CLG-NFUR2.01		Page 1 of 20
Title: Screening and Confirmation of Nitrofuran Metabolites by Liquid Chromatography-Tandem Mass Spectrometry		
Revision: 01	Replaces: CLG-NFUR2.00	Effective: 3/09/2010

Contents

A. INTRODUCTION

1. Theory

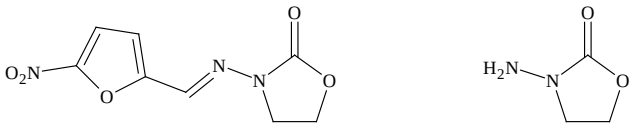
Nitrofuran antibiotics, furazolidone and furaltadone, are analyzed as their respective metabolites, 3-amino-2-oxazolidinone (AOZ) and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ). These metabolites are obtained from blended tissue samples using incubation under acid hydrolysis conditions and simultaneously derivatized using 2-nitrobenzaldehyde. The extract is neutralized, and the derivatized metabolites (2-NP-AOZ and 2-NP-AMOZ) are isolated using liquid-liquid extraction with ethyl acetate followed by screening and confirmation using liquid chromatography-tandem mass spectrometry (LC/MS/MS).

2. Applicability

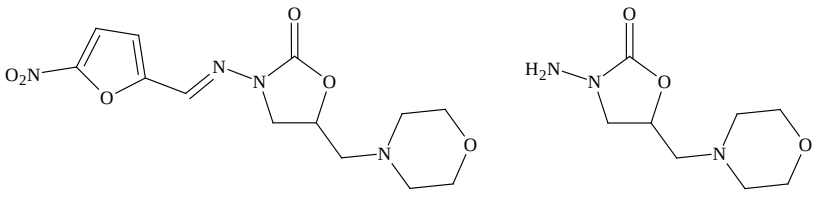
This method is applicable to the screening and confirmation of nitrofuran metabolites in bovine, porcine, and avian (poultry) liver at levels ≥ 5 ppb, and catfish muscle at levels ≥ 1 ppb.

3. Structure

a. Furazolidone and AOZ



b. Furaltadone and AMOZ



B. EQUIPMENT

Note: Equivalent apparatus and instrumentation may be substituted for the following:

1. Apparatus

a. Balance – Top loading, model number PM300, Mettler.

b. Centrifuge Tubes – 50 mL polypropylene, Cat. No. 06-443-19, Fisher Scientific.

c. Centrifuge Tubes – 15 mL polypropylene, Cat. No. 352096, Becton Dickinson. Lab ware or Glass test tube 16 X 100 mm, Kimble No. 73500 16100.

d. Centrifuge, refrigerated – Model No. PR 7000, International Equipment Company.

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

f. Eppendorf pipettors – Variable volumes: single channel, 50-200 µL Cat. No. 022470256, 500-5000 µL Cat. No. 022472151, Brinkmann Instruments.

g. Test tube rack – Cat No. 5930-0020, Nalgene Nunc International.

h. Incubator – Model No. 120-923, Lab-Line Instruments, Inc.

i. N-evap – Model No. 112, Organomation.

j. Syringeless filter device – Mini – UniPrep, 0.45 µm nylon, Cat. No. UN203NPUNYL, Whatman.

k. Volumetric flask – 100 mL amber, class A.

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

2. Instrumentation

a. Mass spectrometer – Thermo Finnigan, TSQ Quantum.

b. HPLC equipped with Thermo Finnigan Surveyor quaternary pump and Thermo Finnigan Surveyor Auto Sampler.

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

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted for the following:

1. Reagents

a. Methanol (MeOH) - HPLC grade, Cat. No. 230-4, Burdick & Jackson.

b. Ethanol (EtOH) - 200 Proof, Cat. No. EM-EX0289-3, VWR.

c. Ethyl acetate (EtOAc) - HPLC grade, Cat. No. 100-4, Burdick & Jackson.

d. Water - Deionized, HPLC grade, Millipore Rx system.

e. 2-Nitrobenzaldehyde - Cat. No. N1080-2, Sigma-Aldrich.

f. Dimethylsulfoxide (DMSO) - Cat. No. 27685-5, Sigma-Aldrich.

g. Hydrochloric acid (HCl) - 1N, Cat. No. SA48-1, Fisher Scientific.

h. Sodium hydroxide (NaOH) - 1N, Cat. No. SS266-1, Fisher Scientific.

i. Ammonium acetate (NH_4OAc), Mass Spectrometric grade - Cat. No. 73594-25G-F, Sigma-Aldrich.

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

k. Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \bullet 7H_2O$) - Cat. No. 431478-50G, Sigma Aldrich.

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. STANDARDS

Note: Equivalent standards/solutions may be substituted for the following.

