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Detection of banned nitrofuran metabolites in animal plasma samples using UHPLC–MS/MS

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ABSTRACT

The use of nitrofurans as veterinary drugs in food-producing animals has been banned in the EU since the 1990s. Monitoring programs in the EU are based on the detection of protein-bound metabolites after slaughter. An UHPLC–MS/MS method was developed and validated for pre slaughter determination of four nitrofuran metabolites (AHD, AOZ, SEM, AMOZ) in animal plasma (bovine, ovine, equine and porcine). This method is proposed as an alternative method for on-farm surveillance. Plasma samples were derivatised with 2-nitrobenzaldehyde and subsequently extracted with organic solvent. Extracts were concentrated and then analysed by UHPLC–MS/MS. The method was validated according to Commission Decision 2002/657/EC. Inter-species recovery for AHD, AOZ, SEM and AMOZ was 72, 74, 57 and 71%, respectively. Decision limits (CCα) were calculated from within laboratory reproducibility experiments to be 0.070, 0.059, 0.071 and 0.054 μg kg^{-1}, respectively. In addition, the assay was applied to incurred plasma samples taken from pigs treated with furazolidone.

1. Introduction

Nitrofurans (NFs) belong to a group of synthetic broad spectrum antibiotics which all contain 5-nitrofuran ring and various substituents in the 2-position. It has been reported that the history of NFs application as pharmacologically active substances starts in 1944, when the antibiotic properties of nitrofurazone (NFZ, Fig. 1) were first confirmed [1]. This discovery subsequently led to the synthesis and evaluation of many structural analogues that were expected to have similar antibiotic properties. Due to their efficiency, availability and relatively cheap production NFs quickly gained worldwide popularity as veterinary drugs and feed additives [2,3]. Four pharmacologically active compounds that achieved significant commercial success, and attracted research interest in the recent decades are furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT) and nitrofurazone (NFZ) (Fig. 1). They all belong to a group azomethine type of NFs (–CH=N– side chain) [3].

Mutagenicity and toxicity of NFs has been revealed through studies in 1970s and 1980s [4–6]. Consequently, the European Union (EU) prohibited the use of FTD, NFT and NFZ in food-producing animals by listing them in Annex IV of the Council Regulation 2377/90 [7]. This annex lists pharmacologically active substances for which no maximum residue levels (MRL) in food can be fixed. FZD was moved to Annex IV in June 1995. Effectively, from January 1997, administration of these NFs to food-producing animals in the EU was no longer allowed [8,9].

It has been found that NF residues monitoring in animal tissue based on identification of parent compounds was not efficient in regulation enforcement. Research showed that parent drugs have short *in vivo* half life and could not be detected in tissue nor blood 24 h after slaughtering [10]. It has been confirmed that NFs metabolize rapidly, and their metabolites bind to tissue protein where they persist for periods post-treatment [11–13]. Therefore, NF metabolites AOZ (3-amino-2-oxazolidone), AMOZ (3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one), AHD (1-aminohydantoin) and SEM (semicarbazide) (Fig. 1) have been established as markers for NF residue testing.

Illegal or accidental contamination with NFs on farms can still occur and requires persistent and efficient monitoring. Review papers on NF residual analyses [14] and trends in sample preparation for veterinary drugs residue analyses [15] reported presence of many validated methods for NF metabolites determination in various animal tissues, such as muscle, retina, liver, yet limited information is available on methods for pre-slaughter monitoring of NF metabolites.

A recent study on pigs treated with FZD indicates existence of correlation between AOZ content in edible tissues and biological fluids [16]. In this study, the AOZ concentrations were monitored in edible tissues, plasma and urine of pigs between 0.5 and 63 days post administration of furazolidone (400 mg/kg in feed for 7 days) showing good correlation between matrices. AOZ residues occurred at highest concentrations in liver (2170 μg kg^{-1})

