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2 **Detection of banned nitrofuran metabolites in animal plasma samples using**

3 **UHPLC-MS/MS**

4

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6

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9

10 **Abstract**

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

25

26 **Keywords**

27 Nitrofuran metabolites; Plasma; Residue determination; UHPLC-MS/MS; Stability
28 study

29

1

2 **1. Introduction**

3 Nitrofurans (NFs) belong to a group of synthetic broad spectrum antibiotics which all
4 contain 5-nitrofuranyl ring and various substituents in the 2-position. It has been
5 reported that the history of NFs application as pharmacologically active substances
6 starts in 1944, when the antibiotic properties of nitrofurazone (NFZ, **Fig. 1**) were first
7 confirmed [1]. This discovery subsequently led to the synthesis and evaluation of
8 many structural analogues that were expected to have similar antibiotic properties.

9 Due to their efficiency, availability and relatively cheap production NFs quickly
10 gained worldwide popularity as veterinary drugs and feed additives [2,3]. Four
11 pharmacologically active compounds that achieved significant commercial success,
12 and attracted research interest in the recent decades are furazolidone (FZD),
13 furaltadone (FTD), nitrofurantoin (NFT) and nitrofurazone (NFZ) [**Fig. 1**]. They all
14 belong to a group azomethine type of NFs (-CH=N- side chain) [3].

15

16 Mutagenicity and toxicity of NFs has been revealed through studies in 1970s and
17 1980s [4-6]. Consequently the European Union (EU) prohibited the use of FTD, NFT
18 and NFZ in food-producing animals by listing them in Annex IV of the Council
19 Regulation 2377/90 [7]. This annex lists pharmacologically active substances for
20 which no maximum residue levels (MRL) in food can be fixed. FZD was moved to
21 Annex IV in June 1995. Effectively, from January 1997, administration of these NFs
22 to food-producing animals in the EU was no longer allowed [8,9].

23 It has been found that NF residues monitoring in animal tissue based on identification
24 of parent compounds was not efficient in regulation enforcement. Research showed
25 that parent drugs have short *in vivo* half life and could not be detected in tissue nor
26 blood 24 h after slaughtering [10]. It has been confirmed that NFs metabolize rapidly,

1 and their metabolites bind to tissue protein where they persist for periods post-
2 treatment [11-13]. Therefore, NF metabolites AOZ (3-Amino-2-oxazolidone), AMOZ
3 (3 -amino-5-morpholinomethyl- 1,3 -oxazolidin-2-one), AHD (1 -aminohydantoin) and
4 SEM (semicarbazide) (**Fig 1.**) have been established as markers for NF residue
5 testing.

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

12 A recent study on pigs treated with FZD indicates existence of correlation between
13 AOZ content in edible tissues and biological fluids [16]. In this study, the AOZ
14 concentrations were monitored in edible tissues, plasma and urine of pigs between 0.5
15 to 63 days post administration of furazolidone (400 mg/kg in feed for 7 days) showing
16 good correlation between matrices. AOZ residues occurred at highest concentrations
17 in liver ($2170 \mu\text{g kg}^{-1}$) followed by plasma ($1969 \mu\text{g kg}^{-1}$), urine ($1260 \mu\text{g kg}^{-1}$),
18 kidney ($1012 \mu\text{g kg}^{-1}$) and muscle ($691 \mu\text{g kg}^{-1}$) at 0.5 days [16]. At the 63 day
19 sampling, AOZ residues were measured at 1.1, 1.2, 1.0, 0.6 and $0.6 \mu\text{g kg}^{-1}$ in liver,
20 plasma, urine, kidney and muscle, respectively. Therefore, this study opens
21 possibilities for pre-slaughter screening and residue monitoring of NF metabolites
22 where plasma is suggested as a suitable matrix for on-farm monitoring of livestock.
23 The same group proposes enzyme linked immunosorbent assay (ELISA) method for
24 screening of AOZ, as it is cheap and relatively easy to use. However performance of
25 enzyme immunoassay methods can be limited when used for confirmatory practice at

1 residue level, which can be observed from reported high standard deviation value at 1
2 $\mu\text{g kg}^{-1}$ [16].

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

7

8

9 **2. Experimental**

10

11 *2.1. Materials and reagents*

12 NF metabolites (AOZ, AMOZ and AHD), nitrophenyl (NP) derivatives: 3-((2-Nitro-
13 benzylidene)-amino)-oxazolidin-2-one (NPAOZ), 5-Morpholin-4-ylmethyl-3-((2-
14 nitro-benzylidene)-amino)-oxazolidin-2-one (NPAMOZ), 1-((2-Nitro-benzylidene)-
15 amino)-imidazolidine-2,4-dione (NPAHD), 2-Nitro-benzaldehyde-semicarbazone
16 (NPSEM) and isotopically labelled internal standards (AMOZ-D₅, AOZ-D₄, ¹³C¹⁵N₂-
17 SEM and ¹³C₃-AHD) were all obtained from Witega, Berlin, Germany. SEM
18 (Vetranal grade), 2-nitrobenzaldehyde (2-NBA), ammonium acetate (MS grade) and
19 99.5% deuterated methanol were purchased from Sigma Aldrich. Ultra-pure water
20 (18.2 MΩ) was generated using a Milli-Q Plus water purification system. Methanol
21 and ethyl acetate (EtOAc) (both HPLC grade) were obtained from BDH Chemicals
22 Ltd. (Poole, UK). 0.1N HCl was prepared by diluting 8.6 mL of conc. HCl to 1000
23 mL with water. 1M NaOH was prepared by dissolving 40 g of sodium hydroxide
24 pellets (Analar Grade, BDH) in water and making up to 1L. Trisodium phosphate
25 buffer 0.3M was prepared by dissolving 11.4 g of Na₃PO₄.12H₂O to 100 mL with

1 water. pH test strips 4.5 – 10.0 were obtained from Sigma Aldrich. A Dispensette®
2 III solvent dispenser (Brand GMBH + CO KG; Wertheim Germany) was used for
3 aliquoting EtOAc; Mistral 3000i centrifuge (MSE; London, UK), TopMix multi-
4 vortexer (Fisher Scientific; Dublin, Ireland), Whatman ReZist™ PTFE syringe filters
5 – 13 mm, 0.22 µm and 0.45 µm were obtained from Fisher Scientific (Dublin,
6 Ireland).

