1. Identification

FADM 594: Determination of Nitrofurans as their tissue bound metabolites in Animal Tissue.

2. Scope

Method is suitable for the confirmatory analysis according to EU criteria [4] of residues of metabolites of nitrofuran drugs in muscle tissue.

3. Description of items to be tested

As outlined in Scope.

4. Parameters/quantities and ranges to be determined

Analyte	Muscle
1-amino-hydantoin (AHD)	0.030 – 5.0 µg/kg
3-amino-2-oxalidinone (AOZ)	0.019 - 5.0 µg/kg
semicarbazide (SEM)	0.20 - 5.0 µg/kg
5-methylmorpholino-3-amino-2-oxalidinone (AMOZ)	0.013 - 5.0 µg/kg
4-Hydroxy-benzhydrazide (HBH)	0.070 - 5.0 µg/kg
3,5-dinitrosalicylic acid hydrazide (DNSAH)	0.058 - 5.0 µg/kg
Oxamic Acid Hydrazide (OAH)	0.20 - 5.0 µg/kg
Aminoguanidine (AGN)	0.017 - 5.0 µg/kg

The reporting limits have been derived from validation data.
The reporting limits shown may be extended upwards by dilution of the sample extract.

5. Apparatus and equipment, including technical performance requirements.

Usual laboratory apparatus not otherwise specified, and the following items:

5.1 Centrifuge Tubes with screw caps polypropylene, 50 mL, 120mm x 36 mm (Sarstedt, P.N. 62.559 or equivalent)

5.2 Centrifuge Tubes with screw caps polypropylene, 15 mL (Sarstedt, P.N. 62.554.502 or equivalent)

5.3 A glass dispenser or equivalent for acetonitrile

5.4 CEM Mars 6 Microwave (240/50 Model No.910905)

5.5 Ceramic Homogenisers (Agilent Technologies, P.N. 5982-9312)

5.6 Hettich Rotanta 460R Centrifuge

5.7 Homogeniser, Polytron

5.8 PTFE Cross Stirrer Bars, 8 x 20 mm (VWR, P.N. 442-0395, or equivalent)

5.9 Merris Minimix vibrational shaker or equivalent

5.10 Multi-vortexer

5.11 Disposable Graduated Transfer pipettes (VWR, P.N. 612-4494, or equivalent)

5.12 Turbovap LV (Caliper Life Sciences)

5.13 Syringe Filters, 0.2 µm PTFE (Agilent PN: 5190-5265) or Mini-Uniprep syringeless filter device, 0.2 µm PTFE (Whatman US503NPEORG) .

5.14 pH Test Strips 4.5-10.0 or equivalent (Sigma Chemical Co., St. Louis, Missouri, USA, P.N. p4536)

5.15 UPLC Glass Vials, 2 mL (Agilent Technologies, P.N. 5182-0714)

5.16 Glass inserts, 400 µL (Agilent Technologies, P.N. 5181-3377)

5.17 UHPLC-MS/MS system comprising a stainless steel analytical column (Agilent ZORBAX Eclipse Plus Phenyl-Hexyl RRHD 1.8 µm particle 2.1 x 50 mm, Part no. 959757-912), fitted with a Waters Critical Clean FRIT (0.2 µm, 2.1 mm), an AB Sciex 5500+ QTRAP Mass Spectrometer, equipped with a TurboV Ion Source, coupled to an Exion LC. The pump is operated at a flow rate of 0.6 ml/min. The UPLC-MS/MS system is controlled by Analyst 1.7.1 software (Sciex).

6. Reference standards and reference materials required

No reference standards/materials are used in this method.

7. Environmental conditions and stabilisation period required

Normal laboratory environmental conditions apply, as specified RP020

8. Safety measures to be observed

No particular safety measures apply.

9. Description of procedure

9.1 Principle

Tissue-bound residues are hydrolysed by the action of dilute hydrochloric acid. The side chains of the nitrofuran drugs are released under these conditions. The residues released and free metabolites alike are derivatised under mild acidic conditions with 2-nitrobenzaldehyde to their corresponding nitrophenyl derivatives. After neutralisation the derivatives are extracted with acetonitrile. The organic solvent is evaporated and the residue is redissolved in a mixture of methanol/water, filtered and transferred to a UPLC vial. Determination is performed by UHPLC-MS/MS analysis. Residues of

metabolites of furazolidone, furaltadone, nitrofurazone, nitrofurantoin nifuroxazide, nifursol, nifuraldezone and nitrovin, are quantified using deuterated analogues and isotope labelled analogues, added as internal standards to the sample before hydrolysis.

9.2 Reagents

All reagents shall be of analytical quality. The water used shall be 18.2 MΩcm water. Where grades/manufacturers are mentioned, these reagents have been found to be suitable. Expiry dates for prepared solutions shall be 12 months unless otherwise indicated.