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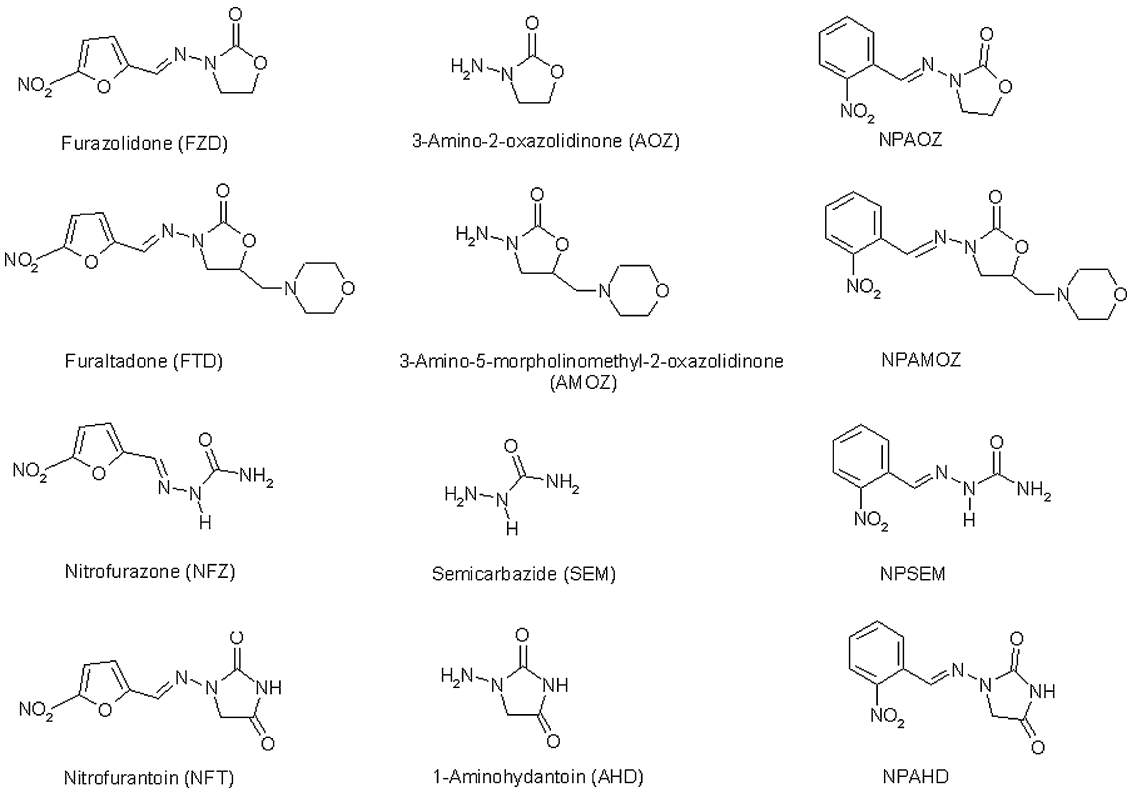

Fig. 1. NF parent compounds, metabolites and NP derivates.

followed by plasma (1969 μg kg^{-1}), urine (1260 μg kg^{-1}), kidney (1012 μg kg^{-1}) and muscle (691 μg kg^{-1}) at 0.5 days [16]. At the 63 day sampling, AOZ residues were measured at 1.1, 1.2, 1.0, 0.6 and 0.6 μg kg^{-1} in liver, plasma, urine, kidney and muscle, respectively. Therefore, this study opens possibilities for pre-slaughter screening and residue monitoring of NF metabolites where plasma is suggested as a suitable matrix for on-farm monitoring of livestock. The same group proposes enzyme linked immunosorbent assay (ELISA) method for screening of AOZ, as it is cheap and relatively easy to use. However, performance of enzyme immunoassay methods can be limited when used for confirmatory practice at residue level, which can be observed from reported high standard deviation value at 1 μg kg^{-1} [16].

Currently no method involving extraction and ultra high performance liquid chromatography tandem mass spectrometry has been published to measure NF metabolites (AHD, AOZ, SEM, AMOZ) in animal plasma. The aim of this study was to provide validation data for this confirmatory method.

2. Experimental

2.1. Materials and reagents

NF metabolites (AOZ, AMOZ and AHD), nitrophenyl (NP) derivatives: 3-((2-nitro-benzylidene)-amino)-oxazolidin-2-one (NPAOZ), 5-morpholin-4-ylmethyl-3-((2-nitro-benzylidene)-amino)-oxazolidin-2-one (NPAMOZ), 1-((2-nitro-benzylidene)-amino)-imidazolidine-2,4-dione (NPAHD), 2-nitro-benzaledehyde-semicarbazone (NPSEM) and isotopically labelled internal standards (AMOZ-D_5, AOZ-D_4, $^{13}C^{15}N_2$-SEM and $^{13}C_3$-AHD) were all obtained from Witega, Berlin, Germany. SEM (Vetranal grade), 2-nitrobenzaldehyde (2-NBA), ammonium acetate (MS grade) and 99.5% deuterated methanol were purchased from Sigma–Aldrich. Ultra-pure water (18.2 MΩ) was generated using a Milli-Q Plus water purification system. Methanol and ethyl acetate (EtOAc) (both HPLC grade) were obtained from BDH Chemicals Ltd. (Poole, UK). 0.1 N HCl was prepared by diluting 8.6 mL of conc. HCl to 1000 mL with water. 1 M NaOH was prepared by dissolving 40 g of sodium hydroxide pellets (Analar Grade, BDH) in water and making up to 1 L. Trisodium phosphate buffer 0.3 M was prepared by dissolving 11.4 g of $Na_3PO_4{\cdot}12H_2O$ to 100 mL with water. pH test strips 4.5–10.0 were obtained from Sigma–Aldrich. A Dispensette® lll solvent dispenser (Brand GMBH + CO KG; Wertheim, Germany) was used for aliquoting EtOAc; Mistral 3000i centrifuge (MSE; London, UK), TopMix multi-vortexer (Fisher Scientific; Dublin, Ireland), Whatman ReZist™ PTFE syringe filters – 13 mm, 0.22 μm and 0.45 μm were obtained from Fisher Scientific (Dublin, Ireland).

2.1.1. Standard solutions

Individual primary stock solutions of NF metabolites and their NP derivatives were prepared at a concentration of 50 mg L^{-1} (free metabolite equivalents) in methanol. Internal standards were prepared at a concentration of 50 mg L^{-1} in deuterated methanol. All standard solutions in this work were stored at −20 °C. Primary stock solutions were found to be stable for one year. Intermediate stock solutions (free metabolites, NP derivatives and unlabelled standards) containing all four target analytes were prepared from individual primary stock solutions at a concentration of 1 mg L^{-1}. Working standards were prepared at a concentration of 50 μg L^{-1} daily from intermediate standard solutions.