7

8 *2.1.1. Standard solutions*

9 Individual primary stock solutions of NF metabolites and their NP derivatives were
10 prepared at a concentration of 50 mg L⁻¹ (free metabolite equivalents) in methanol.

11 Internal standards were prepared at a concentration of 50 mg L⁻¹ in deuterated
12 methanol. All standard solutions in this work were stored at -20°C. Primary stock
13 solutions were found to be stable for one year. Intermediate stock solutions (free
14 metabolites, NP derivatives and unlabelled standards) containing all four target
15 analytes were prepared from individual primary stock solutions at a concentration of 1
16 mg L⁻¹. Working standards were prepared at a concentration of 50 µg L⁻¹ daily from
17 intermediate standard solutions.

18

19 *2.2 Plasma samples*

20

21 *2.2.1 Negative control plasma samples*

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

1 was centrifuged ($2030 \times g$, 20 min) and plasma was transferred into clean glass tube.

2 Plasma samples were stored at -20°C until analysis. Equine plasma samples were
3 supplied by the Irish Equine Centre, Straffan, Co. Kildare and stored as above.

4

5 2.2.2 *Incurred plasma study*

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

24

25 2.3 *Sample preparation*

1 Plasma samples (1 g) were weighed into 50 mL centrifuge tubes and fortified with 40
2 μL of working internal standard ($50 \mu\text{g L}^{-1}$) and let stand for 15 min. 0.1N HCl (9
3 mL) and 100 mM 2-NBA in methanol (100 μL) were sequentially added. Samples
4 were vortexed (1 min) and incubated overnight (16 h, 37°C) in water bath with gentle
5 shaking. After cooling to room temperature, samples are neutralised by adding 0.3 M
6 trisodium phosphate buffer (1 mL) and 1M NaOH (385 μL). pH was checked with test
7 strips (pH 4.5-10) and corrected if necessary to fall in the range pH 6.5 to 7.5.
8 Extraction was performed with EtOAc (18 mL) by shaking samples on mechanical
9 shaker (20 min). The samples were centrifuged ($2030 \times g$, 10 min) and extracts
10 collected into glass tubes. The extraction was repeated with EtOAc (9 mL) and
11 extracts combined. Solvent was evaporated under nitrogen at 40°C . Dry extract was
12 reconstituted in 0.5 mL of injection solution (0.5 mM ammonium acetate and
13 methanol 80:20, v/v) and vortexed (1 min). Extracts were sequentially filtered through
14 $0.45 \mu\text{m}$ and $0.2 \mu\text{m}$ PTFE 13 mm syringe filters into a 200 μL vial. This dual
15 filtration step was required because $0.2 \mu\text{m}$ filters are susceptible to blockage. It is
16 recommended to pass sample extracts through $0.2 \mu\text{m}$ filters to prevent blockage of
17 UHPLC columns.

18 2.4 UHPLC-MS/MS analysis

19 Separation was carried on a Waters Acquity UHPLC system (Milford, MA, USA).
20 The analytical column was an Acquity UHPLC® BEH C_{18} (100 mm \times 2.1 mm, 1.7
21 μm particle size) with Vanguard pre-column C_{18} (5 mm \times 2.1 mm) and maintained at
22 65°C . This temperature is used in most UHPLC-MS/MS applications in our
23 laboratory to prevent column blockage and prolong column life. It is proposed that
24 this blockage is caused by the precipitation of non-polar material at the inlet side of
25 the column during analysis but this risk is reduced by increasing column temperature

1 to 65°C. A binary gradient separation was performed at a flow rate of 0.5 mL min⁻¹.
2 Mobile phase A consisted of aqueous ammonium acetate (0.5 mM) and mobile phase
3 B was MeOH. The gradient profile was as follows: (a) 0 → 0.6 min, 10% B; (b) 2
4 min, 50% B; (c) 2 → 3 min, 50% B; (d) 3.5 min, 80 % B; (e) 3.5 → 5.5 min, 80 % B;
5 (f) 6min, 10 % B; (g) 6 → 9 min, 10 % B. Total run time was 9 min. The UHPLC
6 autosampler was sequentially rinsed using strong and weak washes consisted of
7 H₂O:MeOH (0.5 mL, 10:90, v/v) and MeOH:H₂O (1 mL, 10:90, v/v), respectively.
8 These washes are required to clean the needle and reduce carryover between
9 injections. In addition, the wash conditions are important because they are required
10 for pushing the sample into the injection loop and can impact on the chromatographic
11 peak shape. The sample injection volume was 20 µL. NF metabolites were detected
12 using a Waters Quattro Premier XE triple quadrupole instrument equipped with an
13 electrospray ionisation probe operating in positive mode (Milford, MA, USA).
14 Nitrogen was used for nebulisation, desolvation (1100 L hr⁻¹) and as cone gas (200 L
15 hr⁻¹). The source and desolvation temperatures were 140°C and 400°C respectively.
16 Capillary voltage was 3.0 kV. Argon was used as collision gas. The MS conditions
17 were optimised by tuning the analyte-specific parameters, including cone voltage,
18 collision energy, and collision cell exit potential for each analyte. This was carried
19 out by infusion of a 1 µg mL⁻¹ standard solution and optimising response for the
20 parent and two most intense product ions. The SRM (selected reaction monitoring)
21 windows were time-sectored, and dwell time, inter-scan delay and inter-channel
22 delays were set to get maximum sensitivity from the instrument. Individual transitions
23 and respective collision energies and voltages are listed in **Table 1**. The UHPLC-
24 MS/MS system was controlled by MassLynx™ software and data was processed
25 using TargetLynx™ Software (both from Waters).