9.2.1 Acetonitrile, 200 far UV (Romil Ltd. "SpS" Super Purity Solvent, Cambridge, UK. P.N.: H048)

9.2.2 Ethanol (Absolute, Merck, Germany, P.N. 1.00986.2500)

9.2.3 Trisodium phosphate.dodecahydrate 0.3M, $Na_3PO_4.12H_2O$ (Merck KGaA, P.N. 1.06578.1000). Dissolve 11.4 g in 100 ml water.

9.2.4 2-Nitrobenzaldehyde 100 mM (Sigma, P.N. N10802). Dissolve 1.51 g NBA in 100 ml methanol.

9.2.5 Water (Millipore, 18.2 MΩcm).

9.2.6 Sodium hydroxide 1M (Riedel de Haen, P.N. 30620). Dissolve 40 g NaOH pellets in 1000 ml water.

9.2.7 Ammonium Formate (Puriss, p.a., Sigma Aldrich Ireland, Tallaght, Dublin 24, P.N. 70221-100G-F or equivalent)

9.2.8 Methanol, (Romil Ltd., United Kingdom, PN: H409) or equivalent.

9.2.9 Hydrochloric acid (ACS Reagent, 37%, Sigma Aldrich, 258148-2.5L)

9.2.10 Hydrochloric acid 1M and 0.1 M.

a. **1M:** Dilute 9.8 ml conc. HCl (9.2.8) to 100 ml with water (9.2.4). This gives a 1M solution.
b. **0.1M:** Dilute 9.8ml conc. HCl (9.2.8) to 1000 ml with water (9.2.5). This gives a 0.1M solution.

9.2.11 Propan-2-ol, PN: H625 (Romil Ltd., United Kingdom) or equivalent

9.2.12 Diethyl Ether, PN: 24004-2.5L-M (Sigma now Merck, Germany) or equivalent.

9.2.13 Magnesium Sulphate, anhydrous powder, very fine (Enviro Clean, EMAG002K, UCT or equivalent.)

9.2.14 Sodium Chloride (PanReac Applichem, Darmstadt, Germany, P.N. 131659.1211 or Sigma-aldrich Bio Xtra (S7653)).

9.2.15 UPLC mobile phase A: 5 mM ammonium formate in 90/10 (v/v) water: methanol
Dissolve 0.3153 g of ammonium formate (9.2.7) in 90/10 (v/v) water (9.2.5):methanol (9.2.8). Degas in an ultrasonic bath for 15 min. Prepare daily.

9.2.16 UPLC mobile phase B: 5 mM ammonium formate in 10/90 (v/v) water: methanol. Dissolve 0.3153 g of ammonium formate (9.2.7) in 10/90 (v/v) water (9.2.5):methanol (9.2.8). Degas in an ultrasonic bath for 15 min. Prepare as required.

(NOTE: The composition of the UPLC mobile phase may be varied to ensure separation of analyte peak from matrix peaks; all such changes shall be noted in the appropriate Laboratory Notebook(s), chromatograms and other records).

9.2.17 Needle Wash: methanol (9.2.8)-water (9.2.5), 90/10 (v/v). Degas in an ultrasonic bath for 15 minutes. Prepare every three months as needed.

9.2.18 Seal Wash: water (9.2.5)-propan-2-ol (9.2.11)-, 90/10 (v/v). Degas in an ultrasonic bath for 15 minutes. Prepare as required.

9.2.19 Sample injection solvent: 5 mM ammonium formate (9.2.7) in 90/10 (v/v) water (9.2.5): methanol (9.2.8). Prepare daily.

9.2.20 *Nitrofuran Standard Solutions*

9.2.20.1 3-Amino-2-oxazolidinone (AOZ)
Weigh (0.005g X 100/F) of AOZ (Witega, Germany, NF005) into a 100ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of AOZ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.2 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ).
Dissolve (0.005g X 100/F) AMOZ (Witega, German, NF003) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of AMOZ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.3 1-aminohydantoin (AHD)
Dissolve (0.005g X 100/F) AHD (Witega, Germany, NF001) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of AHD in the particular batch of material in use as shown in the certificate of analysis). This gives a concentration of 50 µg/ml.

9.2.20.4 Semicarbazide (SEM)
Dissolve ((0.005g X 100/F)*factor) SEM (Sigma P.N. 33656) in a 100 ml volumetric flask [the factor is calculated by dividing the molecular weight of SEM.HCl by the molecular weight of SEM]. Make up to the mark with methanol (9.2.8). F is the content (%) of SEM in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.5 3,5 Dinitrosalicylhydrazide (DNSAH)
Dissolve (0.005g X 100/F) DNSAH (Witega, Germany, NF028) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of DNSAH in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml

9.2.20.6 4-Hydroxybenzhydrazide (HBH)
Dissolve (0.005g X 100/F) HBH (Toronto Research Chemicals, Canada, H829100) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of HBH in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml

