Amended, Aug 04, 2022
Implemented, Jan 01, 2023
MOHWV0040.07

Method of Test for Veterinary Drug Residues in Foods - Test of Nitrofuran Metabolites

1. Scope

This method is applicable to the determination of nitrofuran metabolites in honey, milk, eggs, muscle and viscera of livestock, poultry and aquatic products.

2. Method

After hydrolysis, derivatization, extraction and purification, nitrofuran metabolites are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: CORTECS C18, 2.7 μm, 2.1 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Horizontal shaking bath: controlled temperature within ± 1°C.

2.1.4. Centrifuge: centrifugal force ≥ 2600 ×g.

2.1.5. Vortex mixer.

2.1.6. pH meter.

2.1.7. Nitrogen evaporator.

2.2. Chemicals

Methanol, HPLC grade;
Ethyl acetate, HPLC grade;
n-Hexane, HPLC grade;
Dimethylsulfoxide (DMSO), reagent grade;
2-Nitrobenzaldehyde, reagent grade;
Dipotassium hydrogen phosphate, reagent grade;
Sodium chloride, reagent grade;
Sodium hydroxide, reagent grade;
Ammonium acetate, reagent grade;
Hydrochloric acid, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
3-Amino-5-methylmorpholino-2-oxazolidinone (AMOZ), 3-amino-

2-oxazolidinone (AOZ), 1-aminohydantoin hydrochloride (AH-HCl), 3,5-dinitrosalicylic acid hydrazide (DNSAH) and semicarbazide hydrochloride (SC-HCl), reference standards; 3-Amino-2-oxazolidinone-d_4 (AOZ-d_4), 3-amino-5-methylmorpholino-2-oxazolidinone-d_5 (AMOZ-d_5), 1-aminohydantoin-$^{13}C_3$ (AH-$^{13}C_3$), 3,5-dinitrosalicylic acid hydrazide-$^{15}N_2$ (DNSAH-$^{15}N_2$) and semicarbazide-$^{13}C^{15}N_2$ hydrochloride (SC-$^{13}C^{15}N_2$ HCl), isotope-labelled internal standards.

2.3. Apparatus

2.3.1. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.2. Volumetric flask: 50 mL, amber.

2.3.3. Solid phase extraction cartridge: Mega Bond Elut Plexa, 500 mg, 6 mL, or an equivalent product.

2.3.4. Membrane filter: 0.22 μm, PVDF.

2.4. Reagents

2.4.1. 50 mM 2-nitrobenzaldehyde

Dissolve and dilute 0.075 g of 2-nitrobenzaldehyde with methanol to 10 mL. Freshly prepare, and store in an amber flask.

2.4.2. 0.125 M hydrochloric acid

Slowly transfer 10.4 mL of hydrochloric acid into 500 mL of deionized water, and dilute with deionized water to 1000 mL.

2.4.3. 0.8 M sodium hydroxide

Dissolve and dilute 16 g of sodium hydroxide with deionized water to 500 mL.

2.4.4. 0.1 M dipotassium hydrogen phosphate

Dissolve and dilute 17.4 g of dipotassium hydrogen phosphate with deionized water to 1000 mL.

2.4.5. 20% methanol

Dilute 20 mL of methanol with deionized water to 100 mL.

2.4.6. 30% methanol

Dilute 30 mL of methanol with deionized water to 100 mL.

2.5. Mobile phase

2.5.1. Solvent A:

Dissolve and dilute 0.39 g of ammonium acetate with deionized water to 1000 mL, and filter with a membrane filter.

2.5.2. Solvent B: methanol.

2.6. Internal standard solution preparation

Transfer equivalent 5 mg of AOZ-d_4, AMOZ-d_5, AH-$^{13}C_3$, DNSAH-$^{15}N_2$ and SC-$^{13}C^{15}N_2$ isotope-labelled internal standards accurately weighed to each 50-mL volumetric flask, dissolve and dilute with methanol to volume as the internal standard stock solutions. Store in the dark in the freezer. When to use, mix appropriate volume of each internal standard stock solution, and dilute with methanol to 100 ng/mL as the internal standard solution.

2.7. Standard solution preparation

Transfer equivalent 5 mg of AOZ, AMOZ, SC and AH reference standards accurately weighed to each 50-mL volumetric flask, dissolve and dilute with methanol to volume as the standard stock solutions. Store in the dark in the freezer. Transfer about 5 mg of DNSAH reference standards accurately weighed to a 50-mL volumetric flask, dissolve with 2 mL of DMSO, and dilute with methanol to volume as the standard stock solution. Store in the dark in the freezer. When to use, mix appropriate volume of each standard stock solution, and dilute with methanol to 100 ng/mL as the standard solution.