1. Source

a. 3-Amino-2-oxazolidinone (AOZ), ($C_3H_6N_2O_2$), MW 102.09, CAS # 80-65-9, Cat. No. 33347, Riedel-de-Haen through 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, 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, Riedel-de-Haen through 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, Riedel-de-Haen through Sigma-Aldrich.

2. Preparation

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

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

Weigh 2.5 ± 1 mg AOZ and AMOZ 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.

b. AOZ / AMOZ 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.

c. AOZ / AMOZ 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.

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

Weigh 23.0 mg ± 1 mg of 2-NP-AOZ 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. Store at or below -20 ºC.

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

Weigh 16.6 mg ± 1 mg of 2-NP-AMOZ 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. Store at or below -20 ºC.

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

Pipet ~250 µL of 2-NP-AOZ stock standard solution (D.2.d) and ~250 µL of 2-NP-AMOZ stock standard solution (D.2.e), depending on the exact concentrations, into a 100 mL amber volumetric flask. Bring to volume with methanol. Store at or below -20 ºC.

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

Pipet 1.00 mL of 2-NP-AOZ / 2-NP-AMOZ mixed intermediate standard solution (D.2.f.) into a 50 mL amber volumetric flask. Dilute to volume using water. Store at 2 - 8 ºC.

3. Storage and Stability (if not included with preparation)

Stock, intermediate, and fortification standards prepared in methanol are stable for 5 months when stored at or below -20 °C. Working standards (D.2.g.) prepared in deionized HPLC grade water are stable for 48 hours when stored at 2 - 8 °C.

E. SAMPLE PREPARATION

1. Tissue homogenization

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

b. Transfer homogenized sample into a plastic bag and store in a freezer at -10 ºC or colder.

c. Let sample partially thaw prior to analysis.

F. ANALYTICAL PROCEDURE

1. Sample Extraction

a. Weigh 1.0 ± 0.1 g blended tissue into a 50 mL polypropylene tube for each sample, negative control(s) (blank) and positive control(s) (recovery).

b. Add 8 mL MeOH and 1 mL H_2O.

c. Vortex for approximately 10 seconds. A spatula can be used to disperse packed sample.

d. Centrifuge for 5 - 10 minutes at approximately 1600 - 3000 rpm at 3 °C. Adjust centrifuge time and speed so that the tissue should be packed tightly but should easily disperse when vortexed. 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.

h. Prepare positive controls at this time by fortifying known blank samples with the AOZ/AMOZ combined fortification standard solution (D.2.c.). Fortify liver samples with 500 µL for a 5 ppb positive control. Fortify catfish muscle with 100 µL for a 1 ppb positive control.

i. Add 4 mL of deionized water, 0.5 mL 1 N HCl, and 100 µL 10 mM 2-nitrobenzaldehyde solution in DMSO (C.2.b.) to each tube. Vortex for approximately 10 seconds.

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

k. Add 5 mL 0.1M K_2HPO_4, 0.4 mL 1N NaOH, and 5 mL EtOAc.

l. Vortex for approximately 10 seconds.

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

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

o. Wash aqueous portion with another 5 mL EtOAc. Repeat steps F.1.l. and F.1.m. Combine EtOAc layer with previous organic portion.

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

p. Dry EtOAc extract using an N-Evap no higher than 60 °C.

q. Add 1000 µL water to dried residue. Vortex for 10 seconds.

r. Filter reconstituted extract using 0.45 µm nylon Uniprep filter or centrifuge filter. Extract is ready for LC/MS/MS analysis.

2. LC/MS/MS Analysis

a. Instrumental settings

Note: The following instrument parameters may be optimized:

i. HPLC Conditions:

Aqueous Mobile Phase	1 mM aqueous ammonium acetate: methanol (80:20) (C.2.c)
Organic Mobile Phase	Methanol (C1.a.)
Flow Rate	0.2 mL/min
Column Temperature	25 °C
Injection Volume	50 µL
Run Time	18 minutes

ii. HPLC Mobile Phase Gradient Table:

Time	% Aqueous	% Organic
0:00	95%	5%
5:00	50%	50%
10:00	50%	50%
10:10	95%	5%
18:00	95%	5%

iii. Interface Conditions:

Ion Mode	ESI +
Capillary Temperature	350 °C
Spray Voltage	3900 V
Sheath Gas Pressure	40 L/hr
Auxiliary Gas Pressure	18 L/hr

iv. MRM Parameters:

	Precursor Ion (m/z)	Product Ion (m/z)*	Collision Energy (eV)
2-NP-AOZ	236	**134**	18
		104	28
		78	40
2-NP-AMOZ	335	**291**	18
		262	22
		128	30

* Most abundant product ion is in bold.

