CLG-NFUR 3.01		Page 1 of 18
Title: Screening and Confirmation of Four Nitrofuran Metabolites by Liquid Chromatography-Tandem Mass Spectrometry		
Revision: .01	Replaces: CLG-NFUR3.00	Effective: 03/07/2016

Contents

A. INTRODUCTION

1. Summary of Procedure

Nitrofuran antibiotics (furazolidone, furaltadone, nitrofurantoin, and nitrofurazone) are typically analyzed as their respective metabolites: 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydatoin (AHD), and semicarbazide (SEM). Control tissues are washed with methanol and ethanol to remove unbound nitrofuran residues and other material prior to fortification and derivative formation. Sample tissues can either be washed or not washed before extraction to allow for detection of bound or unbound/bound analytes. Metabolites and any unbound residues are obtained from blended tissue samples using incubation under acid hydrolysis conditions and simultaneously derivatized using 2-nitrobenzaldehyde. The extract is neutralized, and the derivatized metabolites (2-NP-AOZ, 2-NP-AMOZ, 2-NP-AHD, and 2-NP-SEM) are isolated using liquid-liquid extraction with ethyl acetate followed by screening and confirmation using liquid chromatography-tandem mass spectrometry (LC/MS/MS). Following the liquid - liquid extraction, the evaporated extract reconstituted in water is washed with hexanes prior to filtration and analysis.

2. Applicability

This method is suitable for the screening and confirmation of nitrofuran metabolites (3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydatoin (AHD), and semicarbazide (SEM)) in avian (poultry) and fish of the order Siluriformes (catfish) muscle at the levels listed in Appendix J. 5.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Balance– Top loading, model number PB302, Mettler.

b. Centrifuge Tubes – 50 mL polypropylene, Cat No REF 352096, BD Falcon.

c. Centrifuge Tubes – 15 mL polypropylene, BD Falcon , Cat. REF 352096 or Glass Disposable 15 mL, Kimble No 73790-15.

d. Centrifuge, refrigerated – Model Rotanta 460R, Andreas Hettich GmbH & Co.

e. Vortexer – Vortexer-2, Cat. No. 58816-123, VWR Scientific.

f. Pipettors – Variable volumes: 0-200 µL, 0-1mL, and 0-5mL .

g. Test tube rack – racks for 15 and 50 mL. tubes. The derivative formation rack holding the 50 mL falcon tubes should be capable of withstanding the temperature of 39° for 16 hours. Nalgene recommended.

h. Incubator –- INNOVA 3100 Water Bath Shaker, New Brunswick Scientific Cat No M1231-000

i. Sample Concentrator- TurboVap LV Biotage Corp.

j. Filters - Xpertek syringe filter 13mm , 0.45µm PTFE, P J Cobert Associates, Cat No. 9445611

k. Syringe- 1 mL plastic Becton, Dickinson and Company , REF 309602

l. High speed microcentrifuge - Model Eppendorf Centrifuge 5417C, Eppendorf North America

m. Centrifugal Filter - 0.2 µm 500µl mofified nylon, VWR P/N 82031-356

n. Autosampler Vial - Qsert amber 300 µL screw cap. Waters Corporation P/N 186002803

o. Autosampler vial caps - Screw cap with pre-split PTFE/Silicone septa, Waters corporation P/N 186000305

p. Volumetric flask – 50 and 100 mL amber, class A.

q. HPLC mobile phase filtering and degassing apparatus – Microfiltration Assembly, 47 mm, Millipore.

2. Instrumentation

a. Mass spectrometer – Thermo Finnigan, TSQ Quantum Access Max.

b. HPLC- Dionex Ultimate 3000 UHPLC Focused equipped with Ultimate 3000 Binary Pump, Autosampler, and Column Oven.

c. LC Column – Luna 5µm C18(2) 100Å, 150 X 2.0 mm.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever is soonest.