1

2 2.5 Calibration

3 Extracted matrix calibration curves were prepared by fortifying negative plasma
4 samples at concentrations of 0.2, 0.5, 1, 2 and 5 $\mu\text{g kg}^{-1}$. Recovery was monitored by
5 analysing plasma samples spiked with NP derivatives at concentrations of 0.25, 0.5, 1
6 and 2 $\mu\text{g kg}^{-1}$ into almost dry extracts and then evaporated to dryness. Regression
7 analyses of the responses (analyte area divided by internal standard area) was
8 performed using TargetLynX™ software.

9 2.6 Method validation

10 The method was validated in house according to Commission Decision 2002/657/EC
11 [17]. Since NFs are banned substances and listed in Annex IV of Council Reg
12 23 77/90, there is no safe limit for presence of these compounds in food. A reference
13 point for action (RPA) for NF metabolites has been established as 1 $\mu\text{g kg}^{-1}$.

14

15 The following parameters were investigated: selectivity, linearity, stability of
16 metabolites in plasma and stability of derivatized metabolites, recovery, within
17 laboratory repeatability (WLR) and reproducibility (WLR), Decision Limit ($CC\alpha$),
18 Detection Capability ($CC\beta$). Selectivity has been assessed through inspection of blank
19 plasma chromatograms for presence of interfering peaks. Additionally bovine plasma
20 samples were fortified with nitrofurans metabolites at 0.2 $\mu\text{g kg}^{-1}$ and with a mixture of
21 38 veterinary anthelmintic compounds and tested routinely to evaluate selectivity in
22 presence of other veterinary substances. Correlation coefficients of over 20 calibration
23 curves were calculated and inspected for linearity. Linearity has also been
24 investigated after testing blank bovine plasma fortified at 0.2, 0.5, 1, 2 and 5 $\mu\text{g kg}^{-1}$
25 with 5 individual replicates at each level.

1 Values of CC_{ct} and CC_{J3} were calculated according to calibration curve procedure
2 from Commission Decision 2002/657/EC [17]. Blank material was fortified at 0.5,
3 0.75 and 1 µg kg⁻¹. Each individual validation run contained seven replicates at each
4 level, five point calibration curve, four recovery controls and a negative control.
5 Testing of samples was performed by three different operators over a period of one.
6 month. This data was used to assess WLR and was included in absolute recovery
7 calculations. WLR was determined from three independent sets of analyses performed
8 by the same operator. Finally method has been applied to equine, ovine and porcine
9 plasma in order to assess accuracy and absolute recovery.

10

11 *2.7 Stability studies in plasma and sample extracts*

12 A stability study of NF metabolites in matrix was performed using blank bovine
13 plasma fortified with nitrofurans metabolites (AHD, AOZ, SEM, AMOZ) at 2 µg kg⁻¹.
14 Plasma has been shaken for 1h on a mechanical shaker to ensure homogeneity and
15 weighed into 30 tubes (1 ± 0.02 g of plasma). Stability of NF metabolites in bovine
16 plasma was evaluated over 1, 2, 3, 4, 8 and 16 weeks storage at -20°C. In addition,
17 the stability of derivatized NF metabolites in extracts prepared for injection was also
18 investigated. A total of 50 blank bovine plasma samples (1 g each) were fortified with
19 internal standard and extracted. After extraction and the evaporation step, they were
20 fortified at 2 µg kg⁻¹ with derivatised metabolites (NPAHD, NPAOZ, NPSEM,
21 NPAMOZ). Extracts were reconstituted in 0.5 ml of injection solution, filtered and
22 placed in 200 µL vials. The stability of derivatised extracts was evaluated at 0, 1, 2,
23 3, and 4 weeks (n=5) under storage at 4 and -20°C.

24

25

1 3. Results and discussion

2

3 3.1. Selectivity

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

11 To confirm selectivity in presence of other veterinary compounds that may be
12 coexisting in plasma, two blank bovine plasma samples were fortified with a mixture
13 of 38 veterinary drugs and their metabolites at $1000 \mu\text{g kg}^{-1}$ level each (albendazole,
14 albendazole-sulphoxide, albendazole-sulphone, albendazole-amino sulphone,
15 cambendazole, fenbendazole, oxfendazole, fenbendazole sulphone, flubendazole,
16 amino-flubendazole, hydroxy-flubendazole, amino-mebendazole, hydroxy-
17 mebendazole, oxibendazole, mebendazole, triclabendazole, triclabendazole-
18 sulphoxide, triclabendazole-sulphone, thiabendazole, 5-hydroxy-thiabendazole,
19 levamisole, bithionol, clorsulon, closantel, morantel, niclosamide, nitroxynil,
20 oxcyclozanide, rafoxanide, coumaphos, coumaphos-oxon, haloxon, abamectin B 1 a,
21 doramectin, emamectin B1a, eprinomectin B1a, ivermectin B1a, moxidectin). No
22 interference has been observed. Additionally, bovine plasma samples fortified with
23 NF metabolites at $0.2 \mu\text{g kg}^{-1}$ showed usual response in presence of all 38 compounds.
24

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

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

14

15 3.2. Linearity

16 Calibration curves have been designed to respond to the current RPA which is set at 1
17 $\mu\text{g kg}^{-1}$ for animal tissue. As there is no level determined for plasma, in our work it
18 has been chosen that this value would present central point in our calibration curve
19 with two calibration points below (0.2 and 0.5 $\mu\text{g kg}^{-1}$) and two above (2 and 5 $\mu\text{g kg}^{-1}$).
20 Correlation coefficients, residual plots and regression statistics were generated
21 from regression analyses of the responses obtained from testing five replicates at each
22 calibration level [20]. Correlation coefficient r and r^2 were higher than 0.995 for all
23 four metabolites, which indicated high degree of correlation between y (response) and
24 x values (concentration). Additionally correlation coefficients of over 20 routine
25 calibration curves were inspected and all were higher than 0.995. Residual plots and

1 variance homogeneity test showed a significant difference for the variance at different
2 concentration levels ($p < 0.05$) and for that reason weighted regression was used ($1/x$)
3 as this is a common case in residual analyses, particularly when for practical reasons
4 chosen calibration points are not equidistant [21]. The analyses of variance (ANOVA)
5 for linear regression produced F and Significance F values (p value). F values were
6 high for all four compounds confirming a good linear relationship and low variability
7 arising from residuals. Significance F values were extremely low and as a probability
8 parameter, it indicated that linear relationship between y and x values for all four
9 analytes is high.