Note: Other instruments may give different relative abundances.

v. MS Parameters:

Segment #	1	2
Starting Retention Time (min)	0.0	9.59
Scan Time (sec)	0.2	0.2

vi. Injection sequence:

(a) 2-NP-AOZ / 2-NP-AMOZ combined working standard solution (D.2.g.)

(b) Positive control

(c) Water Blank

(d) Tissue Blank

(e) Samples

3. Example Chromatograms: Refer to Section K.2.

G. IDENTIFICATION AND CONFIRMATION

1. The retention time must match that of the positive control within 5%.

2. MS/MS Criteria

a. Identification (screening)

i. All product ions for a given analyte must be present. The required ions are listed in F.2.a.iv.

ii. Each ion must have a signal-to-noise ratio ≥ 3.

b. Confirmation

Product ion abundance ratios must match that of the positive control within 20% relative difference. The following are representative ion ratios calculated relative to the most abundant product ion:

	Ratio #1	Ratio #2
2-NP-AOZ	78/134	104/134
2-NP-AMOZ	128/291	262/291

3. A water blank analyzed after the recovery must be negative for all analytes.

4. The tissue blank must be negative for all analytes.

5. The tissue fortification must be positive for all analytes.

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

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Concentrated acids and bases (see above)	See Hazards, above	Neutralize solutions to meet local, state, and Federal guidelines.
Organic Solvents (see above)	See Hazards, above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well

ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. Positive control is positive for all analytes.
   b. Negative control shows no confirmable analytes.

2. Critical Control Points and Specifications

Record		*Acceptable Control*
a.	Sample weight (F.1.a.)	1.0 ± 0.1 g
b.	Standards (D.2.a-g)	Standards should be stored in amber bottles

3. Readiness To Perform
   a. Familiarization
      i. Phase I: Analyze a 5 ppb equivalent mixed external standard (D.2.g.) and a water blank over 3 days to ensure that instrument response is adequate for identification and confirmation.
      ii. Phase II: Fortified samples - Analyze a set of 5 fortified tissues and one tissue blank over 3 different days. Liver shall be fortified at 5 ppb, and catfish muscle at 1 ppb. Different tissues may be run on different days.

      NOTE: Phase I and Phase II may be performed concurrently.

      iii. Phase III: Check samples for analyst accreditation.
         (a) For each tissue of interest, run 6 samples, blind to the analyst. At least one, but no more than two of the six unknowns must be negative. Positive liver shall be fortified at 5 ppb, and positive catfish muscle shall be fortified at 1 ppb.
         (b) Report analytical findings to Supervisor/Quality Assurance Manager (QAM).

(c) Authorization from QAM and supervisor is required to commence official analysis.

b. Acceptability criteria.

Refer to I. 1.

4. Intralaboratory Check Samples

a. System, minimum contents.

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

ii. Records are to be maintained for review.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst with this method.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: bovine, porcine, and avian liver, and catfish muscle.

b. Sample storage:

i. Condition: Frozen, -10 ºC or lower.

ii. Time: Two months.

6. Sample Set

a. Negative control (tissue blank).

b. Positive control (fortified blank).

c. Test samples to be analyzed.

7. Analyst Capability

a. Minimum proficiency level (MPL):

i. For bovine, porcine, and avian liver: 5 ppb.

ii. For catfish muscle: 1 ppb.

J. WORKSHEET

The worksheets on the following pages are examples.

Nitrofuran Worksheet
(AOZ and AMOZ)

Analyst:	
Date Started:	
Date Completed:	
Reviewed by:	
Supervisor reviwed by:	
Sample IDs	

Standards	AOZ	AMOZ	PIP	Amount
Nitrofuran Stock (D.2.a)				
Nitrofuran Intermediate (D.2.b)				
Combined working standard (D.2.c)				500 µL
2NP stock standards (D.2.d,e)				
2NP intermediate stds (D.2.f,g)				
2NP working standards (D.2.h,i)				

Solvent and Reagents	ID	DSP/PIP	Amount
Methanol (F.1.b)			8 mL
H_2O (F.1.b)			1 mL
Methanol (F.1.e)			5 mL
Ethanol (F.1.f,g)			5 mL
H_2O (F.1.i)			4 mL
1N HCL (F.1.i)			0.5 mL
10 mM 2-Nitrobenzaldehyde (F.1.i)			100 µL
1N NaOH (F.1.k)			0.4 mL
Ethyl Acetate (F.1.k,o)			5 mL
Potassium phosphate (F.1.k)			5 mL
H_2O (F.1.q)			1 mL
Mobile Phase			