2.2. Plasma samples

2.2.1. Negative control plasma samples

Bovine and porcine blood samples were collected by inspectors from the Department of Agriculture Fisheries and Food in tubes containing Lithium Heparin anticoagulant. Ovine blood samples in heparinised tubes were obtained from Teagasc Animal & Grassland Research and Innovation Centre, Athenry, Co. Galway. On arrival

Table 1
MS/MS fragmentation conditions for NP derivates of NF and corresponding internal standards.

Target metabolites and related internal standards	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)	Retention times (min)
NP-AHD	248.95 ± 0.5	104.00 ± 0.5	0.023	26	22	2.33
		134.00 ± 0.5	0.023	26	10	2.33
$^{13}C_3$-NP-AHD	251.95 ± 0.5	178.89 ± 0.5	0.023	25	18	2.33
NP-AOZ	235.95 ± 0.5	104.05 ± 0.5	0.020	30	20	2.40
		133.90 ± 0.5	0.020	30	9	2.40
D_4-NP-AOZ	240.10 ± 0.5	134.00 ± 0.5	0.020	25	15	2.40
NP-SEM	208.95 ± 0.5	192.00 ± 0.5	0.020	26	8	2.41
		165.95 ± 0.5	0.020	26	10	2.41
$^{13}C^{15}N_2$-NP-SEM	211.92 ± 0.5	167.68 ± 0.5	0.020	25	10	2.41
NP-AMOZ	335.00 ± 0.5	291.15 ± 0.5	0.100	25	16	2.71
		262.10 ± 0.5	0.100	25	10	2.71
D_5-NP-AMOZ	340.10 ± 0.5	296.1 ± 0.5	0.100	25	12	2.71

blood was centrifuged (2030 × *g*, 20 min) and plasma was transferred into clean glass tube. Plasma samples were stored at −20 °C until analysis. Equine plasma samples were supplied by the Irish Equine Centre, Straffan, Co., Kildare and stored as above.

2.2.2. Incurred plasma study

Incurred plasma samples were obtained from three male castrated pigs weighing approximately 15 kg each, treated with FZD by in-feed administration. The pigs were given a standard pig ration based on barley, wheat and soyabean. The macronutrient diet comprising of protein, lysine, soyabean oil, fibre, calcium and phosphorous was at 19–20%, 1.3%, 4.3%, 3.6%, 0.8% and 0.5%, respectively. The study was accepted by the local ethics committee based at Teagasc, Pig Development Unit. The treatment of the animals was carried out in accordance with licences issued by the competent authorities, which specified, *inter alia*, that no material from the treated animals should be permitted to enter the food chain. Pigs were treated with FZD at a dose of 20 mg kg^{-1} bodyweight per day, for 5 days. The health of the animals was monitored daily throughout the study for general health by qualified personnel supervised by a veterinarian. Animals were slaughtered at 0 (pig B), 3 (pig C) and 10 (pig D) days post-treatment. Blood samples were collected from each animal at slaughter and stored in heparinised tubes. Tubes were subsequently centrifuged at 2030 × *g* and plasma was removed and stored at −20 °C. The AOZ content in incurred samples of plasma was determined to be in the range 400–1000 μg kg^{-1}. As a result, plasma samples were diluted 200-fold with negative bovine plasma and tested accordingly as residue samples in replicates of five.

2.3. Sample preparation

Plasma samples (1 g) were weighed into 50 mL centrifuge tubes and fortified with 40 μL of working internal standard (50 μg L^{-1}) and let stand for 15 min. 0.1 N HCl (9 mL) and 100 mM 2-NBA in methanol (100 μL) were sequentially added. Samples were vortexed (1 min) and incubated overnight (16 h, 37 °C) in water bath with gentle shaking. After cooling to room temperature, samples were neutralised by adding 0.3 M trisodium phosphate buffer (1 mL) and 1 M NaOH (385 μL). pH was checked with test strips (pH 4.5–10) and corrected if necessary to fall in the range pH 6.5–7.5. Extraction was performed with EtOAc (18 mL) by shaking samples on mechanical shaker (20 min). The samples were centrifuged (2030 × *g*, 10 min) and extracts collected into glass tubes. The extraction was repeated with EtOAc (9 mL) and extracts combined. Solvent was evaporated under nitrogen at 40 °C. Dry extract was reconstituted in 0.5 mL of injection solution (0.5 mM ammonium acetate and methanol 80:20, v/v) and vortexed (1 min). Extracts were sequentially filtered through 0.45 μm and 0.2 μm PTFE 13 mm syringe filters into a 200 μL vial. This dual filtration step was required because 0.2 μm filters are susceptible to blockage. It is recommended to pass sample extracts through 0.2 μm filters to prevent blockage of UHPLC columns.

