Application Note: 361

Determination of Trace Level Nitrofuran Metabolites in Crawfish Meat by Electrospray LC-MS/MS on the TSQ Quantum Discovery MAX

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Key Words

- TSQ Quantum Discovery MAX™
- Surveyor™ HPLC
- Food Residue Analysis
- SRM
- Veterinary Drugs

Introduction

Nitrofurans (furazolidione, furaltadone, nitrofurazone and nitrofurantoin) are a group of veterinary antibiotics banned in many countries because of human health concerns. The ban has stimulated significant interest in development of analytical methods for detecting trace levels of these drug residues in animal products.

Due to the rapid *in vivo* metabolism of the parent drugs, detection of nitrofurans in meat products relies on determination of their corresponding tissue-bound metabolites: 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD). These metabolites were removed from tissues by hydrolysis in acidic solution and derivatized to nitrobenzyl- (NB-) derivatives with 2-nitrobenzyladehyde (2-NBA). Figure 1 illustrates the transformation. LC-MS/MS utilizing selected reaction monitoring (SRM) of the corresponding four metabolite derivatives has become the method of choice.

In this note we describe a sensitive and selective LC-MS/MS method for detecting trace level nitrofuran metabolites in crawfish using a Thermo Scientific TSQ Quantum Discovery MAX triple quadrupole mass spectrometer coupled to a Thermo Scientific Surveyor HPLC module. The limit of quantitation (LOQ) as low as <0.05 µg/kg has been clearly demonstrated in fortified crawfish meat for all four nitrofuran metabolites. This LOQ represents twenty-fold better than the Minimum Required Performance Limit (MRPL) established by European Union (EU) in 2003.

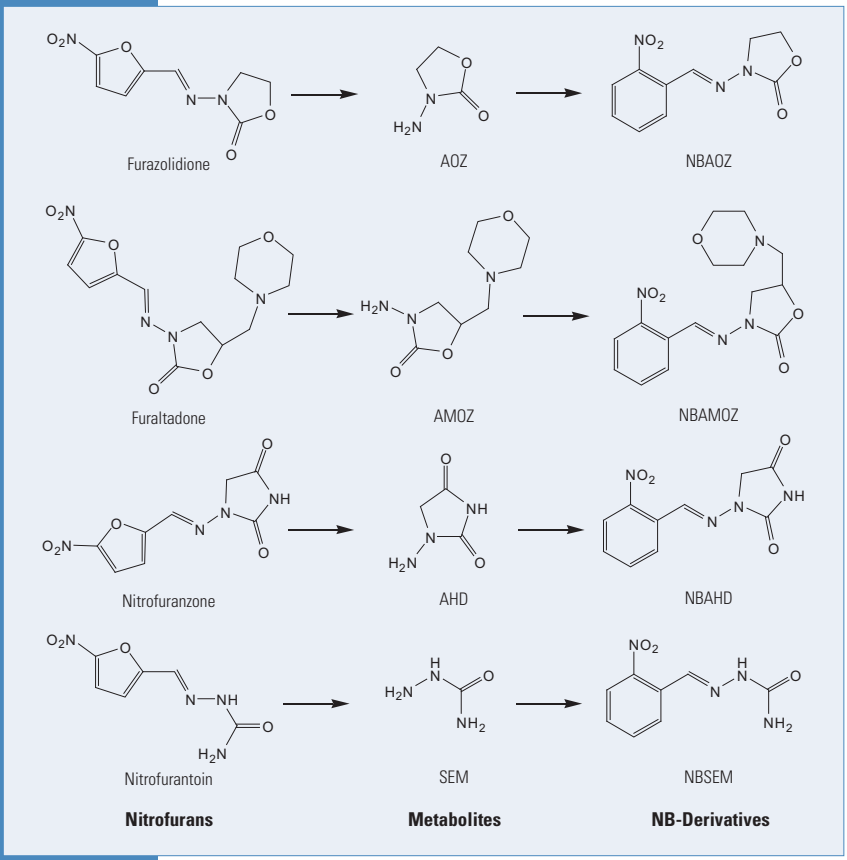

Figure 1: Nitrofurans, their metabolites, and 2-NBA derivatives

Experimental Conditions

Standards and Reagents

The following are a list of chemicals used in this work, and unless specified all chemicals are of at least reagent grade.