Instruments and Setting		
Incubator (F.1.j)		
Start date/time/temp (F.1.j)		
Stop date/time/temp (F.1.j)		
Incubation time (F.1.j)		
Refrigerator		
Freezer		
Centrifuge (F.1.d,m)		
Centrifuge speed	d.	m.
Centrifuge time	d.	m.
Centrifuge temp	d.	m. Room temp
Centrifuge rotor	d.	m.
Balance (F.1.a)		
Timer		
Log Book		
N-Evap/Temp (F.1.p)		
Comments:		

K. APPENDIX

1. References

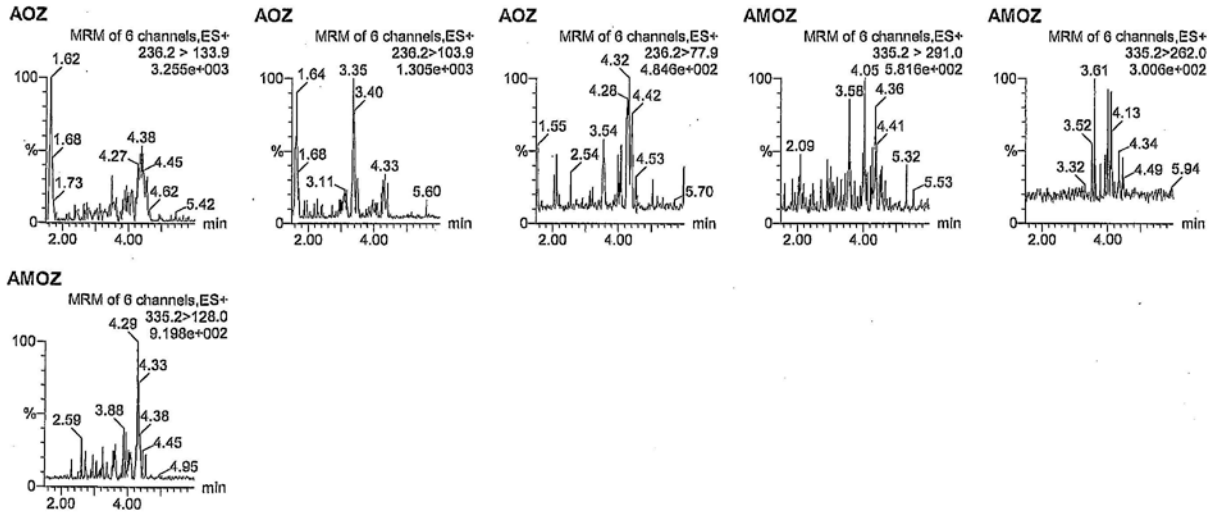
Cooper KM, Mulder PP, van Rhijn JA, Kovacsics L, McCracken RJ, Young PB, Kennedy DG. Depletion of four nitrofuran antibiotics and their tissue-bound metabolites in porcine tissues and determination using LC-MS/MS and HPLC-UV. Food Addit Contam. 2005 May;22(5):406-14.

*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.