2.8. Sample solution preparation

2.8.1. Hydrolysis and derivatization

Transfer about 2 g of the fine-cut and homogenized muscle and visceral sample accurately weighed; remove eggs' shells, and transfer about 2 g of the mixed egg white and yolk sample accurately weighed; transfer about 2 g of the well-mixed honey sample accurately weighed; accurately transfer 2 mL of milk sample into a 50-mL centrifuge tube(note), and add 50 μL of the internal standard solution. After standing for 15 min, add 10 mL of 0.125 M hydrochloric acid and 0.4 mL of 50 mM 2-nitrobenzaldehyde, vortex-mix for 15 sec, and horizontally shake at 80 rpm in a water bath at 37°C for 16 hr in the dark.

Note: When SC in the muscle or the visceral sample was detected higher than 0.5 ppb, the washing step should be conducted after sampling. The step is as follows: Transfer about 2 g of the fine-cut and homogenized sample accurately weighed into a 50-mL centrifuge tube, add 10 mL of 50% methanol, vortex-mix for 30 sec, centrifuge at 2600 ×g for 5 min, and discard the supernatant. Wash the precipitate with 10 mL of 75% methanol, 10 mL of methanol and 5 mL of deionized water in order, and follow the above procedure. Discard the supernatant, add 50 μL of the internal standard solution to the precipitate, stand for 15 min, and follow the hydrolysis and derivatization procedures described in section 2.8.1.

2.8.2. Extraction and purification

2.8.2.1. Muscle, viscera, eggs and honey

Cool the derivatized solution from section 2.8.1 to room temperature, add 1 mL of 0.1 M dipotassium hydrogen phosphate and 1 mL of 0.8 M sodium hydroxide, vortex-mix for 15 sec, and adjust pH to 7.3 ± 0.2 with 0.8 M sodium hydroxide or 0.125 M hydrochloric acid. Wash the electrode with deionized water, collect the washing liquid into the original centrifuge tube, and dilute with deionized water to 20 mL. Vortex-mix for 15 sec, centrifuge at 2600 ×g for 5 min, and collect the supernatant into another centrifuge tube. Add 3 mL of deionized water to the residue, and repeat the extraction step once. Combine the supernatants, add 0.5 g of sodium chloride and 12 mL of ethyl acetate, vortex-mix for 1 min, centrifuge at 2600 ×g for 5 min, and collect the upper layer (ethyl acetate layer) to a 15-mL centrifuge tube. Evaporate to dryness by gently flushing with a stream of nitrogen in a water bath at 40°C. Add 1 mL of 20% methanol to the residue, vortex-mix to dissolve, and add 1 mL of *n*-hexane. After mixing, centrifuge at 2600 ×g for 5 min, collect the lower layer, and filter with a membrane filter. Take the

filtrate as the sample solution.

2.8.2.2. Milk

Cool the derivatized solution from section 2.8.1 to room temperature, add 1 mL of 0.1 M dipotassium hydrogen phosphate and 1 mL of 0.8 M sodium hydroxide, vortex-mix for 15 sec, and adjust pH to 7.3 ± 0.2 with 0.8 M sodium hydroxide or 0.125 M hydrochloric acid. Wash the electrode with deionized water, collect the washing liquid into the original centrifuge tube, and dilute with deionized water to 20 mL. Vortex-mix for 15 sec, centrifuge at 2600 ×g for 5 min, and collect the supernatant. Add 3 mL of deionized water to the residue, and repeat the extraction step once. Combine the supernatants, and transfer into a solid phase extraction cartridge prerinsed with 5 mL of methanol and 5 mL of deionized water, and discard the eluent. Wash the cartridge with 3 mL of deionized water and 3 mL of 30% methanol, and discard the eluent. Dry the cartridge by vacuum suction, add 6 mL of methanol to the cartridge, and collect the eluent. Evaporate the eluent to dryness by gently flushing with a stream of nitrogen in a water bath at 40°C. Dissolve and dilute the residue with 1 mL of 20% methanol. Filter with a membrane filter, and take the filtrate as the sample solution.