2.4. UHPLC–MS/MS analysis

Separation was carried on a Waters Acquity UHPLC system (Milford, MA, USA). The analytical column was an Acquity UHPLC® BEH C_{18} (100 mm × 2.1 mm, 1.7 μm particle size) with Vanguard pre-column C_{18} (5 mm × 2.1 mm) and maintained at 65 °C. This temperature is used in most UHPLC–MS/MS applications in our laboratory to prevent column blockage and prolong column life. It is proposed that this blockage is caused by the precipitation of non-polar material at the inlet side of the column during analysis but this risk is reduced by increasing column temperature to 65 °C. A binary gradient separation was performed at a flow rate of 0.5 mL min^{-1}. Mobile phase A consisted of aqueous ammonium acetate (0.5 mM) and mobile phase B was MeOH. The gradient profile was as follows: (a) 0 → 0.6 min, 10% B; (b) 2 min, 50% B; (c) 2 → 3 min, 50% B; (d) 3.5 min, 80% B; (e) 3.5 → 5.5 min, 80% B; (f) 6 min, 10% B; (g) 6 → 9 min, 10% B. Total run time was 9 min. The UHPLC autosampler was sequentially rinsed using strong and weak washes consisted of H_2O:MeOH (0.5 mL, 10:90, v/v) and MeOH:H_2O (1 mL, 10:90, v/v), respectively. These washes are required to clean the needle and reduce carryover between injections. In addition, the wash conditions are important because they are required for pushing the sample into the injection loop and can impact on the chromatographic peak shape. The sample injection volume was 20 μL. NF metabolites were detected using a Waters Quattro Premier XE triple quadropole instrument equipped with an electrospray ionisation probe operating in positive mode (Milford, MA, USA). Nitrogen was used for nebulisation, desolvation (1100 L h^{-1}) and as cone gas (200 L h^{-1}). The source and desolvation temperatures were 140 °C and 400 °C, respectively. Capillary voltage was 3.0 kV. Argon was used as collision gas. The MS conditions were optimised by tuning the analyte-specific parameters, including cone voltage, collision energy, and collision cell exit potential for each analyte. This was carried out by infusion of a 1 μg mL^{-1} standard solution and optimising response for the parent and two most intense product ions. The SRM (selected reaction monitoring) windows were time-sectored, and dwell time, inter-scan delay and inter-channel delays were set to get maximum sensitivity from the instrument. Individual transitions and respective collision energies and voltages are listed in Table 1. The UHPLC–MS/MS system was controlled by MassLynx™ software and data was processed using TargetLynx™ Software (both from Waters).

2.5. Calibration

Extracted matrix calibration curves were prepared by fortifying negative plasma samples at concentrations of 0.2, 0.5, 1, 2 and

5 μg kg^{-1}. Recovery was monitored by analysing plasma samples spiked with NP derivatives at concentrations of 0.25, 0.5, 1 and 2 μg kg^{-1} into almost dry extracts and then evaporated to dryness. Regression analyses of the responses (analyte area divided by internal standard area) were performed using TargetLynx^{TM} software.

2.6. Method validation

The method was validated in house according to Commission Decision 2002/657/EC [17]. Since NFs are banned substances and listed in Annex IV of Council Reg 2377/90, there is no safe limit for presence of these compounds in food. A reference point for action (RPA) for NF metabolites has been established as 1 μg kg^{-1}.

The following parameters were investigated: selectivity, linearity, stability of metabolites in plasma and stability of derivatised metabolites, recovery, within laboratory repeatability (WLr) and reproducibility (WLR), decision limit (CCα), detection capability (CCβ). Selectivity has been assessed through inspection of blank plasma chromatograms for presence of interfering peaks. Additionally, bovine plasma samples were fortified with nitrofuran metabolites at 0.2 μg kg^{-1}and with a mixture of 38 veterinary anthelmintic drugs and their metabolites and tested routinely to evaluate selectivity in presence of other veterinary substances. Correlation coefficients of over 20 calibration curves were calculated and inspected for linearity. Linearity was also investigated after testing blank bovine plasma fortified at 0.2, 0.5, 1, 2 and 5 μg kg^{-1} with 5 individual replicates at each level.

Values of CCα and CCβ were calculated according to calibration curve procedure from Commission Decision 2002/657/EC [17]. Blank material was fortified at 0.5, 0.75 and 1 μg kg^{-1}. Each individual validation run contained seven replicates at each level, five point calibration curve, four recovery controls and a negative control. Testing of samples was performed by three different operators over a period of one month. This data was used to assess WLR and was included in absolute recovery calculations. WLr was determined from three independent sets of analyses performed by the same operator. Finally method has been applied to equine, ovine and porcine plasma in order to assess accuracy and absolute recovery.

2.7. Stability studies in plasma and sample extracts

A stability study of NF metabolites in matrix was performed using blank bovine plasma fortified with nitrofuran metabolites (AHD, AOZ, SEM, AMOZ) at 2 μg kg^{-1}. Plasma has been shaken for 1 h on a mechanical shaker to ensure homogeneity and weighed into 30 tubes (1 ± 0.02 g of plasma). Stability of NF metabolites in bovine plasma was evaluated over 1, 2, 3, 4, 8 and 16 weeks storage at −20 °C. In addition, the stability of derivatised NF metabolites in extracts prepared for injection was also investigated. A total of 50 blank bovine plasma samples (1 g each) were fortified with internal standard and extracted. After extraction and the evaporation step, they were fortified at 2 μg kg^{-1} with derivatised metabolites (NPAHD, NPAOZ, NPSEM, NPAMOZ). Extracts were reconstituted in 0.5 mL of injection solution, filtered and placed in 200 μL vials. The stability of derivatised extracts was evaluated at 0, 1, 2, 3, and 4 weeks (n = 5) under storage at 4 and −20 °C.