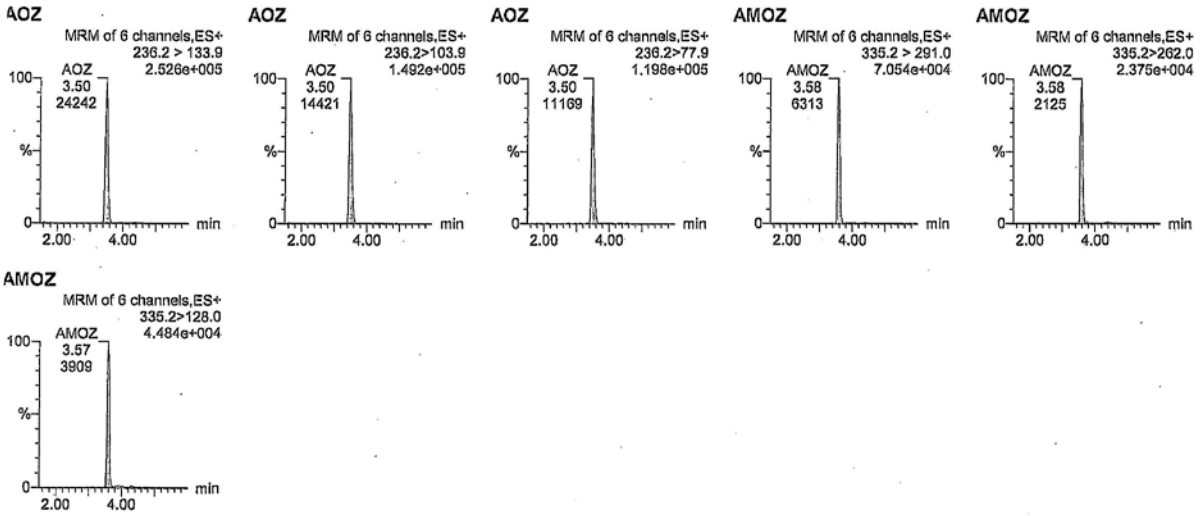
*for proposed MS fragmentation patterns

2. Example Chromatograms

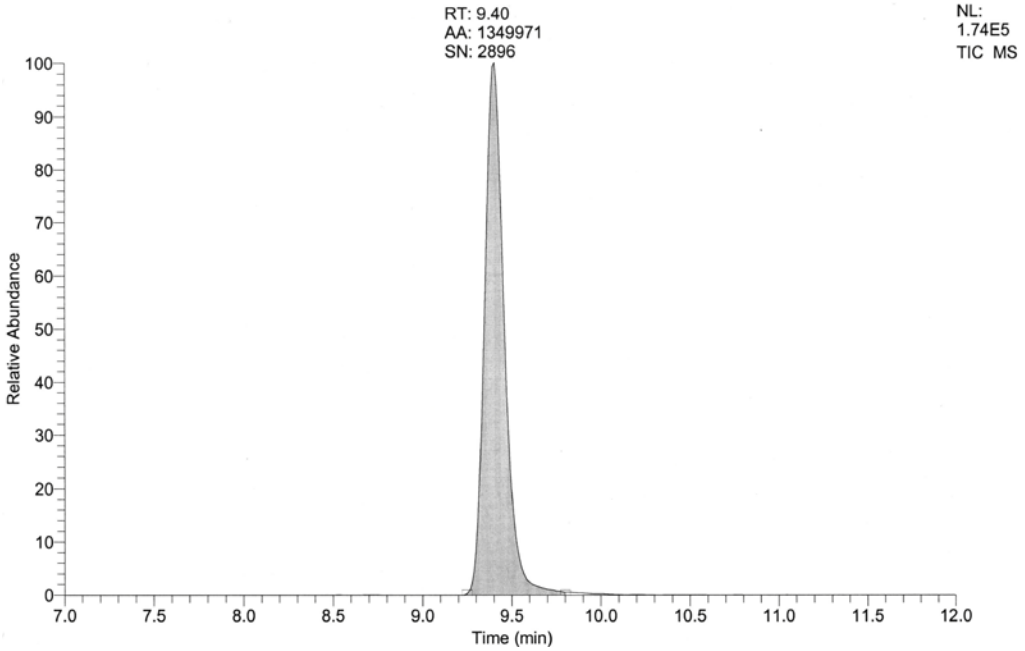
a. Blank catfish (UPLC Chromatograms)



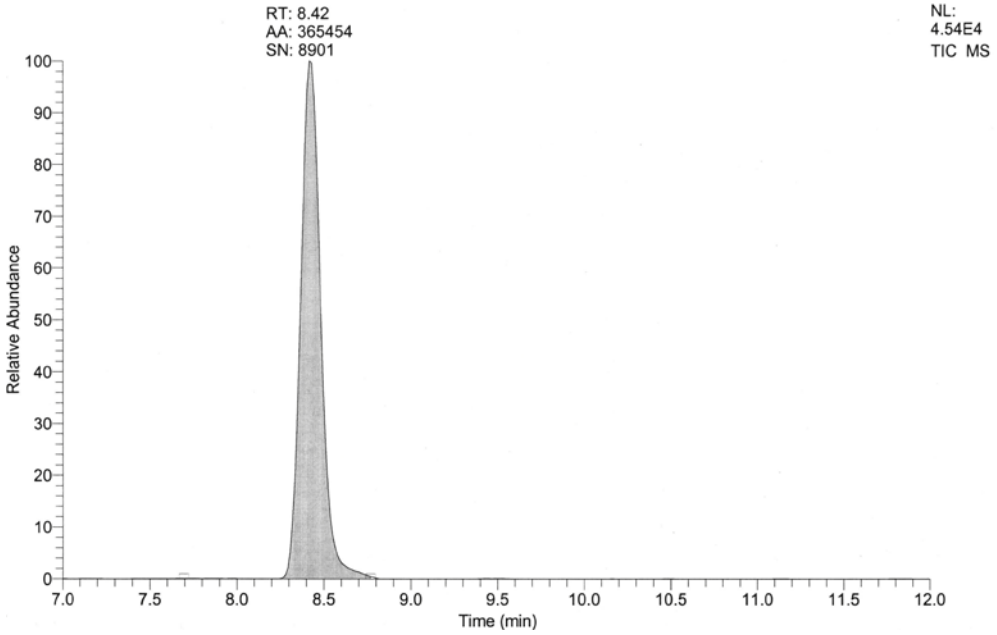
b. Catfish spiked at 1 ppb (UPLC Chromatograms)