9.2.20.7 Oxamic Acid Hydrazide (OAH)
Dissolve (0.005g X 100/F) OAH (Witega, Germany, NF036) in a 100 mL volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of OAH in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.8 Aminoguanidine (AGN)
Dissolve ((0.005g X 100/F)*factor) AGN (Sigma Aldrich, Germany, 396494) in a 100 mL volumetric flask. [the factor is calculated by dividing the molecular weight of AGN.HCl by the molecular weight of AGN]. Make up to the mark with methanol (9.2.8). F is the content (%) of AGN in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.9 3-((2-nitrophenyl)methylene)-amino-2-oxazolidinone (NPAOZ)
Dissolve (0.005g X 100/F) NPAOZ (Witega, Germany, NF013) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of NPAOZ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.10 5-Methylmorfolino-3-((2-nitrophenyl)methylene)-3-amino-2-oxazolidinone (NPAMOZ)
Dissolve (0.005g X 100/F) NPAMOZ (Witega, Germany, NF011) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of NPAMOZ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.11 1-((2-nitrophenyl)methylene)-amino-2-hydantoin (NPAHD, NP009)
Dissolve (0.005g X 100/F) NPAHD (Witega, Germany) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of NPAHD in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.12 (2-nitrophenyl)methylene-semicarbazide (NPSEM)
Dissolve (0.005g X 100/F) NPSEM (Witega, Germany, NF015) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content

(%) of NPSEM in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.13 3,5-(2-nitrophenyl)-Dinitrosalicyclhydrazide (NPDNSAH)
Dissolve (0.005g X 100/F) NPDNSAH (Witega, Germany, NF035) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of NPDNSAH in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.14 4-(2-nitrophenyl)-Hydroxybenzhydrazide (NPHBH)
Dissolve (0.005g X 100/F) NPHBH (Witega, Germany, NF031) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of NPHBH in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.15 (2-nitrophenyl)-Oxamic Acid Hydrazide (NPOAH)
Dissolve (0.005g X 100/F) NPOAH (Within lab synthesis) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of NPOAH in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.16 (2-nitrophenyl)-Aminoguanidine (NPAGN)
Dissolve (0.005g X 100/F) NPAGN (Witega, Germany, NF032) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of NPAGN in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.17 3-Amino-2-oxazolidinone-d_4 (AOZ-d_4)
Weigh (0.005g X 100/F) of AOZ-d_4 (Witega, Germany, NF006) into a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of AOZ-d_4 in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.18 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one-d_5 (AMOZ-d_5).
Dissolve (0.005g X 100/F) AMOZ-d_5 (Witega, Germany, NF004) in a 100 ml volumetric flask. Make up to the mark with methanol (9.2.8). F is the content (%) of AMOZ-d_5 in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.19 Semicarbazide (SEM-$^{15}N_2\,C^{13}$)
Dissolve ((0.005g X 100/F)*factor) SEM-$^{15}N_2\,C^{13}$ (Witega, Germany, NF008) in a 100 ml volumetric flask [the factor is calculated by dividing the molecular weight of SEM.HCl-$^{15}N_2\,C^{13}$by the molecular weight of SEM-$^{15}N_2\,C^{13}$]. Make up to the 100 ml mark with methanol (9.2.8). F is the content (%) of SEM-$^{15}N_2\,C^{13}$ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.20 1-aminohydantoin (AHD-$^{13}C_3$)

Dissolve (0.005g X 100/F) AHD-$^{13}C_3$ (Witega, Germany, NF002) in a 100 ml volumetric flask. Make up to the 100 ml mark with methanol (9.2.8). F is the content (%) of AHD $^{13}C_3$ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.21 3,5 Dinitrosalicylhydrazide (DNSAH-$^{13}C_6$)
Dissolve (0.005g X 100/F) DNSAH-$^{13}C_6$ (Witega, Germany, NF029) in a 100 ml volumetric flask. Make up to the 100 ml mark with methanol (9.2.8). F is the content (%) of DNSAH-$^{13}C_6$ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.22 4-Hydroxybenzhydrazide (HBH-$^{13}C_6$)
Dissolve (0.005g X 100/F) HBH-$^{13}C_6$ (Witega, Germany, NF033) in a 100 ml volumetric flask. Make up to the 100 ml mark with methanol (9.2.8). F is the content (%) of HBH-$^{13}C_6$ in the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.23 Oxamic Acid Hydrazide (OAH-$^{15}N_3$)
Dissolve (0.005g X 100/F) OAH-$^{15}N_3$ (Witega, German, NF037) in a 100 ml volumetric flask. Make up to the 100 ml mark with methanol (9.2.8). F is the content (%) of OAH-$^{15}N_3$ the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

9.2.20.24 Aminoguanidine (AGN-$^{13}C^{15}N_4$)
Dissolve (0.005g X 100/F) AGN-$^{13}C^{15}N_4$ (Witega, Germany, NF039) in a 100 ml volumetric flask. Make up to the 100 ml mark with methanol (9.2.8). F is the content (%) of AGN-$^{13}C^{15}N_4$ the particular batch of material in use as shown in the certificate of analysis. This gives a concentration of 50 µg/ml.

Note: A factor is used to correctly calculate the amount of standard material needed when the standard material is, for example, in the form of a salt (.HCl) or contains water of crystallization (.H_2O). The factor is calculated by dividing the molecular weight of the standard material being weighed by the molecular weight of the nitrofuran residue being tested for. Where a factor is required, this is specified in points 9.2.20.1 to 9.2.20.23 above.