1. Reagents

a. Methanol (MeOH) - HPLC grade, Cat. No. A456-4, Fisher Scientific.

b. Ethanol (EtOH) - 200 Proof, Cat. No. 493546-1L, Sigma Aldrich.

c. Ethyl acetate (EtOAc) - HPLC grade, Cat. No. E196-4, Fisher Scientific .

d. Hexanes - HPLC grade, Cat. No. H303-4, Fisher Scientific

e. Water - Deionized, HPLC grade, ELGA Pure Lab Ultra system.

f. 2-Nitrobenzaldehyde - Cat. No. 72780-50G, Fluka Analytical.

g. Dimethylsulfoxide (DMSO) - Cat. No. 154938-1L, Sigma-Aldrich.

h. Hydrochloric acid (HCl) - Concentrated, Cat. No. A144S-500, Fisher Scientific.

i. Sodium hydroxide (NaOH) - Pellets, 97+% A.C.S. reagent, Cat. No. 221465-500G, Sigma Aldrich..

j. Ammonium acetate (NH_4OAc), Analytical reagent grade - Cat. No. 3272-04, Macron Chemicals.

k. Potassium phosphate dibasic anhydrous (K_2HPO_4) - Cat. No. P288-500, Fisher Scientific.

2. Solutions

a. 0.1M K_2HPO_4 :

Weigh 17.41 g of K_2HPO_4 into a 1 L volumetric flask or graduated cylinder. Dilute to volume with deionized water.

b. 10 mM 2-nitrobenzaldehyde in DMSO:

Add 8 mg ± 0.6 mg of 2-nitrobenzaldehyde into 5 mL of DMSO. Prepare daily.

c. Aqueous Mobile Phase - 1 mM aqueous ammonium acetate: methanol (80:20):

Weigh 0.0617 ± 0.0020 g NH_4OAc (Mass Spec grade) and transfer to a 1000 mL graduated cylinder. Add 800 mL deionized HPLC grade water. Bring to 1000 mL with methanol. It is optional to vacuum filter through a 0.45 µm or 0.2 µm nylon filter.

d. Organic Mobile Phase - 1 mM aqueous ammonium acetate: methanol (10:90):

Weigh 0.0617 ± 0.0020 g NH4OAc (Mass Spec grade) and transfer to a 1000 mL graduated cylinder. Add 100 mL deionized HPLC grade water. Bring to 1000 mL with methanol. It is optional to vacuum filter through a 0.45 µm or 0.2 µm nylon filter.

e. 10.0 mM Aqueous Ammonium Acetate:

Weigh 0.0617 ± 0.002g NH4OAc (mass Spec grade) and transfer to a 100 mL graduated cylinder bring to 100 mL with water.

f. 1 N Hydrochloric acid:

Using a graduated cylinder measure 83 mL of concentrated hydrochloric acid.

Add the 83 m L of acid into a 1L volumetric flask containing water. Mix and bring to volume with water.

g. 1 N Sodium hydroxide:

Dissolve 20.0 g sodium hydroxide in water and transfer to a 500 mL volumetric flask. When cool bring to volume with water and mix.

Caution this is an exothermic reaction: let solution cool before adjusting to final volume. Store solution in a plastic container.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever ends sooner.

1. Standard Information

a. 3-Amino-2-oxazolidinone (AOZ), ($C_3H_6N_2O_2$), MW 102.09, CAS # 80-65-9, Cat. No. 33347-50MG-R, Sigma-Aldrich.

b. 3-(2-Nitrobenzylidenamino)-2-oxazolidinone (2-NP-AOZ), ($C_{10}H_9N_3O_4$), MW 235.20, CAS # 19687-73-1, Cat. No. 33868-10MG-R, Riedel-de-Haen through Sigma-Aldrich.

c. 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), ($C_8H_{15}N_3O_3$), MW 201.22, CAS # 43056-63-9, Cat. No. 33349-50MG-R, Sigma-Aldrich.

d. 5-(Morpholinomethyl)-3-(2-nitrobenzylidenamino)-2-oxazolidinone (2-NP-AMOZ), ($C_{15}H_{18}N_4O_5$), MW 334.33, CAS # 183193-59-1, Cat. No. 33869-10MG-R, Sigma-Aldrich.

e. 3-Amino-5-morpholinomethyl-2-oxazolidinone-d5, or 4,4,5,α,α-Pentadeutero-3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ-d5),($C_8H_{10}D_5N_3O_3$), MW 206.25, CAS#1017793-94-0, Cat. No. 33881-10MG-R, Sigma-Aldrich.