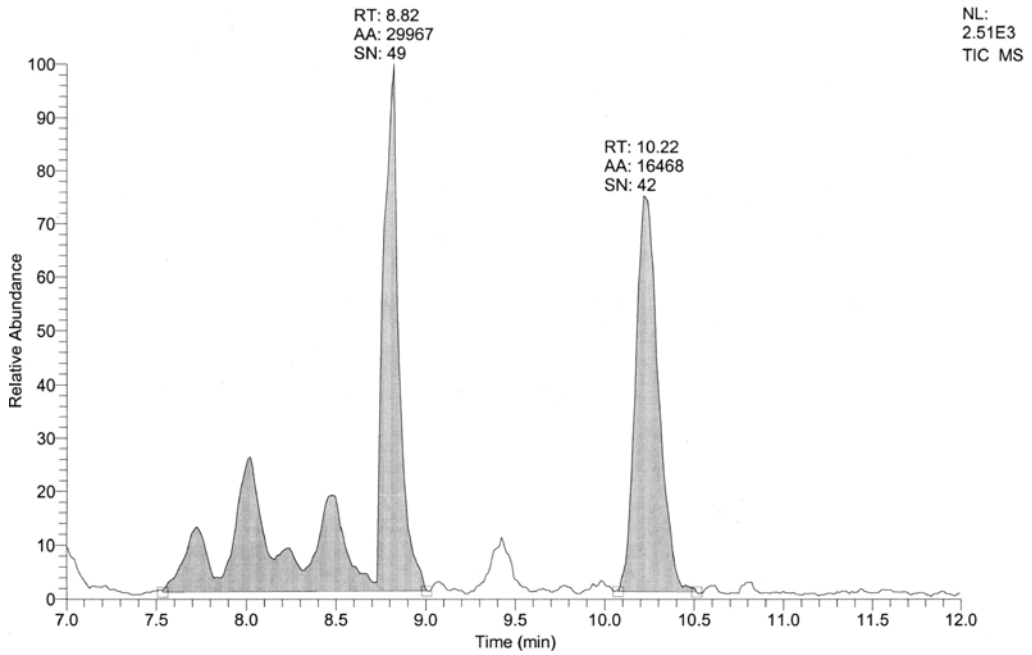
c. 2-NP-AMOZ standard, equivalent to 5 ppb AMOZ (HPLC Chromatograms)



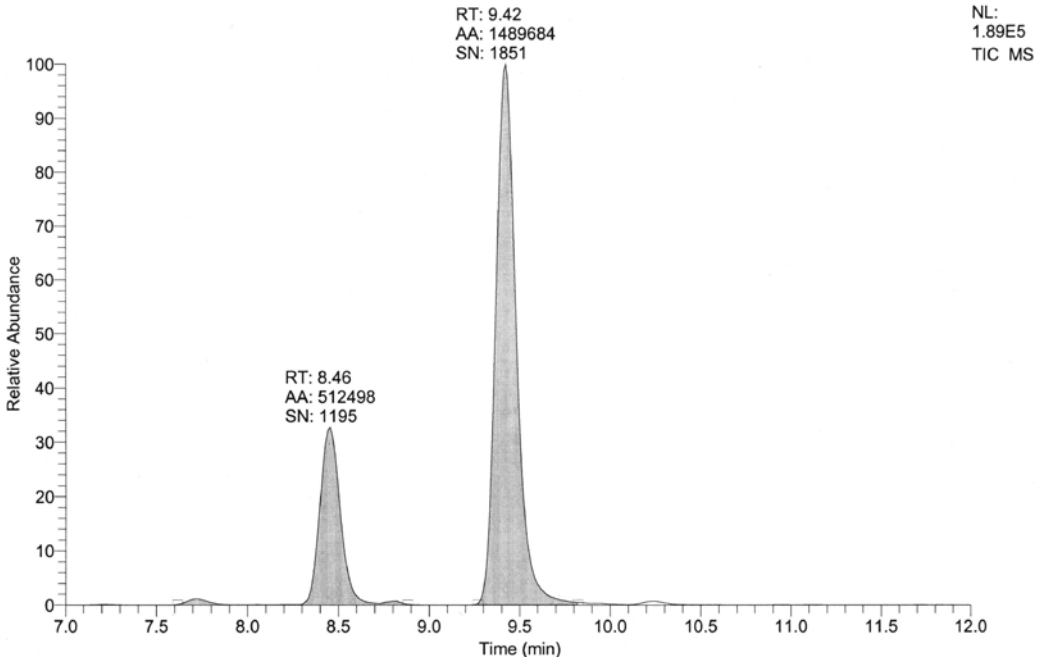
d. 2-NP-AOZ standard, equivalent to 5 ppb AOZ (HPLC Chromatograms)



