Laboratory Methods: Detection of Nitrofuran Metabolites in Shrimp

<< Back to Drug & Chemical Residues Methods Main Page (/Food/FoodScienceResearch/LaboratoryMethods/ucm2006950.htm)

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A. SCOPE

This method is used for total residues screening of 3-amino-2-oxazolidinone (AOZ), 5-methyl-morpholino-3-amino-2-oxazolidinone (AMOZ), semicarbazide (SC), and 1-aminohydantoin (AH), (which are metabolites of furazolidone, furaltadone, nitrofurazone, and nitrofirantion, respectively) in shrimp.

The method involves overnight acid hydrolysis and simultaneous derivatization with 2-nitrobenzaldehyde (2-NBA), pH adjustment, liquid-liquid extraction, and detection of 2-NBA derivatives of AOZ, AMOZ, SC, and AH by LC-MS-MS (liquid chromatography tandem mass spectrometry).

B. APPARATUS

1. Adjustable pipettors (Eppendorf)
2. Balance (AND HM-202)
3. Balance (Mettler PM 4800)
4. Water bath with shaker with a homemade platform (New Brunswick Scientific, Innova 3100)
5. Centrifuge (SORVALL, RC 5C Plus)
6. pH meter (ORION model 401 Aplus)
7. Vortex mixer (Vortex-Genie 2 mixer)
8. Solid phase extraction manifolder 24 port (Supelco/Sigma-Aldrich)
9. Evaporator: TurboVap with a 15 ml test tube rack (Zymark, Hopkinton, MA)
10. Disposable 50 ml centrifuge tubes (VWR, 21008-240)
11. Disposable 20 ml reservoirs (part No.12131011, Varian)
12. Disposable frits (part No.12131023, Varian)
13. Disposable 15 ml tube (part No. 3131-340, LabCon)
14. Disposable 3 ml syringe (part No. 309595, B.D.)
15. Disposable 2 µm syringe filter (GelmanLab, No. 2003-09)
16. 1.8 ml autosampler vial (Finnigan, No. A4954-010)

C. LC-MS-MS EQUIPMENT

1. LC/Triple Quadrupole MS/MS:
2. Injection loop on injection port in the autosampler: 50 µl
3. Syringe in the autosampler: 250 µl
4. Sample transfer tube (Part # 60053-60012S) in AS: 17 µl (dead volume)
5. Standard tray (P/N F118-010): in two rows, 2x20
6. LC column: Inertsil ODS-3 5µ 130 x 2.0 mm (Varian Part # 0396-150X021)
7. Guard column: 2.0 mm ODS-3 5µ (Varian Part # 0396-MG2)
8. MetaSaver Precolumn Filter: 2µ Disposable (Varian Part # 6007)

D. CHEMICALS and SOLVENTS

1. Methanol (MeOH) (High purity solvent, Burdick & Jackson)
2. Ethyl Acetate (EtOAc) (High purity solvent, Burdick & Jackson)
3. Hydrochloric Acid (HCl) (Concentrated, Fisher Scientific)
4. Formic acid (class IIIA, 90%, Fisher Scientific)
5. Sodium Chloride (NaCl,)(Certified A.C.S, Fisher Scientific)
6. Dipotassium hydrogen phosphate trihydrate (K_2HPO_4) (Certified A.C.S, Fisher Scientific)
7. Solid and dry pellet sodium hydroxide (NaOH) (Mallinckrodf)
8. 2-Nitrobenzaldehyde (Sigma-Aldrich)
9. Distilled and deionized water generated in house
10. NBA-SC, NBA-AH, NBA-AMOZ, and NBA-AOZ (2-NBA derivatives of SC, AH, AMOZ, and AOZ, respectively; 10 mg analytical standards, identity confirmed by NMR, HPLC purity > 99% (Sigma-Aldrich cat. # 33868, 33869, 33870, and 33871, respectively)

E. SAFETY

Safety procedures for handling, storage, and disposal of chemical solvent and their wastes should be strictly followed. The safety precautions for running instruments and apparatus should be strictly followed. Protective clothing, gloves, and safety glasses should be worn at all times when handling solvents or chemicals, and when performing procedure. Fume hood should be used when handling hazardous solvents and chemicals. The material safety data sheet (MSDS) for chemicals used should be followed.

F. REAGENTS

1. **0.125M HCl**
   Add 5.2 ml of conc. HCl to approximately 200 ml of water in a 500 ml volumetric flask. Add water to mark and mix by inverting.
2. **0.1M K_2HPO_4**
   Dissolve 17.4 g K_2HPO_4 in less than 1 L of water. Transfer the solution into 1L volumetric flask and add water to the mark.

3. **0.8M NaOH**
   Dissolve 3.2 g NaOH in less than 100 ml of water. Transfer the solution in 100 ml volumetric flask and add water to the mark.
4. **0.125M NaOH**
   Dilute 10 ml of 0.8 M NaOH into 630 ml of water.
5. **0.1% formic acid**
   Add 1 ml of formic acid into less than 1 L of water in a 1 L water volumetric flask and add water to the mark.
6. **50 mM 2-NBA**
   Dissolve 0.07555g 2-NBA in 10 ml MeOH.
7. **50/50 v/v water/MeOH**
   Add 50 ml MeOH into 50 ml of water.