f. 1-Aminohydantoin hydrochloride (AHD),($C_3H_5N_3O_2$·HCl), MW 151.55, CAS# 2827-56-7, Cat. No. 33655-100MG-R, Sigma-Aldrich.

g. 1-(2-Nitrobenzylidenamino)-2,4-imidazolidinedione (2-NP-AHD), ($C_{10}H_8N_4O_4$), MW 248.19, Cat. No. 33870-10MG-R, Sigma-Aldrich.

h. Semicarbazide hydrochloride (SEM),($NH_2CONHNH_2$ · HCl), MW 111.53, CAS# 563-41-7, Cat. No. 33656-100MG-R, Sigma-Aldrich.

i. 2-Nitrobenzaldehyde semicarbazone (2-NP-SEM),($C_8H_8N_4O_3$) MW 208.17, CAS# 16004-43-6, Cat. No. 33871-10MG-R, Sigma-Aldrich.

2. Preparation of Standard Solution(s)

Note: Different solution concentrations may be prepared as long as fortification volumes are adjusted accordingly.

a. AOZ, AMOZ, AHD, and SEM Stock standard solutions (~25 µg/mL):

Weigh 2.5 ± 1 mg AOZ, AMOZ, AHD, and SEM into separate 100 mL amber volumetric flasks. Dissolve and bring to volume with methanol. Calculate the exact concentration of the stock solutions taking the actual weight and purity into account. This standard will expire in 5 months when stored at < -10 ºC.

b. AOZ/AMOZ/AHD/SEM Combined Intermediate standard solution (250 ng/mL):

Pipet ~1.0 mL (depending on the exact concentration) of each stock standard (D.2.a) into a 100 mL amber volumetric flask and bring to volume with methanol. This standard will expire in 5 months when stored at < -10 ºC.

c. AOZ/AMOZ/AHD/SEM Combined fortification standard solution (10 ng/mL):

Pipet 2.0 mL of the Intermediate standard solution (D.2.b.) into a 50 mL amber volumetric flask and bring to volume with methanol. This standard will expire in 5 months when stored at < -10 ºC.

d. 2-NP-AOZ Stock Standard Solution (equivalent to ~100 µg/mL AOZ):

Weigh 2.30 mg ± 1 mg of 2-NP-AOZ into a 10 mL amber volumetric flask. Dissolve and bring to volume with methanol. Calculate the exact concentration of the stock solutions taking the actual weight and purity into account. This stock standard will expire in 5 months when stored at < -10 ºC.

e. 2-NP-AMOZ Stock Standard Solution (equivalent to ~100 µg/mL AMOZ):

Weigh 1.66 mg ± 1 mg of 2-NP-AMOZ into a 10 mL amber volumetric flask. Dissolve and bring to volume with methanol. Calculate the exact concentration of the stock solutions taking the actual weight and purity into account. This stock standard will expire in 5 months when stored at < -10 ºC.

f. 2-NP-AHD Stock Standard Solution (equivalent to ~ 100 µg/mL AHD):

Weigh 2.16 mg ± 1 mg of 2-NP-AHD into a 10 mL amber flask .Dissolve and bring to volume with methanol. Calculate the exact concentration ofthe stock solutions taking the actual weight and purity into account. This stock standard will expire in 5 months when stored at < -10 °C.

g. 2-NP-SEM Stock Standard Solution (equivalent to 100 µg/mL SEM):

Weigh 2.77 mg ± 1 mg of 2-NP-SEM into a 10 mL amber flask. Dissolve and bring to volume with methanol. Calculate the exact concentration of the stock solutions taking the actual weight and purity into account. This stock will expire in 5 months when stored at < -10 °C.

h. 2-NP-AOZ / 2-NP-AMOZ / 2-NP-AHD / 2-NP-SEM mixed intermediate standard solution (equivalent to 250 ng/mL AOZ, 250 ng/mL AMOZ, 250 ng/mL AHD, and 250 ng/mL SEM):

Pipet ~250 µL of 2-NP-AOZ stock standard solution (D.2.d), ~250 µL of 2-NP-AMOZ stock standard solution (D.2.e), ~250 µl 2-NP-AHD stock standard Solution (D.2.f), and ~ 250 µl 2-NP-SEM (D.2.g) depending on the exact concentrations, into a 100 mL amber volumetric flask. Bring to volume with methanol. This standard will expire in 5 months when stored at < -10 ºC.