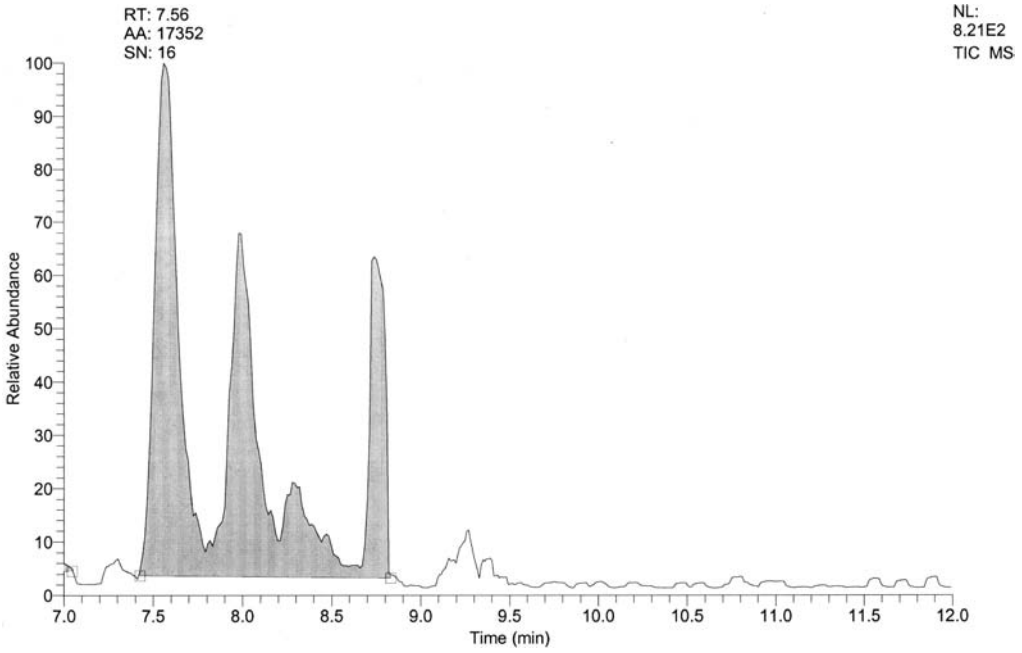
e. Poultry liver negative control (blank) (HPLC Chromatograms)



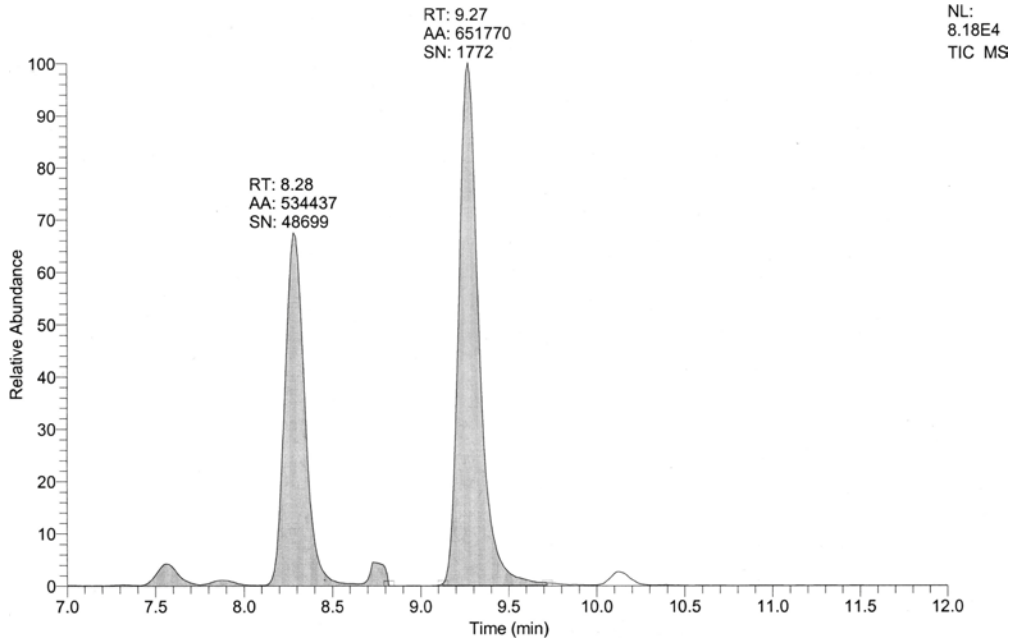
f. Poultry liver positive control (recovery), equivalent to 5 ppb AOZ and AMOZ



