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New Reagent for Trace Determination of Protein-Bound Metabolites of Nitrofurans in Shrimp Using Liquid Chromatography with Diode Array Detector

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The synthesis of derivatives of metabolites from furazolidone, furaltadone, nitrofurazone, and nitrofurantoin using a new derivatizing reagent, 2-naphthaldehyde (NTA), is described. The reaction product was used in liquid chromatography with diode array detector (LC-DAD) for determination of protein-bound metabolites of nitrofurans in shrimp followed by two steps of liquid−liquid extraction. Derivatives of nitrofuran metabolites are well separated from NTA remaining in the extract upon separation on a ChromSpher 5 Pesticide (250 × 4.6 mm, 5 μm) column at 40 °C with acetonitrile/5 mM ammonium acetate adjusted to pH 7.5 gradient as the mobile phase and DAD detection at 308 nm except for naphthyl derivative of 1-aminohydantoin at 310 nm. The high absorptivity of these derivatives makes simultaneous screening of these metabolites in shrimp at 1 μg/kg possible for the first time using LC-DAD. The method was validated using blank shrimp fortified with all four metabolites at 1, 1.5, and 2 μg/kg. Recoveries were >86% with relative standard deviations of <14% for all four metabolites. Comparison between LC-DAD and APCI-MS/MS shows very good agreement for shrimp samples.

KEYWORDS: Protein-bound metabolites of nitrofurans; HPLC; DAD; shrimp; APCI-MS/MS

INTRODUCTION

Nitrofurans have been used previously in livestock for the treatment and prevention of various gastrointestinal infections caused by bacteria or protozoa and also as growth promoter (*1*). The most common nitrofurans are furazolidone, furaltadone, nitrofurazone, and nitrofurantoin. Because of their potentially carcinogenic and mutagenic effects on human health, the use of these compounds in food-producing animals is prohibited in various countries, except for topical applications. These compounds are rapidly metabolized in vivo, leading to metabolites that are accumulated in protein called protein-bound metabolites and stable over considerable periods of time (*2, 3*). These metabolites are markers for the detection of their parent compounds. The structures of nitrofuran parent drugs and their metabolites are shown in **Figure 1**.

Although in 2005 the scientific panel of the European Food Safety Authority (EFSA) on food additives, flavoring, processing aids, and materials in contact with food has stated that at the low concentration of semicarbazide (SEM) present in food the issue of carcinogenicity is not of concern for human health (*4*),

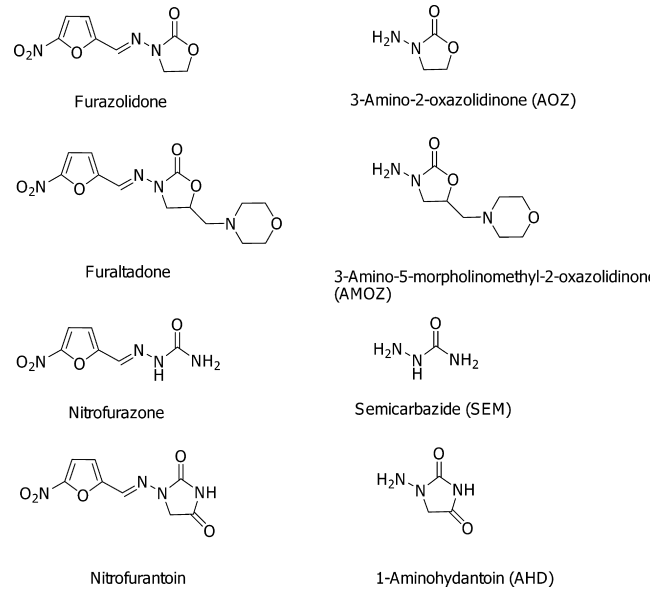

Figure 1. Structures of nitrofurans and their metabolites.

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Table 1. Optimized Parameters for MS/MS Transitions of the Derivatives of Nitrofurans[a]

period	analyte	MS/MS transition	DP (V)	FP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
2	NTAHD	251.7/99.1	−16	−350	4.5	−14	−20	−6
		251.7/153.3					−20	−6
3	NTAOZ	241.0/88	21	360	−12.0	12	25	11
		241.1/126.7					41	32
3	NTAOZ-d_4	244.9/91.9	21	360	−10.5	12	29	13
		244.9/126.8					43	30
3	NTSEM	214.1/127.1	16	360	−10.0	14	43	6
		214.1/154.4					29	18
4	NTAMOZ	340.2/112.1	6	360	−9.5	16	30	15
		340.2/267.1					21	14

[a] Durations for periods 2, 3, and 4 are 0.798, 1.194, and 3.001 min, respectively. DP, declustering potential; FP, focusing potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision cell energy; CXP, collision cell exit potential.

Table 2. APCI Source Parameters[a]

	period 2	period 3	period 4
ionization mode (APCI)	negative	positive	positive
curtain gas (CUR, psi)	17	15	20
corona discharge needle current (NC, μA)	−6	6	5
collision gas (CAD, psi)	7	4	4
source gas 1 (GS1, psi)	90	80	80
source gas 2 (GS2, psi)	10	20	10
temperature (°C)	550	550	550

[a] Durations for periods 2, 3, and 4 are 0.798, 1.194, and 3.001 min, respectively.