10

11 3.3. *Stability*

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

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

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

4

5 3.4. *Within laboratory reproducibility, repeatability and method recovery*

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

15 Inter-species validation results for equine, ovine, porcine and bovine plasma are
16 shown in **Table 3**. Results showed recovery data for equine, ovine and porcine were
17 similar to bovine plasma. Recovery values for AHD, AOZ, SEM and AMOZ were 62-
18 73, 65-75, 54-63 and 68-73, respectively.

19

20 3.4. *Decision limit ($CC\alpha$) and detection capacity (CCJ)*

21 Calculated values for decision limit ($CC\alpha$) and detection capacity (CCJ) are reported
22 in **Table 3**. $CC\alpha$ values were calculated using inter-species within-laboratory
23 reproducibility results for AHD, AOZ, SEM and AMOZ, and were 0.070, 0.059,
24 0.071 and 0.054 $\mu\text{g kg}^{-1}$. Results obtained could not be compared to other studies, as
25 currently there is no data available for determination of NF metabolites in animal

1 plasma by this method. Comparison with other $CC\alpha$ and $CC\beta$ values obtained in NF
2 residue analyses in different tissues (meat, kidney) shows that our data belong to the
3 same order of magnitude, which is considered a positive indicator of this validation
4 study in plasma matrix.

5

6 *3.5 Application to incurred samples*

7 The performance of the assay was assessed using plasma from pigs treated with a
8 FZD and slaughtered at different withdrawal periods. No plasma was available from
9 bovine animals at the time. Porcine plasma samples were diluted 200-fold with
10 negative bovine plasma to fall in the range of the calibration curve. Samples were
11 tested ($n = 5$) and the mean content of AOZ found in samples B, C and D was 4.65;
12 4.59 and 2.03 $\mu\text{g kg}^{-1}$ respectively (**Table 4**). RSDs associated with these results were
13 2.2, 2.3 and 2.0 % respectively.

14

15 *3.6 Routine application of the method*

16 The method has been applied in official food inspection to over 200 samples of
17 bovine plasma on an annual basis. The results of this surveillance work show that the
18 majority of samples are found to be free of NF residues. In a few cases, residues of
19 FZD have been detected in herds [24]. In all these cases animals were prevented from
20 entering the food chain because early detection had been made on-farm. It is
21 highlighted that these positives are sporadic and less likely to occur in future years
22 because NF based drugs are banned since 1997. However, these few positive findings
23 justify the need for the on-farm monitoring.

24

25

1 4. Conclusion

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

12

13

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16

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18

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

Target metabolites and related internal standards	Parent ion (<i>m/z</i>)	Daughter ion (<i>m/z</i>)	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-AMTZ	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-AMTZ	340.10±0.5	296.1±0.5	0.100	25	12	2.71

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

Analyte	Concentration ($\mu\text{g kg}^{-1}$)					
	0.50	0.75	1.0	0.50	0.75	1.0
	Accuracy WLR (%)			RSD WLR (%)		
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
	Accuracy WLR (%)			RSD WLR (%)		
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

Table 3. Inter-species plasma validation results

Analyte	Concentration ($\mu\text{g kg}^{-1}$)						Recovery (%)	
	0.50	0.75	1.0	0.50	0.75	1.0		
	Accuracy (%)			RSD (%)				
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	
AMAZ	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	
AMAZ	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	
AMAZ	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	
AMAZ	85	90	92	9.4	7.0	4.9	72	
	Inter-species WLR Accuracy (%)			Inter-species WLR RSD (%)			CCa ($\mu\text{g kg}^{-1}$)	CCJ3 ($\mu\text{g kg}^{-1}$)
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
AMAZ	98	98	97	10.0	7.8	5.8	0.054	0.093

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

Animal	Replicate 1	Replicate2	Replicate3 ($\mu\text{g kg}^{-1}$)	Replicate4	Replicate 5	Mean	RSD (%)
A-control	-	-	-	-	-	-	-
B	4.626	4.578	4.620	4.602	4.83 1	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

Fig 1. NF parent compounds, metabolites and NP derivatives

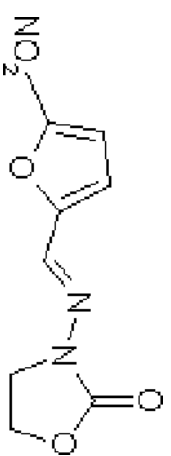
Fig 2. MRM chromatograms of blank bovine plasma at expected RT for AHD, AOZ, SEM and AMOZ

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

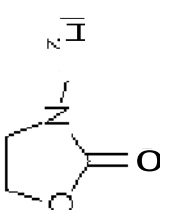
Fig 4. Stability of nitrofurans metabolites in bovine plasma stored at -20°C

Fig 5a. Stability of derivatised metabolites in bovine plasma extract stored at 4°C .

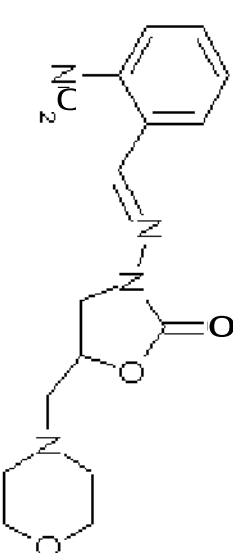
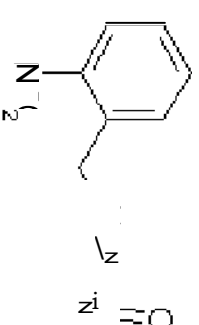
Fig 5b. Stability of derivatised metabolites in bovine extract stored at -20°C .