3. Results and discussion

3.1. Selectivity

The selectivity of the method was evaluated during the validation study through application to 20 negative plasma samples of each species (bovine, ovine, porcine and equine) supplied from different sources. LC–MS/MS traces were found to be free of interference at the retention time of analytes. Typical chromatograms of negative and positive plasma samples fortified at 0.2 μg kg^{-1} level are shown in Figs. 2 and 3. Different batches of blanks were used to prepare calibration curves during validation to increase the ruggedness of the study.

To confirm selectivity in the presence of other veterinary compounds that may be coexisting in plasma, two blank bovine plasma samples were fortified with a mixture of 38 veterinary drugs and their metabolites at 1000 μg kg^{-1} level each (albendazole, albendazole-sulphoxide, albendazole-sulphone, albendazole-amino sulphone, cambendazole, fenbendazole, oxfendazole, fenbendazole sulphone, flubendazole, amino-flubendazole, hydroxy-flubendazole, amino-mebendazole, hydroxy-mebendazole, oxibendazole, mebendazole, triclabendazole, triclabendazole-sulphoxide, triclabendazole-sulphone, thiabendazole, 5-hydroxy-thiabendazole, levamisole, bithionol, clorsulon, closantel, morantel, niclosamide, nitroxynil, oxyclozanide, rafoxanide, coumaphos, coumaphos-oxon, haloxon, abamectin B1a, doramectin, emamectin B1a, eprinomectin B1a, ivermectin B1a, moxidectin). No interference has been observed. Additionally, bovine plasma samples fortified with NF metabolites at 0.2 μg kg^{-1} showed usual response in the presence of all 38 compounds.

The identity of analytes in matrix was confirmed by following: adequate retention times, monitoring ion ratio of two product ions for each analyte (with acceptable tolerances as described in Commission Decision 2002/657/EC), and signal to noise ratio of the transitions better than 3. The mean ion ratio was obtained from extracted matrix calibrants from 0.2 to 5 μg kg^{-1}. For AHD, AOZ and SEM acceptable tolerance was 20%, and for AMOZ it was 25%.

The potential contribution of laboratory consumables to false positive results was also investigated. It has been previously highlighted that the contact of solvents with certain blown plastics can be a source of trace diazocarbondiamide that can give false signal for SEM content when exposed to heating [18,19]. Whenever possible, glassware was used. Additionally pipette tips, septas, paper tissues in the lab were tested for potential SEM content before being put in use. A reagent blank was introduced as part of every analyses to keep this factor under control.

3.2. Linearity

Calibration curves have been designed to respond to the current RPA which is set at 1 μg kg^{-1} for animal tissue. As there is no level determined for plasma, in our work it has been chosen that this value would present central point in our calibration curve with two calibration points below (0.2 and 0.5 μg kg^{-1}) and two above (2 and 5 μg kg^{-1}). Correlation coefficients, residual plots and regression statistics were generated from regression analyses of the responses obtained from testing five replicates at each calibration level [20]. Correlation coefficient r and r^2 were higher than 0.995 for all four metabolites, which indicated high degree of correlation between y (response) and x values (concentration). Additionally correlation coefficients of over 20 routine calibration curves were inspected and all were higher than 0.995. Residual plots and variance homogeneity test showed a significant difference for the variance at different concentration levels ($p < 0.05$) and for that reason weighted regression was used ($1/x$) as this is a common case in residual analyses, particularly when for practical reasons chosen calibration points are not equidistant [21]. The analyses of variance (ANOVA) for linear regression produced F and significance F values (p value). F values were high for all four compounds confirming a good linear relationship and low variability arising from residuals. Significance F values were extremely low and as a probability parameter, it indicated that linear relationship between y and x values for all four analytes is high.