9.2.20.24 Mixture of AOZ, AMOZ, AHD, SEM, DNSAH, HBH, OAH, AGN - **MM1**
Transfer 200 µl of 50 µg/ml stock solutions of AOZ (9.2.20.1), AMOZ (9.2.20.2), AHD (9.2.20.3), SEM (9.2.20.4) DNSAH (9.2.20.5), HBH (9.2.20.6), OAH (9.2.20.7) and AGN (9.2.20.8) into a 10 ml volumetric flask and make up to the mark with methanol (9.2.8). This gives a concentration of 1 mg/l.

9.2.20.25 Mixture of AOZ, AMOZ, AHD, SEM, DNSAH, HBH, OAH, AGN - **MM2**
Transfer 50 µl of the standard mixture MM1 (9.2.20.24) (1 mg/l) to a glass culture tube. Add 950 µl methanol (9.2.8) and vortex. This gives a concentration of 50 µg/l. Prepare daily.

9.2.20.26 Mixture of AOZ, AMOZ, AHD, SEM, DNSAH, HBH, OAH, AGN – **MM3**

Transfer 100 µl of the standard mixture MM2 (9.2.20.25) (50 µg/l) to a glass culture tube. Add 900 µl methanol (9.2.8) and vortex. This gives a concentration of 5 µg/l. Prepare daily.

9.2.20.27 Mixture of AOZ, AMOZ, AHD, SEM, DNSAH, HBH, OAH, AGN – **MM4**
Transfer 100 µl of the standard mixture MM3 (9.2.20.26) (5 µg/L) to a glass culture tube. Add 900 µl methanol (9.2.8) and vortex. This gives a concentration of 0.5 µg/L. Prepare daily.

9.2.20.28 Mixture of NPAOZ, NPAMOZ, NPAHD, NPSEM, NPDNSAH, NPHBH. NPOAH, NPAGN - **NP1**
Transfer 461 µl of 50 µg/ml NPAOZ (9.2.20.9), 332 µl of 50 µg/ml NPAMOZ (9.2.20.10), 431 µl of 50 µg/ml NPAHD (9.2.20.11), 555 µl of 50 µg/l NPSEM (9.2.20.12), 310 µl of 50 µg/ml NPDNSAH (9.2.20.13), 375 µl of 50 µg/ml NPHBH (9.2.20.14), 458 µl of 50 µg/ml NPOAH (9.2.20.15) and 559 µl of 50 µg/ml NPAGN (9.2.20.16) into a 10 ml volumetric flask and make up to the mark with methanol (9.2.8). This gives a concentration of 1 mg/l based on free metabolites

9.2.20.29 Mixture of NPAOZ, NPAMOZ, NPAHD, NPSEM, NPDNSAH, NPHBH. NPOAH, NPAGN - **NP2**
Transfer 1 ml of the standard mixture NP1 (1 mg/l) (9.2.20.28) into a 100 ml volumetric flask and make up to the mark with methanol (9.2.8). This gives a concentration of 10 µg/l based on free metabolites.

9.2.20.30 Mixture of AOZ-d4, AMOZ-d5, SEM-$^{15}N_2$ C^{13}, AHD-$^{13}C_3$, DNSAH-$^{13}C_6$, HBH-$^{13}C_6$, OAH-$^{15}N_3$, AGN-$^{13}C^{15}N_4$-**IS1**
Transfer 200 µl of AOZ-d4 (50µg/ml) (9.2.20.17), AMOZ-d5 (50 µg/ml) (9.2.20.18), SEM-$^{15}N_2$ C^{13} (50 µg/ml) (9.2.20.19), AHD-$^{13}C_3$ (50µg/ml) (9.2.20.20), DNSAH-$^{13}C_6$ (50 µg/ml) (9.2.20.21), HBH-$^{13}C_6$ (50 µg/ml) (9.2.20.22), OAH-$^{15}N_3$ (50 µg/ml) (9.2.20.23) and AGN-$^{13}C^{15}N_4$ (50 µg/ml) (9.2.20.24) into a 10 ml volumetric flask and make up to the mark with methanol (9.2.8). This gives a concentration of 1 mg/l.

9.2.20.31 Mixture of AOZ-d4, AMOZ-d5, SEM-$^{15}N_2$ C^{13}, AHD-$^{13}C_3$, DNSAH-$^{13}C_6$, HBH-$^{13}C_6$, OAH-$^{15}N_3$, AGN-$^{13}C^{15}N_4$ - **IS2**
Add 100µL of the standard mixture IS1 1 mg/l (9.2.20.30) to 1.9 ml of methanol (9.2.8) in a glass culture tube. This gives a concentration of 50 µg/l. Prepare daily.

9.2.20.32 Mixture of AOZ-d4, AMOZ-d5, SEM-$^{15}N_2$ C^{13}, AHD-$^{13}C_3$, DNSAH-$^{13}C_6$, HBH-$^{13}C_6$, OAH-$^{15}N_3$, AGN-$^{13}C^{15}N_4$ – **IS3**
Add 100µL of the standard mixture IS2 50 µg/l (9.2.20.31) to 0.9 ml of methanol (9.2.8) in a glass culture tube. This gives a concentration of 5 µg/l. Prepare daily.