Furazolidone (FZD)



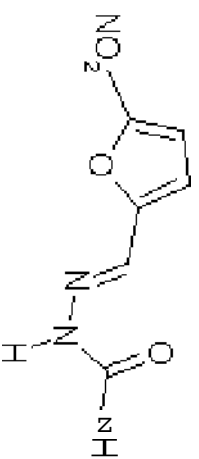
3-Amino-2-oxazolidinone (AOZ)



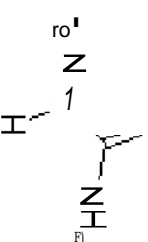
Furaltadone (FTD)

Ar is a phenolino methyl-2-oxazolidinone (AMOT)

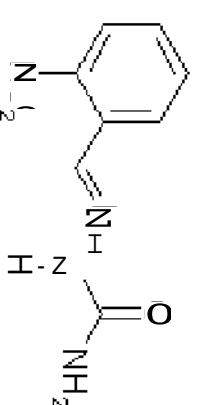
NPSEM



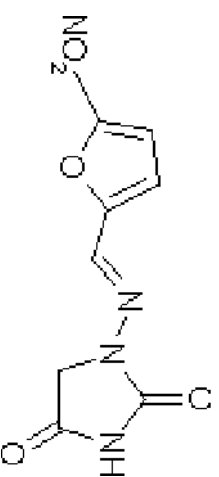
Nitrofurazone (NFZ)



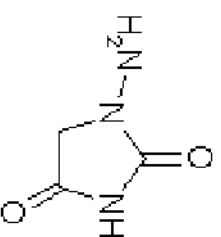
Semicarbazide (SEM)



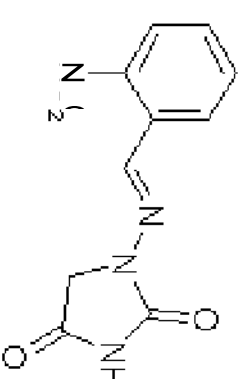
NPSEM



Nitrofurantoin (NFT)



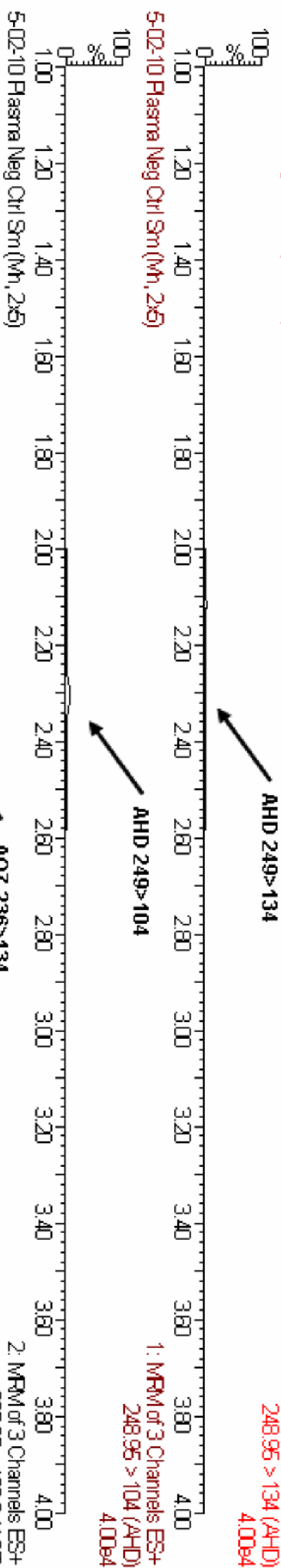
1-Aminohydantoin (AHD)



NPSEM

0 ug/kg

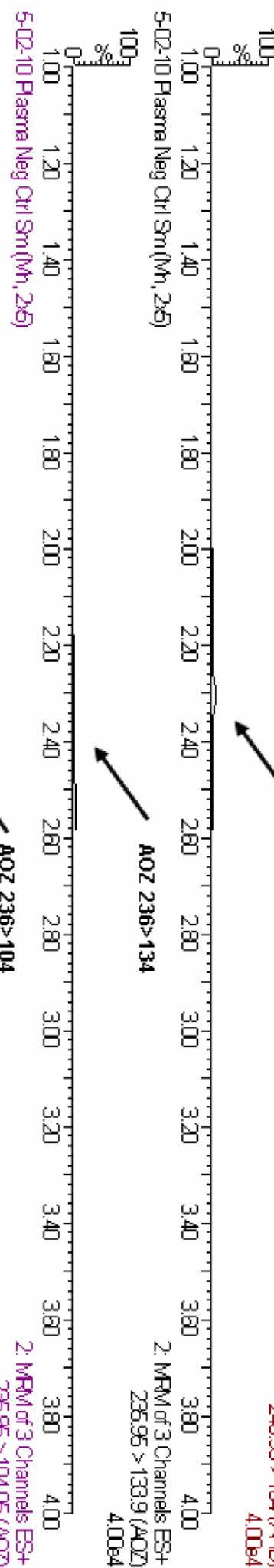
5-02-10 Plasma Neg Ctrl Sm (Mh, 2x5)



AHD 249>134

1. MRM of 3 Channels ES+
248.95 > 134 (AHD)
4.00e4

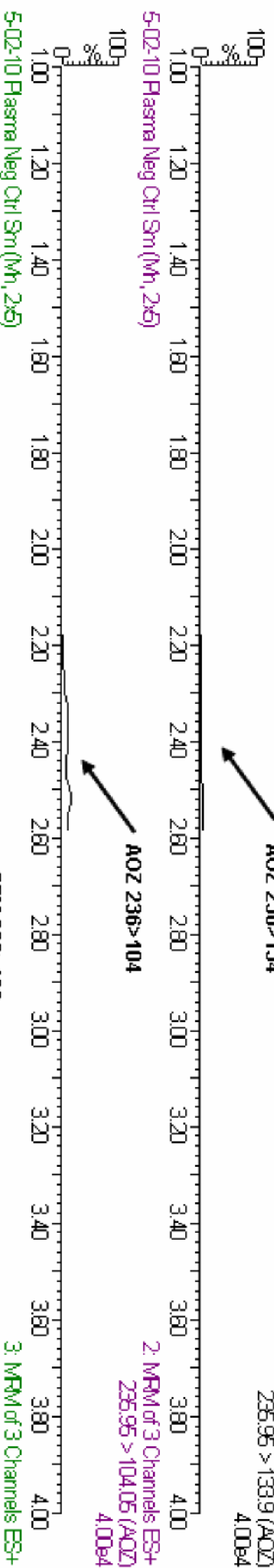
5-02-10 Plasma Neg Ctrl Sm (Mh, 2x5)