i. 2-NP-AOZ / 2-NP-AMOZ / 2-NP-AHD / 2-NP-SEM combined working standard solution (equivalent to 5 ng/mL AOZ, 5 ng/mL AMOZ, 5 ng/mL AHD, and 5 ng/mL SEM):

Pipet 1.00 mL of 2-NP-AOZ / 2-NP-AMOZ / 2-NP-AHD / 2-NP-SEM mixed intermediate standard solution (D.2.h.) into a 50 mL amber volumetric flask. Dilute to volume using water. This working standard will expire in 48 hours when stored at 2 - 8 ºC.

j. AMOZ-d5 Internal Standard (IS) Stock Solution (~25µg/mL).

Weigh 2.5 ± 1 mg AMOZ-d5 into a 100 mL amber volumetric flask. Dissolve and bring to volume with methanol. Calculate the exact concentration of the stock solutions taking the actual weight and purity into account. This standard will expire in 5 months when stored at < -10 ºC.

k. AMOZ-d5 Internal Standard (IS) Intermediate Solution (250 ng/mL)

Pipet ~1.0 mL (depending on the exact concentration) of the AMOZ-d5 stock standard (D.2.j) into a 100 mL amber volumetric flask and bring to volume with methanol. This standard will expire in 5 months when stored at < -10 ºC.

l. AMOZ-d5 Internal Standard (IS) Fortification Solution (10 ng/mL)

Pipet 2.0 mL of the Intermediate standard solution (D.2.k.) into a 50 mL amber volumetric flask and bring to volume with methanol. This standard will expire in 5 months when stored at < -10 ºC.

E. SAMPLE PREPARATION

1. Cut tissue sample into smaller pieces and homogenize in a blender or food processor.

2. Transfer homogenized sample into a plastic bag and store in a freezer at < -10 ºC.

3. Let sample partially thaw prior to analysis.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

   a. Weigh 2.0 ± 0.1 g tissue into a 50 mL polypropylene centrifuge tube, allow tissues to thaw and do the following described below:

      i. Screening - Prepare one each for a blank (negative control), a decision level 0.5 ng/g recovery, a 0.5 ng/g recovery, and a check sample, if necessary.

      ii. Confirmation - Prepare one each for a blank, a 0.5 ng/g recovery, a 1 ng/g recovery, a 2 ng/g recovery, a recovery comparable to the target concentration, and, if necessary, a check sample.

      iii. Samples - Prepare one for each sample. Continue with F.1.b to analyze for bound analytes only. Skip steps F.1.b through F.1.g and continue with F.1.h.iii to analyze for both bound and unbound analytes.

   b. Add 8 mL MeOH and 1 mL H_2O.

   c. Vortex for approximately 30 seconds. A spatula can be used to disperse packed sample.

   d. Centrifuge for 10 minutes at approximately 3400 rpm at ~3 °C. Discard supernatant.

      Note: Refrigeration for centrifuging is not required.

   e. Add 5 mL MeOH, and repeat steps F.1.c. and F.1.d.

   f. Add 5 mL EtOH, and repeat steps F.1.c. and F.1.d.

   g. Repeat step F.1.f. Then redisperse the packed sample as described in F.1.c.

   h. Prepare positive controls at this time by fortifying known blank samples with the AOZ/AMOZ/AHD/SEM combined fortification standard solution (D.2.c.) and all samples with the AMOZ-d5 Internal Standard (IS) Fortification Solution (D.2.l).

      i. Screening

         Add 100 µL for a 0.5 ng/g decision level and analyst recovery.

      ii. Confirmation

         Add 100 µL for a 0.5 ng/g recovery.

         Add 200 µL for a 1.0 ng/g recovery.

         Add 400 µL for a 2.0 ng/g recovery.

      iii. Add 200 µL internal standard to all controls and samples.