9.2.20.33 Working standard solution of nitrophenyl derivatives- **WS1**
Transfer 1 ml of the standard mixture NP2 10 µg/l (9.2.20.29) to a 10 ml volumetric flask and make up to the mark in sample injection solvent (9.2.19).

This gives a concentration of 1 µg/l. Adjust volumes depending on quantity needed. Prepare daily.

9.3 Sample

9.3.1 Proceed from the total sample provided for analysis.

9.3.2 Test portions for tissue samples are weighed by taking a minimum of three aliquots from different parts of the tissue sample, combining, chopping and mixing them and then weighing from this combined portion.

9.3.3 Store the sample in such a way (normally frozen) that deterioration and change in composition are prevented.

9.4 Procedure

9.4.1 Prewashing (TISSUE SAMPLES ONLY)

9.4.1.1 Weigh 1.0 ± 0.01 g of tissue into a 50 ml centrifuge tube (5.1). Return to the freezer prior to analysis.

9.4.1.2 Add 1 ml of water (9.2.5) followed by 8 ml of ice cold methanol (9.2.8). Homogenise (5.7) at full speed for not more than 1 min. Note: if more than one rack of samples are being analysed then, following addition of water and methanol, one rack is kept in the fume cupboard for the samples to be homogenised. The others are placed in the freezer until homogenisation. This ensures that the methanol remains ice cold.

9.4.1.3 Centrifuge (5.6) at 2000 rpm (4° C) for 10 min. Pour off and discard the supernatant.

9.4.1.4 Wash the tissue pellet by adding 4 ml of ice cold methanol (9.2.8) and vortex (5.10) for 10 sec.

9.4.1.5 Centrifuge at 2000 rpm (4° C) for 10 min. Pour off and discard the supernatant.

9.4.1.6 Repeat steps (9.4.1.4 – 9.4.1.5) twice, each time with 4 ml portions of ice-cold methanol (9.2.8).

9.4.1.7 Repeat steps (9.4.1.4 – 9.4.1.5) twice, each time with 4 ml of ice-cold ethanol (9.2.2).

9.4.1.8 Repeat steps (9.4.1.4 – 9.4.1.5) twice, each time with 4 ml of diethyl ether (9.2.12)

9.4.1.9 Cover samples loosely in order to leave ether evaporate overnight.

9.4.2 *Hydrolysis and derivatization*

9.4.2.1 Break up sample pellet with a metal spatula. Add 100 µl of standard solution IS3 (9.2.20.32) to the sample pellet. Fortify the matrix calibrants as per Table 1. Leave all samples for 15 minutes.

Table 1: Preparation of fortified samples (Matrix Calibrants)

Concentration in sample (µg/kg)	Standard mixture MM (9.2.20.25-.27) (µl)	Internal standard Mixture IS3 (9.2.20.32) (µl)
	Tissue	Tissue
0.02	40 µL MM4	100
0.04	80 µL MM4	100
0.2	40 µL MM3	100
0.5	100 µL MM3	100
1	200 µL MM3	100
2	40 µL MM2	100
5	100 µL MM2	100

9.4.2.2 Add 100 µl of 100 mM NBA solution (9.2.4) and 9 mL of 0.1 M HCl (9.2.10), close the tube and shake sample by hand to disperse pellet. Add a magnetic stirring bar (5.8) to each tube.

9.4.2.3 Vortex (5.10) for 1 minute.

9.4.2.4 Place samples in the CEM microwave (5.4) for the derivatisation reaction, ramping from room temperature to 60 °C over 4 min, followed by a 120 min hold at 60 °C. Up to 49 (plus one temperature probe sample) can be handled at one time.

9.4.3 *Neutralisation*

9.4.3.1 After removal from the microwave, add 1ml of 0.3 M trisodium phosphate (9.2.3) and shake gently by hand.

9.4.3.2 Add 540 µl of 1 M NaOH (9.2.6) and swirl/shake gently by hand. Check pH is between 6.5 and 7.5 using pH test strips (5.14). If not, adjust pH accordingly with either 1 M HCL (9.2.10) or 1 M NaOH (9.2.6). Remove the stirring bars by sliding a magnetic rod up the outside of the tubes.

9.4.5 *Extraction*

9.4.5.1 For tissue samples, add 10 mL of acetonitrile (9.2.1) using a dispenser (5.3) to each tube.

9.4.5.2 Add approximately 1 g of sodium chloride (9.2.14) to each tube and vortex (5.10) for 1 min.

9.4.5.3 Add approximately 4 g of $MgSO_4$ (9.2.13) and a ceramic homogeniser (5.5) to each tube and shake on the minimix vibrational unit (5.9) for 5 min. Up to 36 samples may be handled at a time.