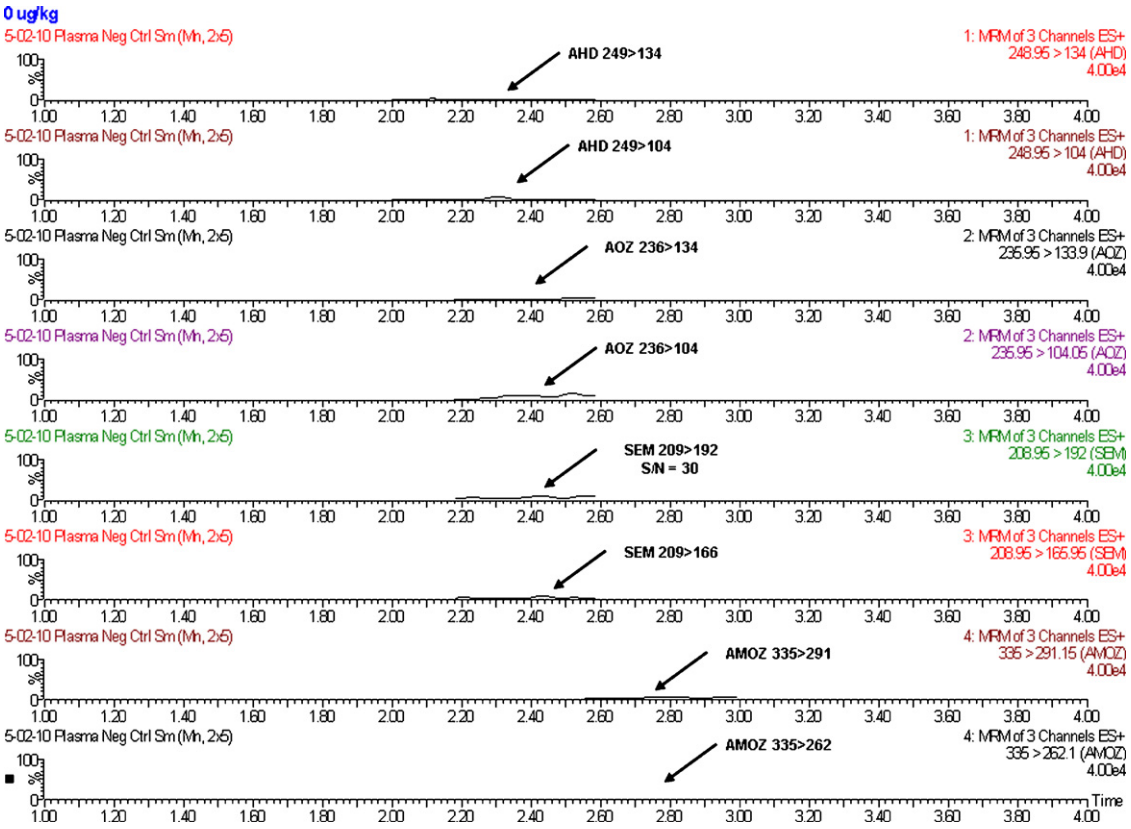

Fig. 2. MRM chromatograms of blank bovine plasma at expected RT for AHD, AOZ, SEM and AMOZ.

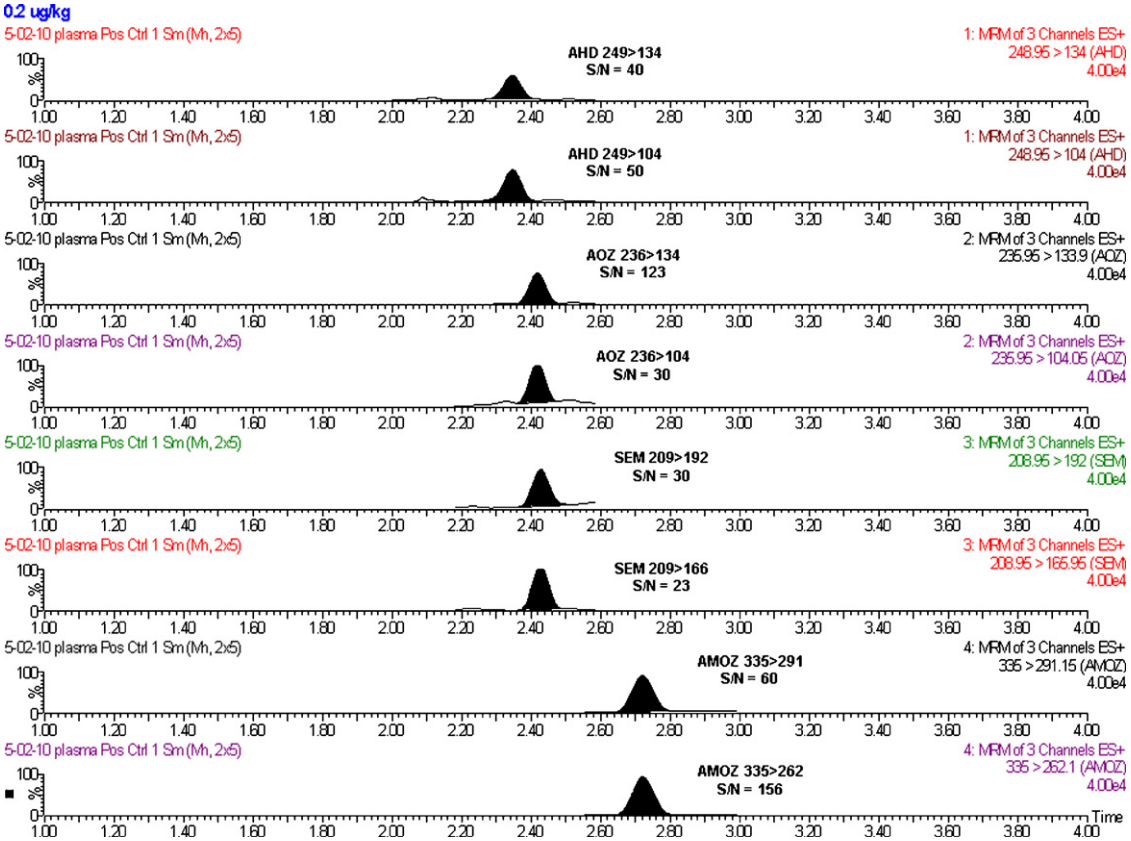

Fig. 3. MRM of bovine plasma fortified with each metabolite at 0.2 $\mu g\,kg^{-1}$ (the lowest calibration point).