2. Extraction Procedure

   a. Add 4 mL of deionized water, 0.5 mL 1 N HCl, and 200 µL 10 mM 2-nitrobenzaldehyde solution in DMSO (C.2.b.) to each tube. Vortex for approximately 30 seconds.

   b. Incubate at 35 - 39 °C for at least 16 hours.

   c. Add 5 mL 0.1M K2HPO4, 0.4 mL 1N NaOH, and 5 mL EtOAc.

   d. Vortex for approximately 30 seconds.

   e. Centrifuge for 10 minutes at approximately 3400 rpm at room temperature.

   f. Transfer EtOAc layer to 15mL polypropylene tube or 16 X 100 mm glass test tube.

   g. Wash aqueous portion with another 5 mL EtOAc. Repeat steps F.2.d. and F.2.e. Combine EtOAc layer with previous organic portion.

   h. Centrifuge the sample transferred into the 15 mL tube at 2000 rpm for 10 minutes. Transfer the EtOAC layer from the centrifuged tube into a clean dry 15 mL tube

      Stopping point: Extracts may be stored in the refrigerator for up to 1 week.

   i. Dry EtOAc extract using a TurboVap no higher than 60 °C.

   j. Add 900 µL water to dried residue. Vortex for 30 seconds.

   k. Add 5 mL hexanes to the 15 mL tube containing the 900 µL of sample extract.

   l. Wash the extract by vortexing for 10 seconds, centrifuge at 1000 rpm for 10 minutes. Remove and discard the hexanes layer.

   m. Add 5 mL hexanes and repeat step F.2.l.

   n. Evaporate hexanes residuals left in the 15 mL tube using the TurboVap.

      Note: Do not evaporate the sample, this step will only take a few minutes.

   o. Add 100 µl of 10 mM ammonium acetate into the 900 µL sample extract.

      Note: Continue either with steps p - q (option 1) or steps r - s (option 2). Resume either path with step t.

   p. Option 1: Centrifuge for 10 minutes at approximately 2000 rpm at room temperature.

   q. Option 1: Filter this extract through a 0.45 µm 13mm PTFE syringe filter into an autosampler vial.

r. Option 2: Filter by transferring approximately 500 µL of this extract into a 0.2 µm 500 µL centrifugal filter.

s. Option 2: Centrifuge in a microcentrifuge at 10,000 rpm for 15 minutes or until most of the extract has passed through the filter.

t. Transfer the supernatant to 300 µL amber glass autosampler vials.

3. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

a. HPLC Conditions:

Aqueous Mobile Phase	1 mM aqueous ammonium acetate: methanol (80:20) (C.2.c)
Organic Mobile Phase	Organic Mobile Phase - 1 mM aqueous ammonium acetate: methanol (10:90) (C.2.d)
Flow Rate	0.2 mL/min
Column Temperature	25 °C
Injection Volume	25 µL
Run Time	18 minutes

b. HPLC Mobile Phase Gradient Table

Time	% Aqueous	% Organic
0:00	95%	5%
12:00	5%	95%
12:10	95%	5%
18:00	95%	5%

c. Interface Conditions:

Ion Mode	ESI +
Capillary Temperature	350 °C
Spray Voltage	3900 V
Sheath Gas Pressure	40

Auxiliary Gas Pressure	15
Vaporizer Temperature	250°C

d. MRM Parameters:

	Precursor Ion (m/z)	Product Ion (m/z)*	Collision Energy (eV)
2-NP-AOZ	236.0	**134.0** 104.1 78.2	17 21 19
2-NP-AMOZ	335.1	**291.1** 262.0 128.2 100.2	7 14 20 28
2-NP-AHD	249.1	**134.0** 104.1	10 20
2-NP-SEM	209.0	**166.0** 192.0 149.0	8 8 13
2-NP-AMOZ-d5	340.2	296.4	19

* Most abundant product ion is in bold.

Note: Other instruments may give different relative abundances

4. Injection sequence
   a. Screening Set
      i. External Standard(s) (optional)
      ii. Blank
      iii. 0.5 ng/g Decision Level Recovery
      iv. 0.5 ng/g Recovery
      v. Check sample (if necessary)
      vi. Blank or solvent blank
      vii. Up to 21 Samples
      viii. External standard or positive control

b. Confirmation Set

i. External Standard(s) (optional)

ii. Blank

iii. Recovery Curve (0.5 ng/g, 1 ng/g, and 2 ng/g)

iv. Recovery

v. Check sample (if necessary)

vi. Blank or solvent blank

vii. Up to 19 Samples

viii. External or positive control

G. CALCULATIONS / IDENTIFICATION

1. Screening

a. The screening ion for a given analyte must be present. The required ion for each compound is listed in F.3.d.