9.4.5.4 Centrifuge (5.6) at 3500 rpm for 12 min at 4 ºC.

9.4.5.5 Carefully transfer the supernatant using a plastic Pasteur pipette (5.11) to a 15 mL tube (5.2).

9.4.5.6 For test samples and matrix calibrants, evaporate the acetonitrile to dryness on a Turbovap LV (5.12) at a temperature not exceeding 40 ± 2 ºC, under nitrogen, staring at 5 psi and increasing to 18 psi as the volume decreases.

9.4.5.7 For recovery control samples, evaporate the acetonitrile to 1-2 mL on a Turbovap LV (5.12) at a temperature not exceeding 40 ± 2 ºC, under nitrogen. Fortify recovery control samples as per Table 2 and continue evaporation to dryness.

Table 2: Preparation of fortified samples (Recovery controls)

Concentration in sample (µg/kg)	Standard mixture NP2 (9.2.20.29) (µl)
	Tissue
0.25	25
0.5	50
1	100
2	200

9.4.5.8 Reconstitute all extracts in 0.5 mL of sample injection solvent (5.2.19) and vortex for 60 sec.

9.4.5.9 Filter the resulting turbid extracts through a 13 mm, 0.2 µM PTFE filter directly into a UPLC vial (5.15), fitted with a 300 µL insert (5.16). Apply gentle pressure to ensure that the extract is collected in a drop wise fashion. Alternatively Mini Unipreps (5.13) may be used to filter and inject the samples from.

9.4.6 *Determination*

9.4.6.1 Determine acceptable performance of the UPLC-MS/MS system by injecting the WS-1 1 µg/l standard (9.2.20.33). The signal to noise ratio for each of the

16 transitions described in Table 3 is measured (not the internal standards), this ratio should be at least 3:1. The retention time is also checked for each transition to ensure that it falls approximately in the centre of the window in which the ion is acquired. If not, the retention windows described in Table 3 are changed so that each transition occurs approximately in the centre of that window.

9.4.6.2 Analyse the sample extracts on the UPLC-MS/MS system (5.17), together with appropriate standards (9.2.20). Inject 10 µl of sample extracts and standards. A check sample should be injected at evenly spaced intervals throughout the run. This sample is used to monitor both retention time and detector response and ensure that each stay constant throughout the run. For retention time, each check must be within ± 2% of the mean retention time value. For detector response, the RSD of the checks must be ≤ 15%. A retention time check sheet will be included with the data pack for each analytical run. The detector response will be recorded on the system suitability sheet (EQ-05) for the analytical run and this will be stored with the data pack.

9.4.6.3 A peak determined in a sample shall be deemed to be the analyte if it meets all the criteria set out in Section 10, Expression of Results. The sample may be reanalysed to offer further confirmation of the presence of an analyte in said sample.

9.5 Controls

Negative control samples, matrix calibrants and recovery control samples, as described below, are incorporated in each batch of samples. The calibrants used in this test method are extracted matrix matched calibrants.

9.5.1 A 1 g aliquot of a control sample (i.e. a sample not containing nitrofuran residues) is analysed using the same procedure as for the test samples (9.4.1-9.4.6). Where a measurable response is obtained for the control tissue sample, this is subtracted from the response for the fortified samples (9.5.2, 9.5.3) in calculating recovery.

9.5.2 Extracted Matrix Matched Calibrants: control sample, fortified with AOZ, AMOZ, AHD, SEM, DNSAH, HBH, OAH and AGN. From a proven blank material of the same matrix as the test samples, seven test portions of 1.0 ± 0.01 g are weighed into 50 mL centrifuge tubes (5.1). For tissue samples, pre-washing – steps 9.4.1.2 to 9.4.1.9 - is carried out. For all matrices, following step 9.4.2.1, these blank samples are fortified as indicated in Table 1, left to equilibrate for 15 minutes, and analysed using the same procedure as for the test samples (9.4.2.2).

9.5.3 Recovery controls: blank sample fortified with the nitro-phenyl derivatives of AOZ, AMOZ, AHD, SEM, DNSAH, HBH, OAH and AGN. From a proven blank material of the same matrix as the test samples, four test portions of 1.0 ± 0.01 g are weighed into 50 ml centrifuge tubes (5.1). For tissue samples, pre-washing – steps 9.4.1.2 – 9.4.1.9 – is carried out. For all matrices, 100 µl of internal standard mixture IS3 (9.2.20.32) is added to these samples; they are let equilibrate for 15 minutes and then analysed using the same procedure as for the test samples (9.4.2.2).

After evaporation of the acetonitrile phase to 1-2 ml (9.4.5.7) the samples are fortified, according to Table 2, and the evaporation is continued to dryness. The procedure is then continued with reconstitution (9.4.5.8) and further.

9.5.4 Reagent blanks: derivatisation reagents are pipetted into an empty tube (5.1) at the same time as they are added to the samples (9.4.2.2). This mix is injected onto the UPLC-MS/MS to ensure no signal results from the presence of the derivatisation reagents.