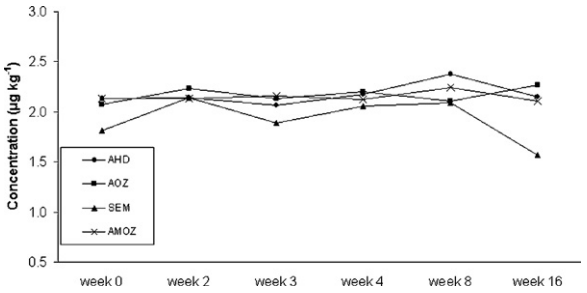

Fig. 4. Stability of nitrofuran metabolites in bovine plasma stored at −20 °C.

3.3. Stability

Stability of methanol solutions of four NF metabolites has been previously assessed by others [22]. It has been shown that stock standard solutions at 1 mg mL^{-1} are stable over the period of 10 months if stored at 4 °C. However, the same study showed that low concentration SEM standards in methanol (10 μg L^{-1}) decomposes at a rate of 5% in 3.9 months. As a result, all working standards were prepared on a daily basis.

In this study, the stability of NF metabolites in bovine plasma samples fortified at a level of 2 μg kg^{-1} was investigated at a storage temperature of −20 °C. This study showed that AHD, AOZ and AMOZ were stable during the period of our study of 16 weeks (Fig. 4). Results showed that non-bound SEM residues were stable for eight weeks but degraded after 16 weeks. Results are in agreement with previous report on SEM instability [23]. Therefore, it is suggested that plasma samples should be tested for NF metabolites within two months of sampling, to avoid potential losses in SEM content.

The stability of derivatised metabolites in purified and filtered matrix extracts were also assessed at 4 and −20 °C. This study showed that NPAHD, NPAOZ, NPSEM and NPAMOZ were stable for at least four weeks at both storage temperatures (Fig. 5a and b).

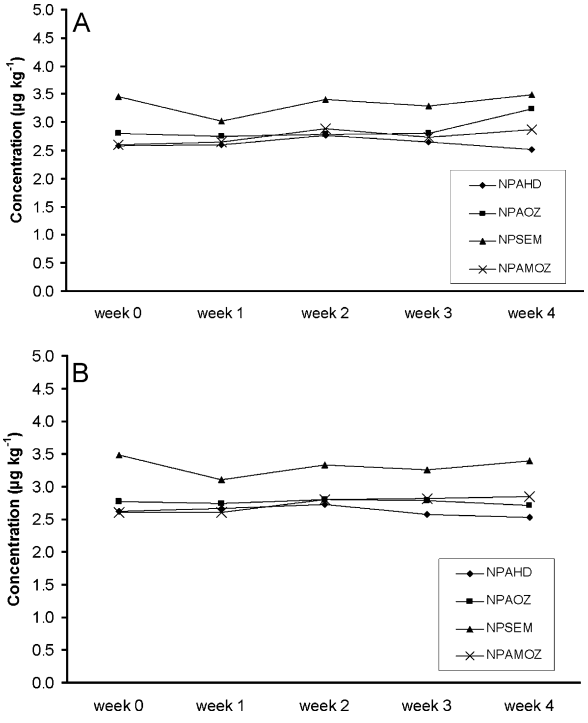

Fig. 5. (A) Stability of derivatised metabolites in bovine plasma extract stored at 4 °C. (B) Stability of derivatised metabolites in bovine extract stored at −20 °C.

Table 2
Within-laboratory repeatability and reproducibility results for bovine plasma.

Analyte	Accuracy WLr (%)			RSD WLr (%)		
	0.50[a]	0.75[a]	1.0[a]	0.50[a]	0.75[a]	1.0[a]
AHD	102	100	105	6.8	5.7	8.9
AOZ	103	100	102	4.9	4.2	4.3
SEM	103	99	105	8.8	4.9	6.5
AMOZ	104	100	103	4.5	3.9	5.6
Analyte	Accuracy WLR (%)			RSD WLR (%)		
	0.50[a]	0.75[a]	1.0[a]	0.50[a]	0.75[a]	1.0[a]
AHD	94	94	98	12.1	8.8	5.8
AOZ	101	98	103	14.0	5.2	5.9
SEM	96	97	100	8.2	7.1	9.1
AMOZ	98	96	102	12.3	7.9	7.6

[a] Concentration (μg kg^{-1}).

3.4. Within laboratory reproducibility, repeatability and method recovery

Results of within laboratory reproducibility (WLR) and repeatability (WLr) studies at three fortification levels (0.5, 0.75 and 1.0 μg kg^{-1}) in bovine plasma are shown in Table 2. Overall accuracy ranged from 94 to 104% for all four analytes. Precision varied between 3.9 and 14%. Recovery values for AHD, AOZ, SEM and AMOZ were 83, 84, 63 and 72%, respectively (Table 3). Recovery was determined by comparing the slope of calibration curves prepared in plasma samples fortified pre and post derivatisation. Recovery results were obtained from the average of 10 analytical runs from three different operators over a period of several months. RSD of absolute recovery ranged from 10.2 to 15.9%.