2.9. Calibration standard curve

Take a blank sample, add 10-100 μL of the standard solution and 50 μL of the internal standard solution respectively, and follow the procedures described in section 2.8 to obtain the calibration standard solutions. Operate LC-MS/MS according to the following conditions. Establish the calibration standard curve of each nitrofuran metabolite by the ratios of peak area of each nitrofuran metabolite to that of the internal standard vs. the added concentrations in the range of 1-10 ng/mL of each nitrofuran metabolite.

LC-MS/MS operating conditions (note):

Column: CORTECS C18, 2.7 μm, 2.1 mm i.d. × 10 cm.

Mobile phase: a gradient program of solvent A and solvent B is as

follows.

Time (min)	A (%)	B (%)
0.0 → 1.0	80 → 80	20 → 20
1.0 → 9.0	80 → 0	20 → 100
9.0 → 12.0	0 → 0	100 → 100
12.0 → 13.0	0 → 80	100 → 20
13.0 → 17.0	80 → 80	20 → 20

Flow rate: 0.3 mL/min.

Injection volume: 20 μL.

Ion spray voltage: ESI^+, 5.5 kV; ESI^-, -4.5 kV.

Turbo heater temperature: 550°C.

Nebulizer gas (GS1): 30 psi.

Heated gas (GS2): 55 psi.

Curtain gas: 20 psi.

Collision gas: High.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, declustering potential and collision energy are as follows:

Analyte	Ionization mode	Ion pair	Declustering potential (V)	Collision energy (eV)
		Precursor ion (*m/z*)> product ion (*m/z*)		
SC	ESI^+	209 > 192*	40	16
		209 > 166		15
		209 > 134		16
AOZ	ESI^+	236 > 134*	60	17
		236 > 104		30
		236 > 149		20
AH	ESI^+	249 > 134*	80	18
		249 > 104		30
		249 > 178		21
AMOZ	ESI^+	335 > 128*	60	30
		335 > 262		16
		335 > 291		23
DNSAH	ESI^-	374 > 182*	-70	-28
		374 > 226		-28
		374 > 183		-28
SC-$^{13}C^{15}N_2$ (I.S.)	ESI^+	212 > 168	65	15
AOZ-d_4 (I.S.)	ESI^+	240 > 134	60	18

AH-$^{13}C_3$ (I.S.)	ESI^+	252 > 134	80	20
AMOZ-d_5 (I.S.)	ESI^+	340 > 296	60	17
DNSAH-$^{15}N_2$ (I.S.)	ESI^-	376 > 183	-70	-36

*The quantitative ion. The qualitative ion pair should be selected at least one ion pair depending on the matrix.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.10. Identification and quantification

Accurately inject 20 μL of the sample solution and the calibration standard solutions into LC-MS/MS separately, and operate according to the conditions described in section 2.9. Identify each nitrofuran metabolite based on the retention time and the relative ion intensities(note). Calculate the amount of each nitrofuran metabolite in the sample by the following formula.

The amount of each nitrofuran metabolite in the sample (ppb) = $\frac{C \times V}{M}$

Where,

C: the concentration of each nitrofuran metabolite in the sample solution calculated by the calibration standard curve (ng/mL)

V: the final make-up volume of sample (mL)

M: the weight of sample (g or mL)

Note: Relative ion intensities are calculated by peaks areas of qualitative ions divided by peak areas of quantitative ions. Maximum permitted tolerances of relative ion intensities by LC-MS/MS are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≤ 10	± 50

Remark

1. The limits of quantitation (LOQs) for AMOZ, AOZ, AH, DNSAH and SC are all 0.5 ppb.

2. Further validation shall be performed when interfering compounds appear in samples.
3. The binding types of SC naturally occur in the shells of crustaceans at high levels and their surface muscles may be contaminated with SC. Therefore, the surface muscle near the shell should be excluded before analyzing.
4. In addition to the use of nitrofurans causing the residue of SC, azodicarbonamide, one of foaming agents for plastic products, may generate SC during heating process. Some studies indicate that trace amounts of SC may be generated in egg powder, milk powder or honey etc. during processing.