G. STANDARD SOLUTIONS

1. Dissolve 0.43, 0.56, 0.46, and 0.33 mg of NBA-AH, NBA-SC, NBA-AOZ, and NBA-AMOZ, respectively, in 100 ml MeOH. The resulting solutions (**stock mixtures**) contain 4.3 ng/µl, 5.6 ng/ml, 4.6 ng/µl, and 3.3 ng/µl of NBA-AH, NBA-SC, NBA-AOZ, and NBA-AMOZ, respectively. These concentrations correspond to 2 ng/µl of the underivatized metabolites (AH, SC, AOZ, and AMOZ).
2. Dilute the stock mixture 500 times to prepare solution D500: 10 µl of stock solution plus 5 ml of 50/50 (v/v) MeOH/water (this concentration corresponds to 4 pg/µl of the underivatized metabolites).
3. Prepare solution D1000: 2 ml of D500 solution plus 2 ml of 50/50 (v/v) MeOH/water (this concentration corresponds to 2 pg/µl of the underivatized metabolites).
4. Prepare solution D2000: 2 ml of D1000 solution plus 2 ml of 50/50 (v/v) MeOH/water (this concentration corresponds to 1 pg/µl of the underivatized metabolites).
5. Prepare solution D4000: 2 ml of D2000 solution plus 2 ml of 50/50 (v/v) MeOH/water (this concentration corresponds to 0.5 pg/µl of the underivatized metabolites).
6. Prepare solution D8000: 2 ml of D4000 solution plus 2 ml of 50/50 (v/v) MeOH/water (this concentration corresponds to 0.25 pg/µl of the underivatized metabolites).

H. SAMPLE PREPARATION PROCEDURES

1. Weigh 2.0 (± 0.1) g homogenized shrimp sample into the bottom of a 50 ml labeled centrifuge tube (VWR 21008-240). Record the weight of the sample.
2. Add 10 ml of 0.125 M HCl and 400 µl of freshly prepared 50 mM 2-NBA in MeOH to each sample. Vortex-mix the tube with a cap on at setting 70 for 15 seconds.
3. Place samples overnight (16 hours) into a water bath at 37°C with shaker set at 80 rpm.
4. Cool samples to room temperature. Add 1 ml of 0.1M K_2HPO_4 and 1 ml of 0.8 M NaOH. Vortex-mix at setting 70 for 15 seconds.
5. Determine the pH of the sample with a pH meter. Adjust pH to 7.3 ± 0.2 with 0.125 M HCl or NaOH. Record the final pH. Wash the electrode with water. Collect washing water. The final volume of the sample should be no more than 20 ml. Record the final volume.
6. Centrifuge samples at 4°C and 3000 rpm for 5 min.

7. Clean inside and outside of metal tubing on the filtration manifold with water. Attach 20 ml reservoirs (part No.12131011, Varian) to the manifold. Insert a frit (part No. 12131023, Varian) on the bottom of the reservoir. Place the frit rough face up. Put a new labeled 50 ml centrifuge tube under each reservoir.
8. Decant the supernatant into the reservoir. Keep shrimp pellet for washing in next step. Apply vacuum and collect the filtrate.
9. Add 3 ml of water into the shrimp pellet. Vortex-mix at setting 70 for 15 seconds. Centrifuge at 4°C and 3000 rpm for 5 min.
10. Decant the second supernatant into the same reservoir. Apply vacuum and collect the second filtrate in the same 50 ml tube.
11. Add water to adjust the total volume of the filtrate to 20 ml. Add approximately 0.5 g of NaCl and 12 ml of EtOAc to the filtrate. Close the tube with a cap and vortex-mix at setting 70 for 15 seconds.
12. Centrifuge samples at 4°C and 3000 rpm for 5 min.
13. Pipet top layer of EtOAc into a new labeled 15 ml tube. Add 2 ml of water into EtOAc extract. Pipet out the bottom (water) layer and discard it.
14. Evaporate EtOAc extract to dryness using Zymark evaporator at 40°C.
15. Add 1ml of 50/50 (v/v) MeOH/water into each tube with dried sample. Vortex-mix at setting 70 for 15 seconds.
16. Transfer the final solution into a 3 ml syringe (B.D. 309595) with a 2 µm syringe filter (GelmanLab, 2003-09). Filter the solution into a labeled 1.8 ml autosampler vial (Finnigan, No. A3954-010) for LC/MS/MS analysis.

I. LC-MS-MS ANALYSIS PROCEDURES

Equipment set up

1. LC/Triple Quadrupole MS/MS: a Surveyor LC pump, a Surveyor Autosampler (AS), a Surveyor PDA and a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer AM (accurate mass) are connected in series. The injection loop on injection port in the autosampler is 50 µl, the syringe in the autosampler is 250 µl. The sample transfer tube (Part # 60053-60012S) in AS is 17 µl (dead volume). The tray is standard tray (P/N F118-010) able to contain 40 1.8 ml vials with septa and screw cap (Part # A4954-010) in two rows.
2. LC column is an Inertsil ODS-3 5µ 130 x 2.0 mm (Varian Part # 0396-150X021) with a guard column of 2.0 mm ODS-3 5µ (Varian Part # 0396-MG2). A MetaSaver Precolumn Filter 2µ Disposable (Varian Part # 6007) is installed between the guard column and injection port on the autosampler.