9.6 *UPLC-MS/MS Analysis*

UPLC conditions:

Flow:	0.6 ml/min
Injection volume:	10 µl
Column Temperature:	40°C
Sample Temperature:	15°C
Total run time:	9 min

The gradient Timetable contains 10 entries, which are:

Time	Flow	% A	% B
0.0	0.60	95	5
1.0	0.60	95	5
5.0	0.60	60	40
6.7	0.60	60	40
6.8	0.60	50	50
8.0	0.60	50	50
8.1	0.60	0	100
9.5	0.60	0	100
9.6	0.60	95	5
11.0	0.60	95	5

Sciex 5500+: MS conditions:

Ion Source:	TurboV Drive	**CAD Gas:**	8
Ion Spray Voltage Positive:	+1400 V	**Entrance Potential (EP):**	10 V
Ion Spray Voltage Negative:	-1400 V	**Curtain Gas Pressure:**	30 psi
Source Temperature:	650 ºC	**Ion Source Gas 1 (GS1):**	70 psi
Collision Gas:	Nitrogen (N_2)	**Ion Source Gas 2 (GS2):**	70 psi

MS/MS Fragmentation Conditions:

NPAOZ, NPAMOZ, NPAHD and NPSEM, NPDNSAH , NPHBH, NPOAH and NPAGN fragment to structure related product ions (Table 3). The isotope labelled analogues used as internal standards exhibit similar fragmentations.

Table 3: MS/MS fragmentation conditions

Compound Name	Precursor Ion	Product Ion	RT (min)	MRM Window	DP	CE	CXP	Pol.
NPAHD	249.0	134.1	4.62	20	95	18	7	+
NPAHD	249.0	104.0	4.62	20	91	31	13	+
2NPAHD-$^{13}C_3$	252.1	134.1	4.60	30	89	18	7	+
NPAOZ	236.1	134.1	4.81	30	91	19	16	+
NPAOZ	236.1	104.2	4.81	30	87	31	13	+
2NPAOZ-D4	240.1	134.3	4.75	30	90	19	7	+
NPSEM	209.1	192.1	3.72	30	80	16	11	+
NPSEM	209.1	166.2	3.72	30	67	15	9	+
2NPSCA-$^{13}C^{15}N_2$	212.1	168.1	3.72	30	59	16	9	+
NPAMOZ	335.1	291.1	6.45	30	80	18	15	+
NPAMOZ	335.1	262.2	6.45	30	84	25	14	+
2NPAMOZ-D5	340.1	296.1	6.35	30	64	17	17	+
NP-3-5DNSAH	374.0	226.0	7.30	150	-93	-34	-7	-
NP-3-5DNSAH	374.0	182.1	7.30	150	-87	-30	-5	-
NP-3-5DNSAH-$^{13}C_6$	380.1	188.0	7.30	150	-75	-30	-10	-
NPHBH	286.0	121.1	5.75	30	66	27	7	+
NPHBH	286.0	93.0	5.57	30	67	54	11	+
NPHBH-$^{13}C_6$	292.0	127.2	5.75	30	97	31	7	+
NPOAH	237.1	192.1	3.31	30	66	20	10	+
NPOAH	237.1	135.2	3.31	30	59	30	7	+
NPOAH-$^{15}N_3$	240.1	194.1	3.31	30	69	19	10	+
NPAGN	208.1	119.2	2.45	60	87	20	11	+
NPAGN	208.1	149.1	2.45	60	86	28	6	+
NPAGN-$^{13}C^{15}N_4$	213.0	92.2	2.45	60	71	34	16	+

Key: RT = Retention Time, MRM window = multiple reaction monitoring window, DP = Declustering Potential, CE= Collision Energy, CXP= Collision Exit Potential, Pol= Polarity

Table 4: Sciex 5500+: Time Segment

#	Start Time (min)	Scan Type	Divert Valve
1	0.0	Dynamic MRM	To Waste
2	2.1	Dynamic MRM	To MS
3	8.4	Dynamic MRM	To Waste

9.7 Column Care

9.7.1 Pre-Run

9.7.1.1 Prior to injecting samples, the column must be equilibrated by setting the column

temperature to 40 °C, flow rate of 0.1 mL min^{-1}at 100% MPB and gradually building to 0.6 mL min^{-1}.

9.7.1.2 Once the pressure is stable, change the mobile phase composition to 50% MPB until the pressure stabilises.

9.7.1.3 Change the mobile phase composition to 5 % MP. until the pressure stabilises reflecting the starting gradient.

9.7.1.4 Start the equilibration mode on the system and run for a minimum of 40 min before commencing analysis.

9.7.2 Post-Run

9.7.2.1 Wash the column with 50/50 (v/v) water:methanol (Line A2) at 0.6 mL min^{-1} for 20 min ("Post_Wash" acquisition method)

9.7.2.2 Wash the column with 100% acetonitrile (Line B2), at 0.6 mL min-1 for 20 min (Post_Wash_organic" acquisition method).

10 Expression of Results

Calculate the ion ratio (Eq. I) and the relative deviation of the ion ratio (Eq. II) for each analyte compared to the positive control samples.