b. The retention times of the recoveries of QCS samples for the screening ion must match the retention time of the screening ion in the 0.5 ng/g decision level recovery (or external standard) within 5%. Retention time for the screening ions in the samples must match the retention time of the screening ions in a fortified recovery (or external standard) within 5%.

c. The screening ion must have a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.

d. A sample is screened positive for an analyte if the following criteria are met:

i. The fortified recovery of the analyte must exceed 10% of the 0.5 ng/g decision level recovery.

ii. The sample response equals or exceeds the 0.5 ng/g fortified recovery level.

e. The level of the screening ion in the blank must be less than 10% of the 0.5 ng/g decision level recovery.

2. Confirmation

a. Monitored ions for each analyte will be assessed as follows:

i. Recovery retention times must match the retention time of the 0.5 ng/g decision level recovery (or external) standard within 5%. Retention time for the samples must match the retention time of the positive control or

the external standard within 5%.

ii. All product ions specified for ratio matching are present with a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.

iii. One of the following ion ratio matching conditions is met:

Note: Ratios are calculated by dividing the area count of each diagnostic ion by the amount of the base ion. Ion ratios should be less than 1. If the ratio is not less than 1 for a sample set, the inverse of this ratio may be used.

(a) If two product ions are assessed, one sample ion ratio should match the calculated ratio of a recovery, external standard, or average ratio of the recovery standards within a ± 10% absolute difference.

(b) If three product ions are monitored, the presence of two sample ratios should match the ratio of a recovery, external standard, or calculated average ratio of the recovery standards within a ± 20% absolute difference.

b. A sample is confirmed positive for an analyte if the above (G.2.a) and following criteria are met:

i. The fortified recovery of the analyte must exceed 10% of the 0.5 ng/g decision level recovery.

ii. The sample response equals or exceeds the appropriate fortified recovery level.

c. The blank must be less than 10% of the 0.5 ng/g level for the decision level recovery.

Note: For batch processing, analyte area amounts or normalized areas (versus internal standard) may be used for screening and/or sample level assessment. Use a consistent approach (either areas or normalized responses) with each batch.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — safety glasses, lab coat, protective gloves.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Concentrated acids and bases HCl, NaOH	Corrosive. Contact with liquids can result in burns and severe skin, eye, and respiratory irritation	Prepare solutions using these reagents with care in a well-ventilated area such as a fume hood. Wear protective eyewear, gloves, and clothing when handling.
Organic Solvents (EtOAc, MeOH, EtOH, DMSO)	Flammable, vapors are corrosive to the skin, eyes, and respiratory system.	Use only in an efficient fume hood, away from any electrical or heating devices.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

a. For screening:

i. For set acceptance, the four analytes in the fortified recovery must meet screening criteria.

ii. The blank (negative control) must be negative using the criteria in Section G.

b. For confirmation:

i. For set acceptance, the analytes of interest (i.e. analytes to be confirmed) in the fortified recovery (positive control) must meet confirmation criteria.

ii. The blank (negative control) must be negative using the criteria in Section G for the analytes of interest.

2. Critical Control Points and Specifications

Record		*Acceptable Control*
a.	Sample weight (F.1.a.)	2.0 ± 0.1 g
b.	Standards (D.2.a-g)	Standards should be stored in amber bottles

3. Intralaboratory Check Samples

   a. System, minimum contents.

      i. Frequency: One per week per analyst when samples analyzed.

      ii. Records are to be maintained.

   b. Acceptability criteria.

      Refer to I. 1.

      If unacceptable values are obtained, then:

      i. Investigate following established procedures.

      ii. Take corrective action as warranted.

4. Sample Condition upon Receipt

   Cool or Frozen

J. APPENDIX

1. References

Cooper KM, Mulder PP, van Rhijn JA, Kovacsics L, McCracken RJ, Young PB, Kennedy DG. Depletion of four nitrofuran antibiotics and their tissue-bound metabolites in porcine tissues and determination using LC-MS/MS and HPLC-UV. Food Addit Contam. 2005 May;22(5):406-14.