AOZ and SEM•HCl (Sigma-Aldrich, St. Louis, MO, USA)

2-NBA (Sigma-Aldrich)

DMSO (Sigma-Aldrich)

d_4-AMOZ and d_5-AMOZ (Cambridge Isotope Laboratory, MA, USA)

1-Amino-imidazolidin-2,4-dione-[2,4,5-^{13}C] (WITEGA Laboratorien Berlin-Adlershof GmbH, Berlin, Germany)

Semicarbazide hydrochloride-^{13}C, $^{15}N_2$ (WITEGA)

Ammonium Acetate (NH_4Ac), K_2HPO_4, and NaOH (Sigma-Aldrich)

Methanol (HPLC grade, Thermo Fisher Scientific, Pittsburgh, PA, USA)

Water (in-house distilled water, filtered with a 0.45 µm filter)

Analytical Equipment

HPLC: Surveyor HPLC module consisting of an AS Autosampler and MS Pump
Mass spectrometer: TSQ Quantum Discovery MAX

Analytical Standard Preparation

The primary analytical standard solutions of 1 mg/mL were prepared by dissolving the corresponding solid standards into methanol. The working standard solutions were prepared by serial dilution of the primary standard solution with 95:5 water:methanol.

Sample Preparation

Note: Nitrofuran metabolite derivatives are sensitive to light; avoid prolonged exposure of sample to direct light sources during sample preparation.

The extraction and derivatization of the nitrofurans from the crawfish were performed at Food Laboratory of Jiangsu Entry-Exit Inspectional and Quarantine Bureau at Nanjing, China, following the published procedure with some modification:

- To 2 g of homogenized crawfish meat inside a 50-mL glass tube, add 4 mL water, 0.5 mL of 0.5 M HCl solution, and 200 µL of freshly prepared 50 mM 2-NBA in DMSO. Vortex for one minute and store the sample in the dark at 37°C overnight (14-16 hours)
- After cooling the sample to room temperature, add 5 mL 0.1 M K_2HPO_4, adjust the pH of the mixture to 7.0-7.5 with 0.4 M NaOH solution. Centrifuge the mixture and collect the supernatant
- Extract the supernatant twice each time with 7 mL ethyl acetate. Combine the ethyl acetate extracts and evaporate to dryness under N_2 at 40°C
- Reconstitute the residues in 1.0 mL of water:methanol (95:5). Centrifuge the samples and filter the supernatant with 0.2 µm syringe filter prior to injection to LC-MS system

Note that the sample preparation results in a two-fold concentration that will be factored into the calculation of nitrofuran metabolite concentrations in meat samples. For fortified samples, the nitrofuran metabolites and their internal standards were added into the homogenized meat sample prior to the hydrolysis and derivatization. For calibration, the same procedures were followed except that 2 mL of working standard solutions was used instead of the meat samples.

Chromatography Conditions

Analytical column: Thermo Scientific Hypersil GOLD,™ 5 µm, 100 × 2.1 mm
Eluent: A: 0.5 mM Ammonium Acetate in Water;
B: Methanol

Gradient:

Time (min)	% A	% B
0	80	20
8.5	50	50
9.5	50	50
10	80	20
15	80	20

Flow rate: 250 µL/min
Column temperature: Ambient (18-22°C)
Injection volume: 20 µL (with loop)