AHD 249>104

1. MRM of 3 Channels ES+
248.95 > 104 (AHD)
4.00e4

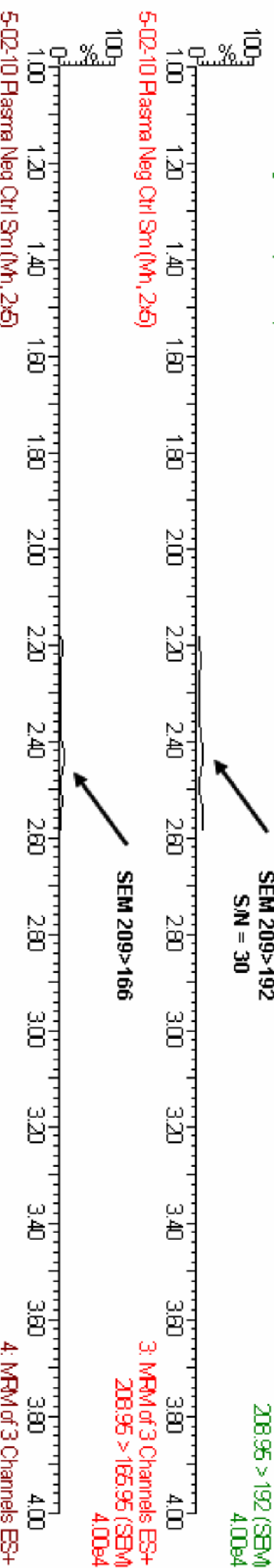
5-02-10 Plasma Neg Ctrl Sm (Mh, 2x5)



AOZ 236>104

2. MRM of 3 Channels ES+
236.95 > 104.05 (AOZ)
4.00e4

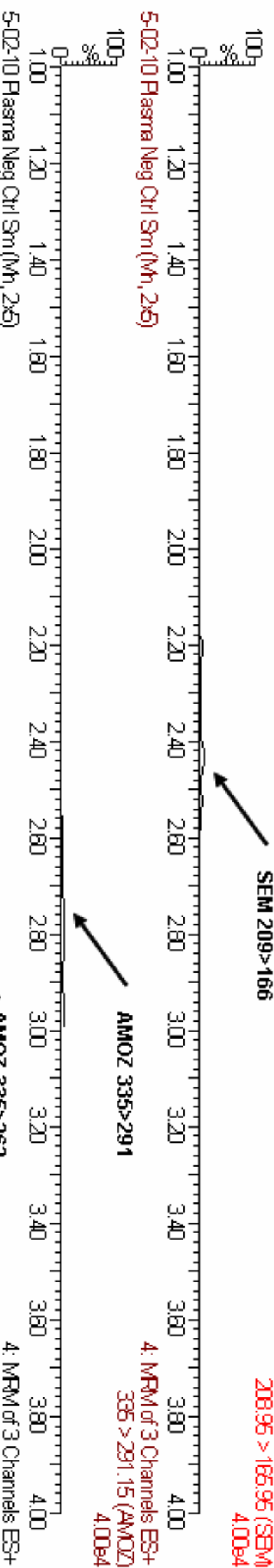
5-02-10 Plasma Neg Ctrl Sm (Mh, 2x5)



SEM 209>192
S/N = 30

3. MRM of 3 Channels ES+
208.95 > 192 (SEM)
4.00e4

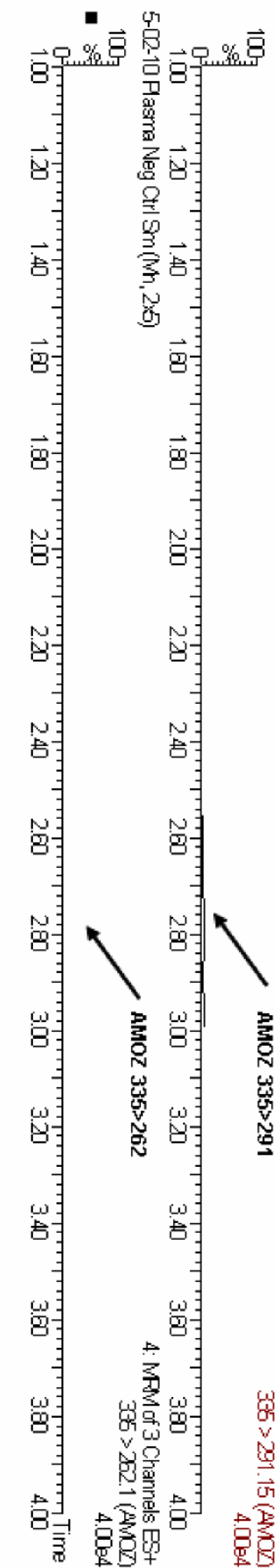
5-02-10 Plasma Neg Ctrl Sm (Mh, 2x5)



SEM 209>166

3. MRM of 3 Channels ES+
208.95 > 165.95 (SEM)
4.00e4

5-02-10 Plasma Neg Ctrl Sm (Mh, 2x5)



AMOZ 335>291

4. MRM of 3 Channels ES+
335 > 291.15 (AMOZ)
4.00e4

5-02-10 Plasma Neg Ctrl Sm (Mh, 2x5)



AMOZ 335>262

4. MRM of 3 Channels ES+
335 > 262.1 (AMOZ)
4.00e4

0.2 ug/kg

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

AHD 249>134
SN = 40

1: MRM of 3 Channels ES+
248.95 > 134 (AHD)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

AHD 249>104
SN = 50

1: MRM of 3 Channels ES+
248.95 > 104 (AHD)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

SEM 209>166
SN = 23

2: MRM of 3 Channels ES+
235.95 > 133.9 (AOZ)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

SEM 209>192
SN = 30

2: MRM of 3 Channels ES+
235.95 > 104.05 (AOZ)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

AMOZ 335>291
SN = 60

3: MRM of 3 Channels ES+
208.95 > 192 (SEM)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

AMOZ 335>291
SN = 60

3: MRM of 3 Channels ES+
208.95 > 165.95 (SEM)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