*Delatour T, Gremaud E, Mottier P, Richoz J, Vera FA, Stadler RH. Preparation of stable isotope-labeled 2-nitrobenzaldehyde derivatives of four metabolites of nitrofuran antibiotics and their comprehensive characterization by UV, MS, and NMR techniques. J Agric Food Chem. 2003, 51, 6371-6379.

*for proposed MS fragmentation patterns

2. Chromatograms/ spectra

   [Reserved]

3. Proposed MS fragmentation patterns for derivatized nitrofuran antibiotics

a. 2-NP-AOZ ($C_{10}H_9N_3O_4$), MW=235

Ion (m/z)	Fragment
236	$[M+1]^+$
134	$[M+1-C_3H_6N_2O_2]^+$
104	$[M+1-C_3H_6N_2O_2-NO]^+$
78	$[M+1-C_3H_6N_2O_2-NO-CO]^+$

b. 2-NP-AMOZ ($C_{15}H_{18}N_4O_5$), MW=334

Ion (m/z)	Fragment
335	$[M+1]^+$
291	$[M+1-CO_2]^+$
262	$[M+1-CO_2-CH_3N]^+$
128	$[M+1-CO_2-CH_3N-C_6H_4NOCO]^+$

c. 2-NP-SEM ($C_8H_8N_4O_3$), MW=208

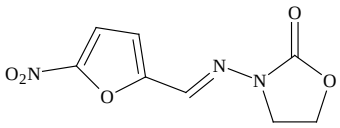
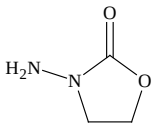
Ion (m/z)	Fragment
209	$[M+1]^+$
192	$[M+1-H_3N]^+$
166	$[M+1-CONH]^+$
149	$[M+1-CO(NH_2)_2]^+$

d. 2-NP-AHD ($C_{10}H_8N_4O_4$), MW=248

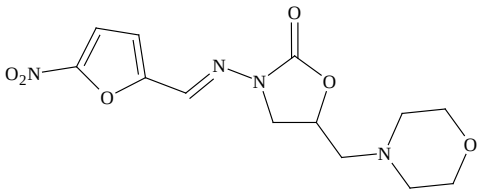
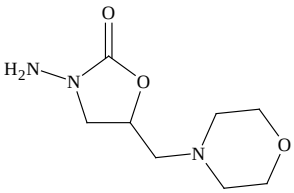
Ion (m/z)	Fragment
249	$[M+1]^+$
134	$[M+1-C_3H_5N_3O_2]^+$
104	$[M+1-C_3H_5N_3O_2-NO]^+$

4. Structures

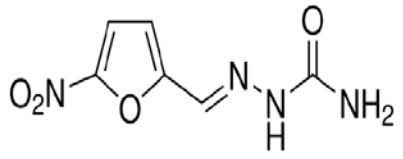
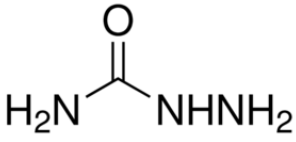
a. Furazolidone and AOZ

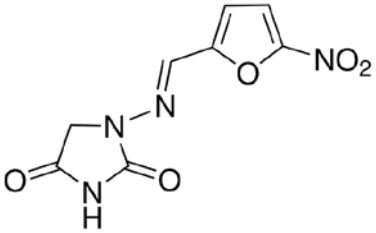
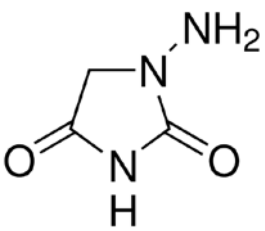
b. Furaltadone and AMOZ

c. Nitrofurazone and SEM

d. Nitrofurantoin and AHD

5. Minimum Level of Applicability

Table 1 - Minimum Level of Applicability for screening

Analyte	Poultry (ng/g)	Catfish (ng/g)
2-NP-AOZ	0.5	0.5
2-NP-AMOZ	0.5	0.5
2-NP-AHD	0.5	0.5
2-NP-SEM	0.5	0.5

Table 2 - Minimum Level of Applicability for confirmation

Analyte	Poultry (ng/g)	Catfish (ng/g)
2-NP-AOZ	0.5	0.5
2-NP-AMOZ	0.5	0.5
2-NP-AHD	0.5	1
2-NP-SEM	0.5	0.5

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Staff.