Mass Spectrometry Conditions

The mass spectrometer was calibrated routinely with 1,3,6-polytryosine, according to the standard operating procedures at the Nanjing laboratory. For method development, a standard solution containing 1 µg/mL of derivatized nitrofuran metabolites including the internal standards was infused at 10 µL/min with 250 µL/min 50:50 (A:B) mobile phase into the ESI source. First, the spray voltage, sheath gas, auxiliary gas and tube lens were optimized with the automated tune of Thermo Scientific Xcalibur™ software. Second, the most abundant fragment ions and their optimized collision energy (CE) values were found in MS/MS optimization. For known SRM transitions, parent and product ions can be input directly to obtain the optimized CE value for each SRM transition. Finally, the Source CID (skimmer offset voltage), collision gas pressure, and ion transfer capillary temperature were adjusted manually for best signal sensitivity. The final operation parameters are summarized as follows:

Ion source (polarity): ESI (+)
Spray voltage: 5000 V
Sheath gas pressure: 30 units
Auxiliary gas pressure: 8 units
Ion transfer capillary temperature: 300°C
Source CID: 10 V
Scan type: SRM
Q1 and Q3 peak width (FWHM): 0.7 Da
Collision gas and pressure: Ar at 1.3 mTorr

For each parent ion, two SRM transitions were used, one for quantitation and one for confirmation, which would give 4 IP (identification points) to meet the EU's criteria for residue analysis in food. Based on the elution order of the nitrofuran metabolite derivatives, the chromatography run was divided into three segments for data acquisition. Table 1 lists SRM transitions and their parameters in each segment.

Results and Discussion

Figure 2 shows representative chromatograms of a 0.050 µg/kg fortified crawfish sample. As shown, all four nitrofuran metabolite derivatives were detected with excellent signal quality as measured by signal-to-noise (S/N) ratio.

The LOQ values reported in literature, ranging from 0.02 to 0.1 µg/kg for different nitrofuran metabolites in meat samples, have mostly been obtained from extrapolation based on S/N =10 of the signals of analytes at higher concentrations in standards or fortified samples. In reality, however, these extrapolated LOQ's often cannot be achieved, because the S/N ratios of the signals deteriorate more than as predicted by the dilution factor. The current data in Figure 2 demonstrates that all four nitrofuran metabolites can be detected in fortified crawfish samples at 0.05 µg/kg level, far lower than the MRPL value of 1 µg/kg required by EU.

Figure 3 shows seven-point calibration curves constructed from data from measuring standard solutions at concentration levels of 0.025, 0.2, 0.5, 1, 2.5, 5.0 and

Segment	Time (min)	Analyte	Parent Ion (*m/z*)	Product Ions (*m/z*)	CE (V)
1	0–7.4	NBAOZ	236.045	104	19
			236.045	134*	22
		d_4-NBAOZ (IS)	240.037	134	14
		NBAHD	249.040	104	22
			249.040	134*	14
		$^{13}C_3$-NBAHD (IS)	252.037	134	14
2	7.4–8.5	NBSEM	209.000	166*	11
			209.000	192	13
		(^{13}C, $^{15}N_2$)-NBESEM (IS)	212.048	168	11
3	8.5–14.5	NBAMOZ	335.092	291*	12
			335.092	262	19
		d_5-NBAMOZ (IS)	340.134	296	12

Table 1: Segments of chromatography separation and SRM transitions

Note: * SRM transition for quantitation; IS : internal standard; CE: Collision Energy. For each segment, Scan Time (s) = 0.1 and Scan Width (*m/z*) = 0.002.