Methods for LC/MS

1. **Surveyor autosampler method**
   Needle height from bottom: 2.0 mm.
   Injection volume and mode: 40 µl, no waste.
   Post-injection valve switch time: 0.0 min
   Loop loading speed: 5 µl/s
   Syringe speed: 8 µl/s
   Flush/Wash source is Wash bottle containing 50/50 (v/v) methanol/water.
   Flush/Wash volume: 400 µl
   Flush speed: 250 µl/s
   Tray temperature control: On. Temperature: 10°C
   Column Oven Control: On. Temperature: 30°C

2. **LC method**
   Mobile phase A: 0.1% v/v formic acid in deionized (DI) water
   Mobile phase B: MeOH
   Allow mobile phase A/B at 45/55 (v/v) to run through HPLC system for at least 30 min at flow rate 0.15 ml/min to equilibrate the system.
   Gradient Program:
   From 0 min to 8 min: 0.15 ml/min flow rate, keep mobile phase A/B at 45/55
   From 8 to 8.5 min: 0.15 ml/min flow rate, mobile phase A/B from 45/55 to 0/100
   From 8.5 to 8.7 min: flow rate from 0.15 to 0.30 ml/min, keep mobile phase A/B at 0/100
   From 8.7 to 15 min: keep flow rate at 0.30 ml/min, keep mobile phase A/B at 0/100
   From 15 to 15.5 min: keep flow rate at 0.30 ml/min, mobile phase A/B from 0/100 to 45/55
   From 15.5 to 20 min: keep flow rate at 0.30 ml/min, keep mobile phase A/B at 45/55
   From 20 to 21 min: flow rate from 0.30 to 0.15 ml/min, keep mobile phase A/B at 45/55
   From 21 to 30 min: keep flow rate at 0.15 ml/min, keep mobile phase A/B at 45/55.
   Pressure min/max: 0/431 bar
   Time/analysis: 30 min

   Retention times and MW

Compound	Retention time (min)	MW
NBA-AMOZ	3.1	334
NBA-AH	5.5	248
NBA-AOZ	5.6	235
NBA-SC	5.7	208

3. **TSQ Quantum Method**
   Acquire time: 30 min
   Ion Source: ESI
   ESI capillary position: 90 degree.
   Calibration File Type: High Resolution

   Ion Source
   I spray voltage: 4000 V
   Sheath gas pressure: 35 (arb)
   Aux gas pressure: 3 (arb)
   Tube Lens Offset: 121V
   Capillary Offset: 35 V
   Capillary temperature: 300°C

   Q2 collision gas: 1.5 mTorr,
   Positive polarity,
   Data type: centroid
   Chrom Filter Peak Width: unchecked
   Scan type: SRM
   Tube Lens: Tuned value
   Q1 power (peak width m/z): 0.7 FWHM

Q3 power (peak width m/z): 0.7 FWHM
Number of segments: 1
Number of events: 2

Event 1 conditions:

SRM (selective reaction monitor) table of scan event 1:

Parent (m/z)	Product (m/z)	Width (m/z)	Time (s)	Collision Energy (V)
209	134	1	0.2	13
209	166	1	0.2	11
209	192	1	0.2	15
Source CID Collision energy: 10				

Event 2 conditions:

SRM table of scan event 2:

Parent (m/z)	Product (m/z)	Width (m/z)	Time (s)	Collision Energy (V)
236	104	1	0.2	15
236	134	1	0.2	12
236	149	1	0.2	13
249	104	1	0.2	15
249	134	1	0.2	13
249	178	1	0.2	13
335	128	1	0.2	8
335	262	1	0.2	10
335	291	1	0.2	12
Source CID Collision energy: 15				

4. **Injection sequence** (injection is always 40 µl)
    1. Inject a 50/50 (v/v) water/MeOH blank as the first and second injection (volume)
    2. Inject method blank as the third injection
    3. Inject a 50/50 (v/v) water/MeOH blank before and after each sample, STD (standard) and method blank.
    4. Inject D8000 standard sample both to start and to end the bracket of samples.

J. CONFIRMATION CRITERIA

1. Retention time of the analytes must agree within 5% of that of standard.
2. The two ion ratios of the analytes must agree within 10% of that of standard.

Comments or questions on method procedures may be directed to:

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Drug & Chemical Residues Methods (/Food/FoodScienceResearch/LaboratoryMethods/ucm2006950.htm)

Elemental Analysis Manual (EAM) for Food and Related Products (/Food/FoodScienceResearch/LaboratoryMethods/ucm2006954.htm)

Macroanalytical Procedures Manual (MPM) (/Food/FoodScienceResearch/LaboratoryMethods/ucm2006953.htm)

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