AMOZ 335>291
SN = 60

4: MRM of 3 Channels ES+
335 > 291.15 (AMOZ)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

AMOZ 335>291
SN = 60

4: MRM of 3 Channels ES+
335 > 262.1 (AMOZ)
4.00e4

5-02-10 plasma Pos CH1 1 Sm (Mh, 2x5)

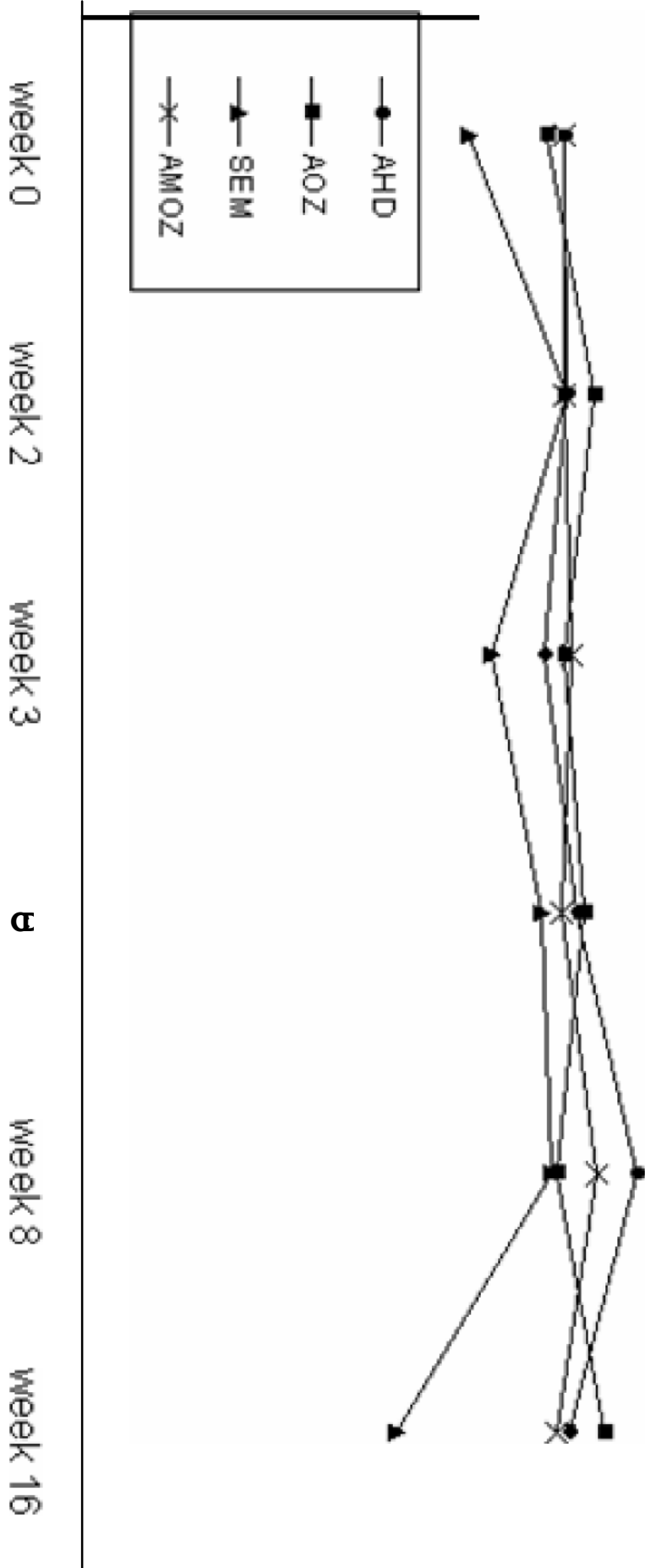
AMOZ 335>291
SN = 60

4: MRM of 3 Channels ES+
335 > 262.1 (AMOZ)
4.00e4

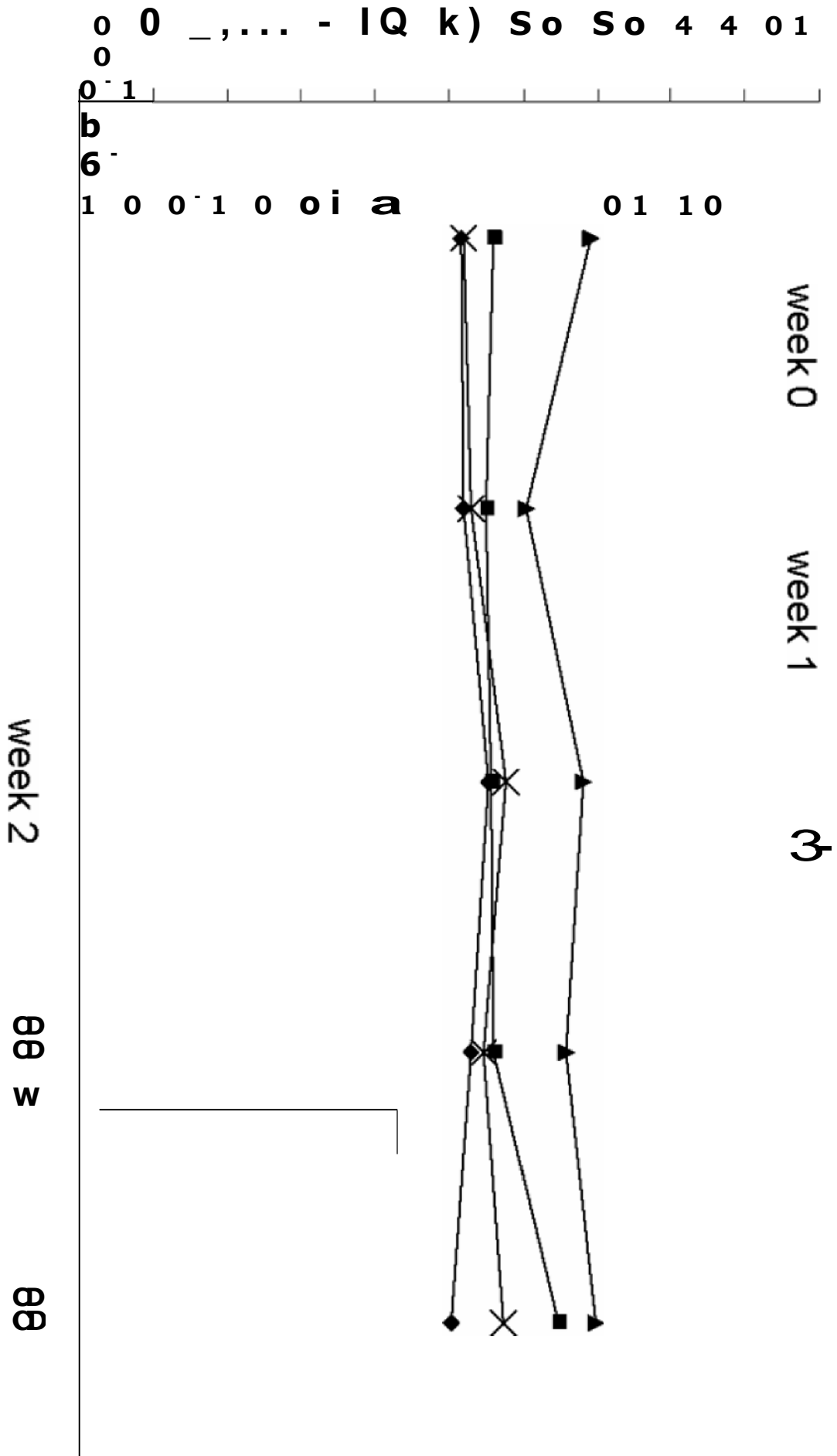
Concentration (pg kg⁻¹)