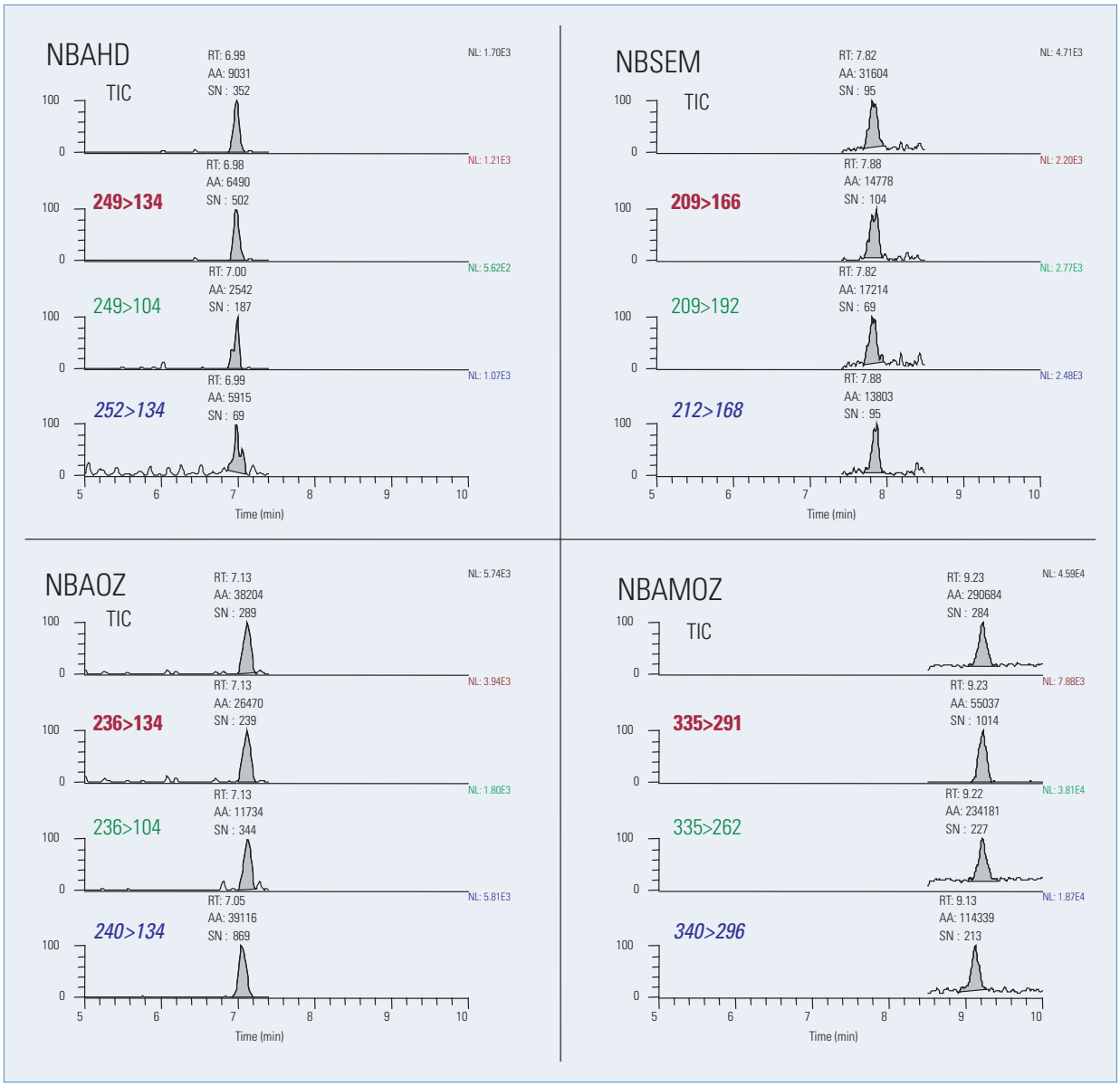

Figure 2: Chromatograms of shrimp meat sample containing 0.050 µg/kg fortified nitrofurans and internal standard in the shrimp meat.
For each panel from the top: TIC (total ion current), SRM for quantitation (bold and red) and confirmation (green), and internal standard (italic and blue).
RT: retention time, AA: peak area counts, SN: signal-to-noise ratio.