Reference

1. Crews, C. 2012. Potential natural sources of semicarbazide in honey. Report for the Food Standards Agency in Scotland. Project code FS241065. The Food and Environment Research Agency, UK.
2. Zhang, P. A., Zhang, J. W., Qiao, M. W. and Tang, G. F. 2010. Determination of metabolites of nitrofuran antibiotics in honey by high performance liquid chromatography-tandem mass spectrometry. J. Zhejiang Agric. Sci. 3: 611-613.
3. Chu, P. S. and Lopez, M. I. 2007. Determination of nitrofuran residues in milk of dairy cows using liquid chromatography−tandem mass spectrometry. J. Agric. Food Chem. 55: 2129-2135.
4. European Commission. 2019. Commission Regulation (EU) 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC. Off. J. Eur. Union L 289: 41-46.
5. Verdon, E., Couedor, P. and Sanders, P. 2007. Multi-residue monitoring for the simultaneous determination of five nitrofurans (furazolidone, furaltadone, nitrofurazone, nitrofurantoine, nifursol) in poultry muscle tissue through the detection of their five major metabolites (AOZ, AMOZ, SEM, AHD, DNSAH) by liquid chromatography coupled to electrospray tandem mass

spectrometry—In-house validation in line with Commission Decision 657/2002/EC. Anal. Chim. Acta 586: 336-347.

Reference chromatogram

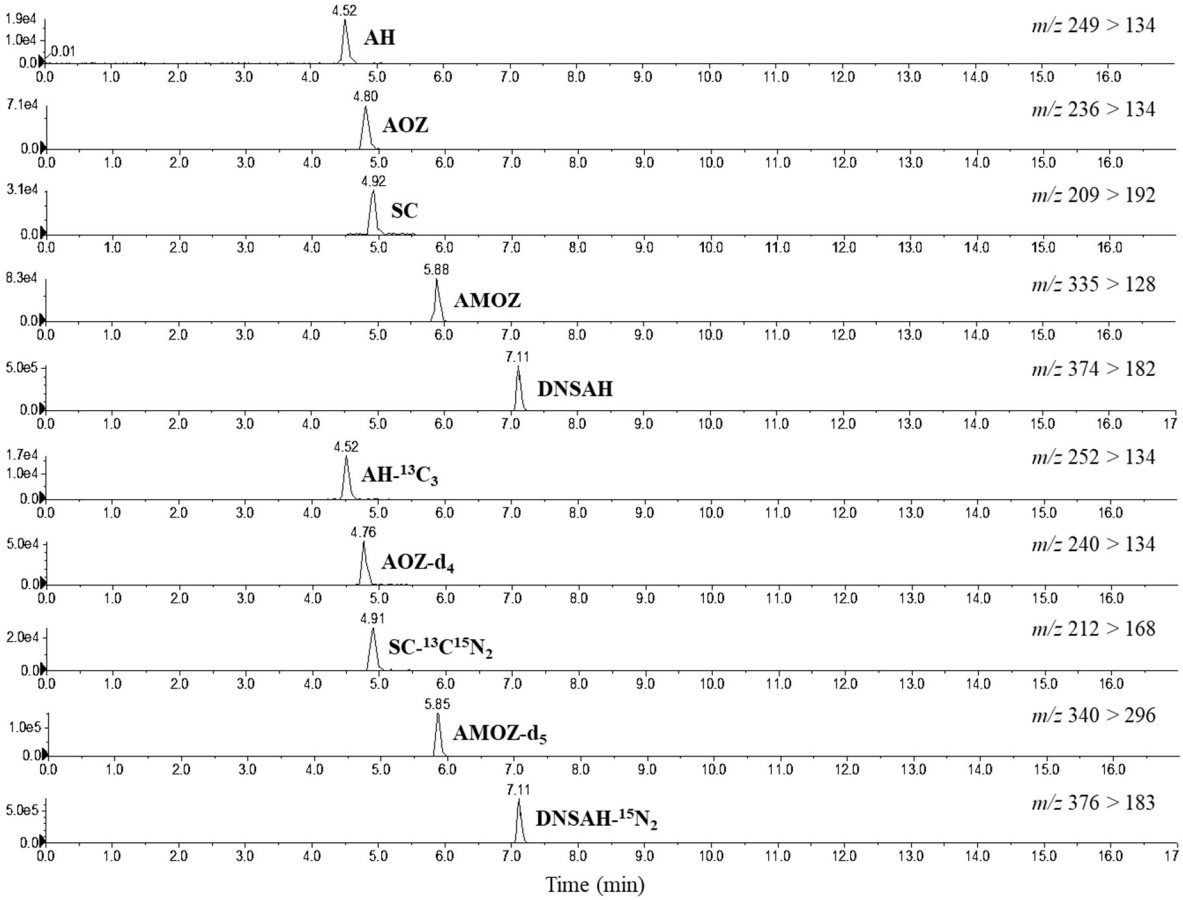

Figure. MRM chromatograms of 5 nitrofuran metabolite standards and their isotope-labelled internal standards analyzed by LC-MS/MS.