Inter-species validation results for equine, ovine, porcine and bovine plasma are shown in Table 3. Results showed recovery data for equine, ovine and porcine were similar to bovine plasma. Recovery values for AHD, AOZ, SEM and AMOZ were 62–73, 65–75, 54–63 and 68–73%, respectively.

3.5. Decision limit (CCα) and detection capacity (CCβ)

Calculated values for decision limit (CCα) and detection capacity (CCβ) are reported in Table 3. CCα values were calculated using inter-species within-laboratory reproducibility results for AHD, AOZ, SEM and AMOZ, and were 0.070, 0.059, 0.071 and 0.054 μg kg^{-1}. Results obtained could not be compared to other studies, as currently there is no data available for the determination of NF metabolites in animal plasma by this method. Comparison with other CCα and CCβ values obtained in NF residue analyses in different tissues (meat, kidney) shows that our data belong to the same order of magnitude, which is considered a positive indicator of this validation study in plasma matrix.

3.6. Application to incurred samples

The performance of the assay was assessed using plasma from pigs treated with a FZD and slaughtered at different withdrawal periods. Porcine plasma samples were diluted 200-fold with negative bovine plasma to fall in the range of the calibration curve. Samples were tested (n = 5) and the mean content of AOZ found in samples B, C and D was 4.65, 4.59 and 2.03 μg kg^{-1}, respectively (Table 4). RSDs associated with these results were 2.2, 2.3 and 2.0%, respectively.

3.7. Routine application of the method

The method has been applied in official food inspection to over 200 samples of bovine plasma on an annual basis. The results of

Table 3
Inter-species plasma validation results.

Analyte	Accuracy (%)			RSD (%)			Recovery (%)
	0.50[a]	0.75[a]	1.0[a]	0.50[a]	0.75[a]	1.0[a]	
Equine							
AHD	97	98	98	8.2	8.1	8.4	62
AOZ	102	94	89	7.6	5.7	5.4	75
SEM	99	95	93	4.4	9.4	5.3	56
AMOZ	103	97	94	5.8	7.5	2.9	69
Ovine							
AHD	100	98	95	5.2	5.3	3.2	71
AOZ	103	99	100	3.8	2.8	5.2	70
SEM	95	97	102	4.7	5.3	9.5	55
AMOZ	105	103	104	4.2	2.9	2.1	68
Porcine							
AHD	100	98	102	6.4	9.0	7.9	73
AOZ	104	105	101	8.1	6.6	6.7	65
SEM	106	108	109	11.3	8.1	8.5	54
AMOZ	100	104	99	6.4	2.9	2.7	73
Bovine							
AHD	82	87	86	12.3	8.6	5.8	83
AOZ	85	95	95	8.0	6.1	3.5	84
SEM	89	95	92	9.4	6.0	5.6	63
AMOZ	85	90	92	9.4	7.0	4.9	72

Analyte	Inter-species WLR accuracy (%)			Inter-species WLR RSD (%)			CCα ($\mu g\,kg^{-1}$)	CCβ ($\mu g\,kg^{-1}$)
	0.50[a]	0.75[a]	1.0[a]	0.50[a]	0.75[a]	1.0[a]		
AHD	95	95	95	11.2	9.0	9.0	0.070	0.117
AOZ	99	98	97	10.6	6.8	7.1	0.059	0.100
SEM	97	99	99	10.1	9.1	10.0	0.071	0.121
AMOZ	98	98	97	10.0	7.8	5.8	0.054	0.093

[a] Concentration ($\mu g\,kg^{-1}$).

Table 4
AOZ content in 200× diluted incurred pig plasma samples.

Animal	Replicate 1[a]	Replicate 2[a]	Replicate 3[a]	Replicate 4	Replicate 5	Mean	RSD (%)
A-control	–	–	–	–	–	–	–
B	4.626	4.578	4.620	4.602	4.831	4.65	2.2
C	4.548	4.726	4.656	4.541	4.464	4.59	2.3
D	2.069	2.076	1.980	2.019	2.015	2.03	2.0

[a] Concentration ($\mu g\,kg^{-1}$).

this surveillance work show that the majority of samples are found to be free of NF residues. In a few cases, residues of FZD have been detected in herds [24]. In all these cases animals were prevented from entering the food chain because early detection had been made on-farm. It is highlighted that these positives are sporadic and less likely to occur in future years because NF based drugs are banned since 1997. However, these few positive findings justify the need for the on-farm monitoring.

4. Conclusion

A UHPLC–MS/MS has been developed and validated to determine residues of four NF residues in animal plasma. The method is proposed as tool for the pre-slaughter monitoring of animal herds. The method has been validated from 0.2 to 5 $\mu g\,kg^{-1}$ using fortified bovine, equine, ovine and porcine plasma. CCα and CCβ were determined according to EU requirements. The method has been comprehensively evaluated through application to incurred plasma and over 200 routine samples per year. Confirmatory testing of NF metabolites in plasma taken from livestock is suggested as a preventative and screening tool that can provide early identification of illegal use on the farm. Early stage recognition is the best option in consumer protection, and preventing product recalls.

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