 1 of ISO 11843-2 – constant standard deviation [5].

The CC and DC were calculated using two calibration curves (at four levels 0.0, 0.5, 1.0, 1.5 and 2.0 μg/kg), from six different experiments on different bovine urine matrices performed on different days. Curves were constructed using analyte/internal standard peak area ratio versus analyte concentration. Calculated CC and DC for different NF metabolites are reported in Table 3. The mean CCα were 0.11 – 0.34 μg/kg and DCβ 0.13 – 0.43 μg/kg. These results are satisfactory since the MRPL is fixed at 1 μg/kg.

Table 3. CCα and CCβ obtained in bovine urine

Analytes	CCα (μg/kg)	CCβ (μg/kg)
AHD	0.27	0.34
AOZ	0.11	0.13
SEM	0.34	0.43
AMOZ	0.14	0.18

The analytical method developed was used to determine the NF metabolites in 26 bovine urine samples. The majority of the studied compounds were however undetectable in these analysed samples. Only AOZ was found in one urine sample at 1.8 μg/kg with a RSD (n=3) of 5.7%.

CONCLUSIONS

The presented LC-ESI-MS/MS method allows simultaneous determination of four nitrofuran metabolite residues in bovine urine. Solid phase extraction with a Strata X cartridge was used to remove interfering substances from urine samples. This method was validated in accordance with Decision 2002/657/EC and is now used for routine analysis at our laboratory. The repeatability and laboratory reproducibility of the method were less than 19% and 24%, respectively. The mean values of decision limit and detection capability were below 1 μg/kg.

Conflict of interest

The authors declare no conflict of interest.

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