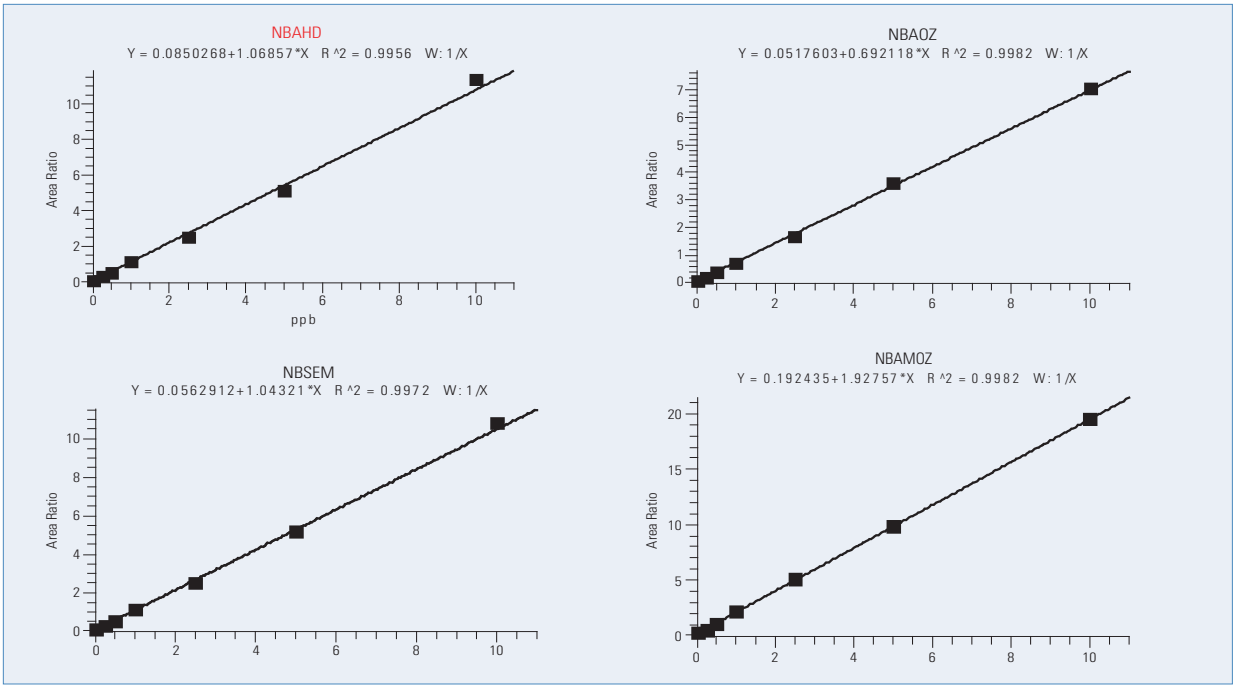

Figure 3: Seven-point calibration curves of nitrofuran metabolite, from 0.025 ng/mL (equivalent to 0.05 ng/mL after sample preparation) to 10 ng/mL

10.0 ng/mL. Excellent linearity was obtained for all four nitrofuran metabolites within the calibration range, with the correlation coefficient $R^2 > 0.995$ (weight factor = 1/X).

The method accuracy and precision were evaluated by performing triplicate preparation and analysis of one batch of homogenized crawfish meat samples fortified with nitrofuran metabolites at three different concentration levels of 0.05, 0.5 and 2.5 µg/kg. The results are given in Table 2. As shown, recovery values in the range of 79%–110% were obtained with standard deviations from 3 to 22%. It should be noted that standard deviations include the errors of both the sample preparation (major contributor) and analytical instrument.

Fortification Level (µg/kg)	AHD	AOZ	SEM	AMOZ
0.05	82 ± 13%	110 ± 22%	89 ± 15%	98 ± 14%
0.5	88 ± 4%	100 ± 11%	100 ± 11%	95 ± 6%
2.5	109 ± 3%	86 ± 18%	79 ± 19%	100 ± 3%

Table 2: Mean recovery values (n=3) of crawfish samples fortified at three levels

Note: values given after ± are standard deviations.

Conclusions

With use of the TSQ Quantum Discovery MAX, a sensitive and reliable LC-MS/MS method using SRM has been developed for detecting trace level nitrofuran metabolites with a quantitation limit of less than 0.050 µg/kg in crawfish. The sample preparation procedure is relatively straightforward and setup of the instrument method is easy and fast.

References

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