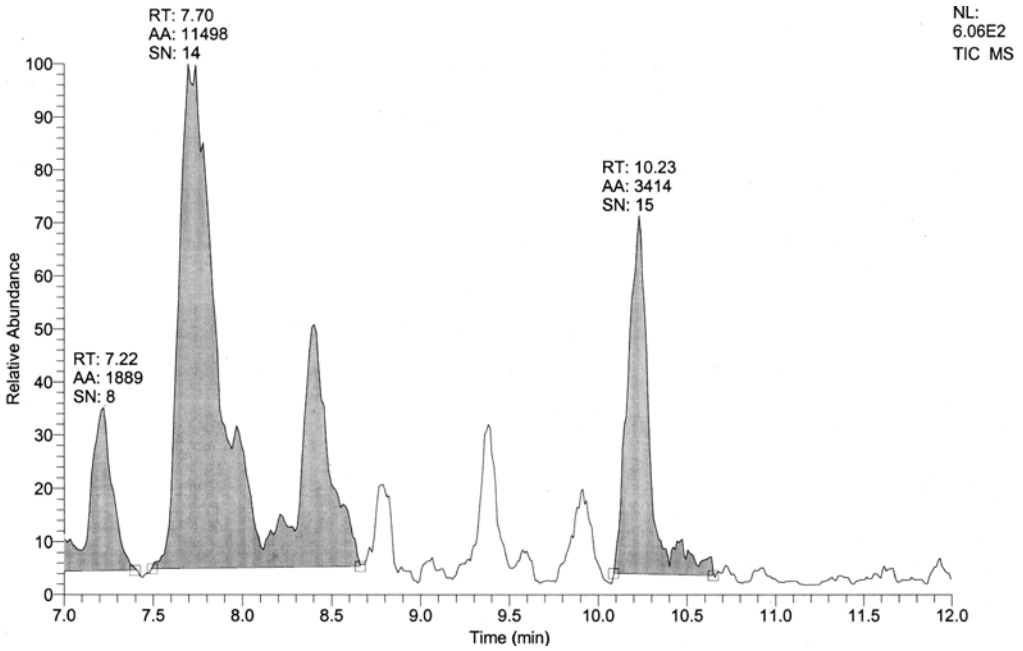
g. Bovine liver negative control (blank) (HPLC Chromatograms)



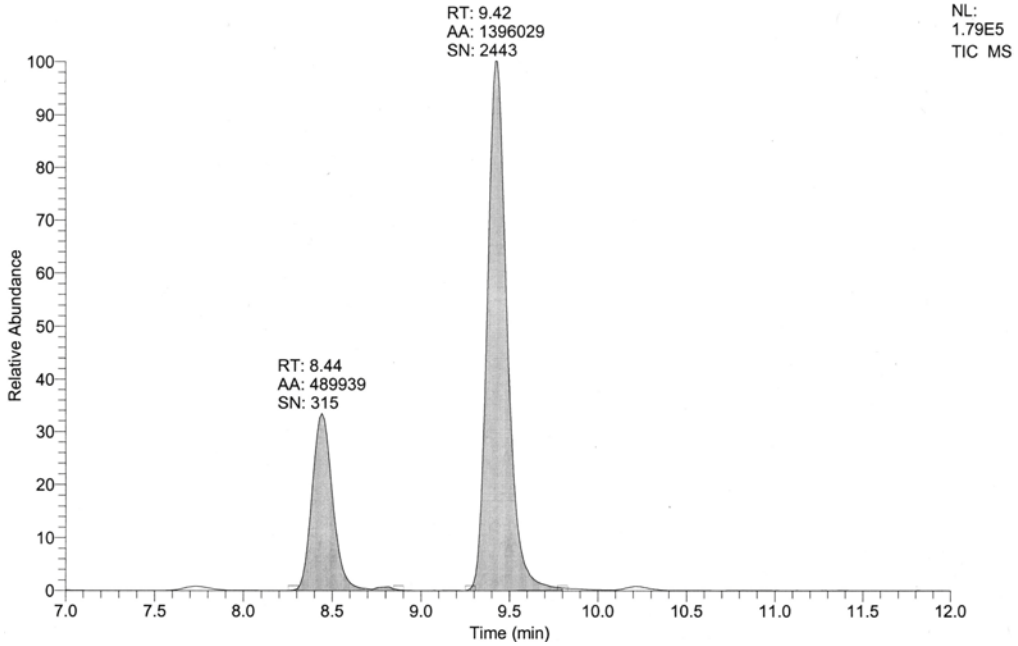
h. Bovine liver positive control (recovery), equivalent to 5 ppb AOZ and AMOZ



i. Porcine liver negative control (blank) (HPLC Chromatograms)



j. Porcine liver positive control (recovery), equivalent to 5 ppb AOZ and AMOZ



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	$[M + 3 - C_3H_6N_2O_2 - NO - CO]^+$

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	$[M + 1 - CO_2 - CH_3N - C_6H_4NOCO]^+$

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

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