The identity of the analyte is confirmed when the EU criteria for ion ratio and retention time similarity to the matrix calibrants are met [4]. The ion ratio should not deviate from the target ion ration by more than +/- 40% for deviation in retention time the value should be less than 1 %.

Calculate the response factor for each analyte (Eq. III). Calculate the amount of analyte present (Eq. IV).

Recovery value for each analyte is calculated by plotting response factor (Eq. III) against concentration for the matrix calibrants and the recovery control samples.

The linearity of the standard curve shall be assessed from the coefficient of determination for the standards used to make up the curve; an acceptable limit for the coefficient of determination is normally ≥ 0.98, residuals should not exceed +/-20%. Sometimes it is necessary to remove points from the calibration curve to achieve adequate linearity. If all samples are to be reported as negative and there is adequate sensitivity at the reporting level then the number of points removed to achieve linearity is incidental. If quantitative results are to be reported a five point calibration is necessary. If this cannot be achieved, analysis of the sample must be repeated.

The recovery is then deemed to be the slope of the matrix calibration curve divided by the slope of the recovery control curve expressed as a percentage.

Equation I: Calculation of the ion ratio (R)

$$R = \left(\frac{A_{lowest\ intensity\ ion}}{A_{highest\ intensityion}} \right)$$

R = ion ratio
$A_{lowest\ intensity\ ion}$ = peak area of the product ion of lowest intensity
$A_{highest\ intensity\ ion}$ = peak area of the product ion of highest intensity

Equation II: Calculation of the relative deviation of the ion ratio (ΔR)

$$\Delta R = \left(\frac{R_{sample} - R_{mean}}{R_{mean}} \right) \times 100\%$$

ΔR = relative deviation of the ion ratio of the analyte in the sample compared to the average ion ratio of the same analyte in the matrix calibrants fortified at 1.0 µg/kg and higher
R_{sample} = ion ratio of the analyte in the sample (Eq.1)
R_{mean} = average ion ratio of the analyte in the matrix calibrants fortified at 1.0 µg/kg and higher (%) (Eq.1)

Equation III: Calculation of the response factor (RF)

$$RF = \left(\frac{Peakarea_{lowest\,intensityion}}{Peakarea_{IS}} \right)$$

RF = response factor
$Peak\ area_{lowest\ intensity\ ion}$ = peak area of the product ion of lowest intensity.
$Peak\ area_{IS}$ = peak area of the product ion of the analyte

Equation IV: Calculation of the amount of analyte present in the sample

$$X = \left(\frac{RF - b}{a} \right)$$

X = amount of analyte in the sample (µg/kg)*
RF = response factor (Eq. III)
b = intercept of the calibration curve obtained from linear regression according to the least-squares-fit method**
a = slope of the calibration curve obtained from linear regression according to the least-squares-fit method**
*Expressed as underivatised metabolite
**Plot the response factor of the matrix calibrants against the fortification level and apply linear regression (least-squares-fit)

11. Criteria and/or requirements for approval/rejection of results

Suitable linearity of the calibration curve (see section 10) and acceptable signal to noise values (signal to noise of the extracted matrix calibrants to be ≥ 10) combined with acceptable recovery of analyte from fortified samples (generally 60-120%, or limits as

calculated from the mean (n = 10) of recovery results ± 2 SD) deem the assay to be satisfactory. However in some cases due to the fact that there is an internal standard added to each sample, recovery values outside this range are acceptable as the internal standard is used to correct for recovery and gives an absolute value for the amount of analyte present. The need for repeat assay is determined from this examination of data.

12. Data to be recorded and method of analysis and presentation

Test report: The test report should show the result obtained (this result is already corrected for recovery, based on internal standard recovery correction).

The test method used should be described as:
"FADM 594: Nitrofurans in animal tissue, eggs and plasma
The samples were prewashed with various organic solvents (not plasma samples) and the tissue-bound nitrofuran metabolite residues were hydrolysed with acid and derivatised with 2-nitrobenzaldehyde. The nitrophenyl derivatives were extracted with acetonitrile and determined by LC-MS/MS, using deuterated analogues as internal standards for quantification."

The test report should mention any operating conditions not specified in this method.

13. Procedure for estimating uncertainty

Where required, derive an estimate for uncertainty of measurement according to the procedure described in RP021

14. References

1. European Agency for the Evaluation of Medicinal Products (EMEA): Furazolidone Summary Report, 1997.

2. E. Horne, A. Cadogan, M. O'Keeffe and L.A.P. Hoogenboom, Analysis of protein-bound metabolites of furazolidone and furaltadone in pig liver by high-performance liquid chromatography and liquid chromatography-mass spectrometry, 1996, *Analyst,* **121**, 1463-1468.

3. R.J. McCracken and D.G. Kennedy. The bioavailability of residues of the furazolidone metabolite 3-amino-2-oxazolidinone in porcine tissues and the effect of cooking upon the residue concentrations, 1997, *Food Additives and Contaminants,* **14**, 507-513.
4. Commission Decision (2002/657/EC) of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and interpretation of results, *O. J. Europ. Comm.* L 221, 8-36.

15. Modification or deviations from standard or non-standard methods.

None.