N . . .) 1

.01



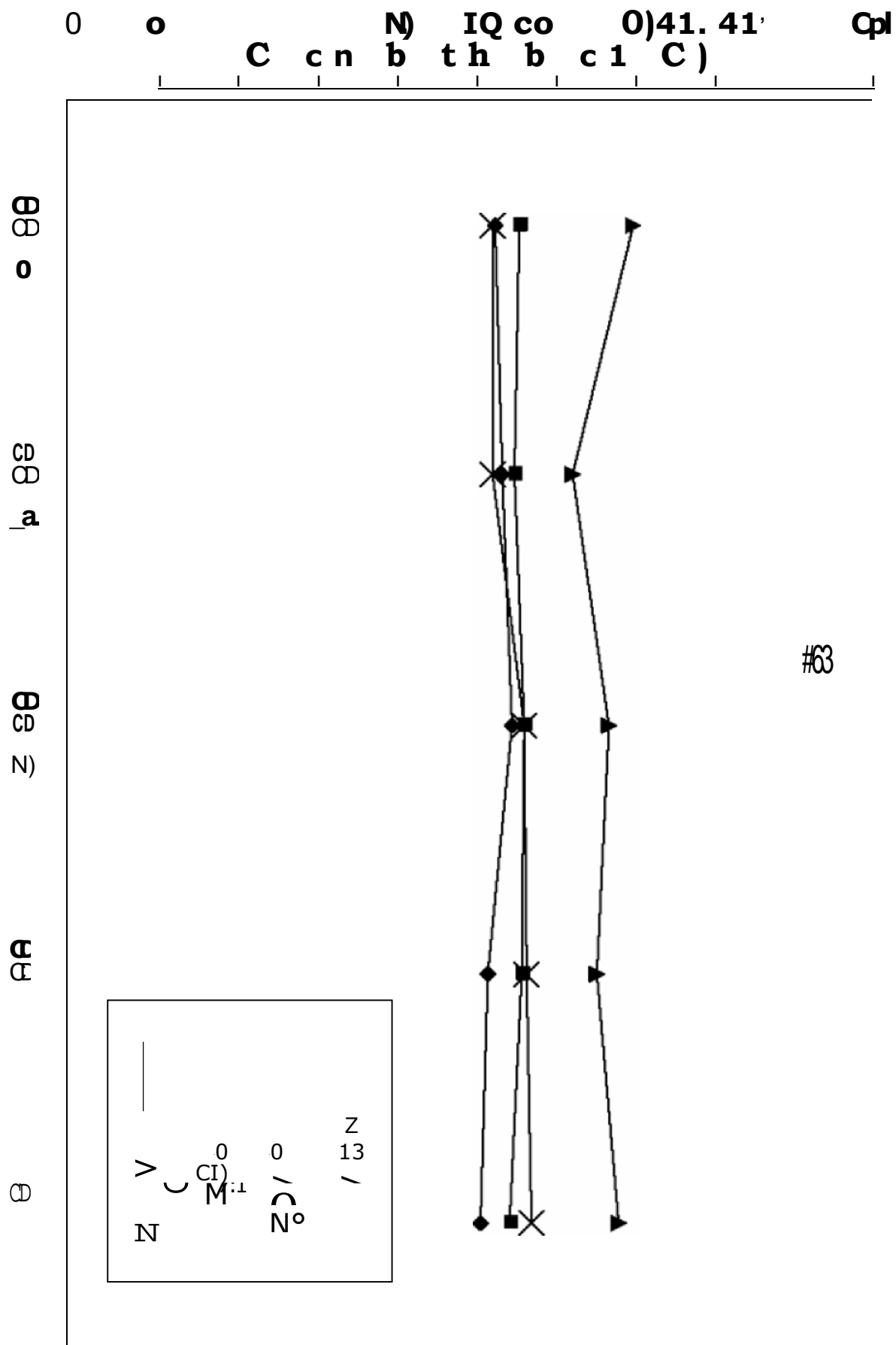
Concentration (pg kg⁻¹)



\mathbf{Z}
 \mathbf{I}
 \mathbf{V}
 \mathbf{N}

\mathbf{z} \mathbf{T} \mathbf{z} \mathbf{a} \mathbf{z}
 (\mathbf{I}) $>$ $:1$
 \mathbf{m} $\mathbf{0}$ $\mathbf{1}$ $>$
 \mathbf{N} $\mathbf{0}$

Concentration (pg kg⁻¹)



#63