SEM remains, however, under EU legislation the marker residue for nitrofurazone.

There have been many confirmatory methods (*5*−*11*) based on LC-MS/MS for the detection of nitrofuran metabolites at minimum required performance limit (MRPL) of 1 μg/kg for each nitrofuran metabolites in compliance with regulation set by the EU Commission Decision of March 13, 2003 (*12*). The first method for determination of all four tissue-bound metabolites in shrimp using LC-MS/MS was reported by Leitner et al. (*5*). They used 2-nitrobenzaldehyde to derivatize metabolites released from tissue under mild acidic condition and clean up the derivatized products by solid phase extraction using Lichrolut before separation on an IntersilODS-3 column and quantified with ESI/MS/MS in multireaction monitoring mode. The use of 2-nitrobenzaldehyde was also applied to determine nitrofuran metabolites in other matrices such as honey (*6*), milk (*7*), shrimp (*8*), poultry muscle and shrimp (*9*), meat (*10*), and fish feeds (*11*).

Because the confirmatory method based on LC-MS/MS is relatively expensive, many alternative screening methods using LC-MS (*13, 14*), LC-UV (*13, 15*), ELISA (*16*−*18*), and micellar electrokinetic capillary chromatography (*19*) have thus been developed. All of these methods except those for ELISA also used 2-nitrobenzaldehyde as a derivatizing agent. Different extensive cleanup methods for removal of this excess reagent were described. Horne et al. (*13*) introduced the hexane cleanup step following ethyl acetate extraction to remove 2-nitrobenzaldehyde present in excess in the analysis of protein-bound metabolites of furazolidone and furaltadone. The limits of detection for fortified control sample was 5 μg of 3-amino-2-oxazolidinone (AOZ)/kg and 10 μg of 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ)/kg of pig liver by HPLC-UV. Solid phase extraction method using an Oasis MAX cartridge to remove 99% of the excess 2-nitrobenzaldehyde in the analysis of AOZ from pig liver was reported and could successfully be automated for increased sample throughput (*15*). However, there is no report describing the simultaneous analysis of all four protein-bound nitrofuran drugs of interest in shrimp at a 1 μg/kg level using HPLC-DAD in one run. In this study, we introduced a new derivatizing agent, namely, 2-naphthaldehyde (NTA), from which naphthyl derivatives of nitrofuran metabolites were formed. These derivatives show 2-fold higher absorptivity than the corresponding 2-nitrophenyl derivatives of nitrofuran metabolites due to the presence of extended conjugated double bond in the naphthalene ring. Furthermore, all four derivatized metabolites are well separated on ChromSpher 5 Pesticide column using the LC-DAD technique. The method has been applied successfully in the determination of all four protein-bound metabolites of nitrofuran in shrimp at a 1 μg/kg level. The comparison of analysis results of shrimp samples using the developed method against LC-APCI-MS/MS was also made.

MATERIALS AND METHODS

Reagents. 1-Aminohydantoin hydrochloride, semicarbazide hydrochloride, and 2-naphthaldehyde were obtained from Aldrich (Milwaukee, WI). 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) and 3-amino-2-oxazolidinone (AOZ) were purchased from Riedel de Haen (Seelze, Germany). The internal standard 3-amino-2-oxazolidinone-d_4 hydrochloride (AOZ-d_4·HCl) was obtained from Chemical Synthesis Services (Belfast, Northern Ireland). All other chemicals were of analytical reagent grade. High-purity water was obtained from a Maxima water purification system (USF-Elga, High Wycombe, Bucks, U.K.). Standard stock solutions of AOZ, AMOZ, 1-aminohydantoin (AHD), and SEM were prepared at 200 μg/mL in methanol by weighing and kept in amber bottles at 4 °C. Working solutions were appropriately diluted with methanol as required.

LC-DAD Instrumentation and Settings. Analyses were performed on a ChromSpher 5 Pesticide column (250 × 4.6 mm, 5 μm, Varian) at 40 °C. An 1100 Agilent autosampler was employed. The eluent was delivered by a gradient system from an Agilent 1100 LC binary pump (Agilent Technology, Waldbronn, Germany). Eluent A was 5 mM ammonium acetate adjusted to pH 7.5, and eluent B was acetonitrile. The gradient elution was started from 10% acetonitrile and increased linearly to 40% over 1 min and then increased linearly further to 48% over 4 min, after which it was held for 10 min and increased linearly again to 100% and held for a further 10 min. The column flow rate was 1 mL/min, and equilibration time was 15 min prior to the next injection. The detector wavelength was at 308 nm for naphthyl derivatives of SEM, AOZ, and AMOZ and at 310 nm for naphthyl derivative of AHD. The injection volume was 10 μL.

LC-APCI-MS/MS Instrumentation and Settings. Separation was carried out on a ChromSpher 5 C18 column (250 × 4.6, 5 μm, Varian). Eluent A was 0.2 mM ammonium formate, and eluent B was methanol. The gradient elution started with 40% methanol and increased linearly

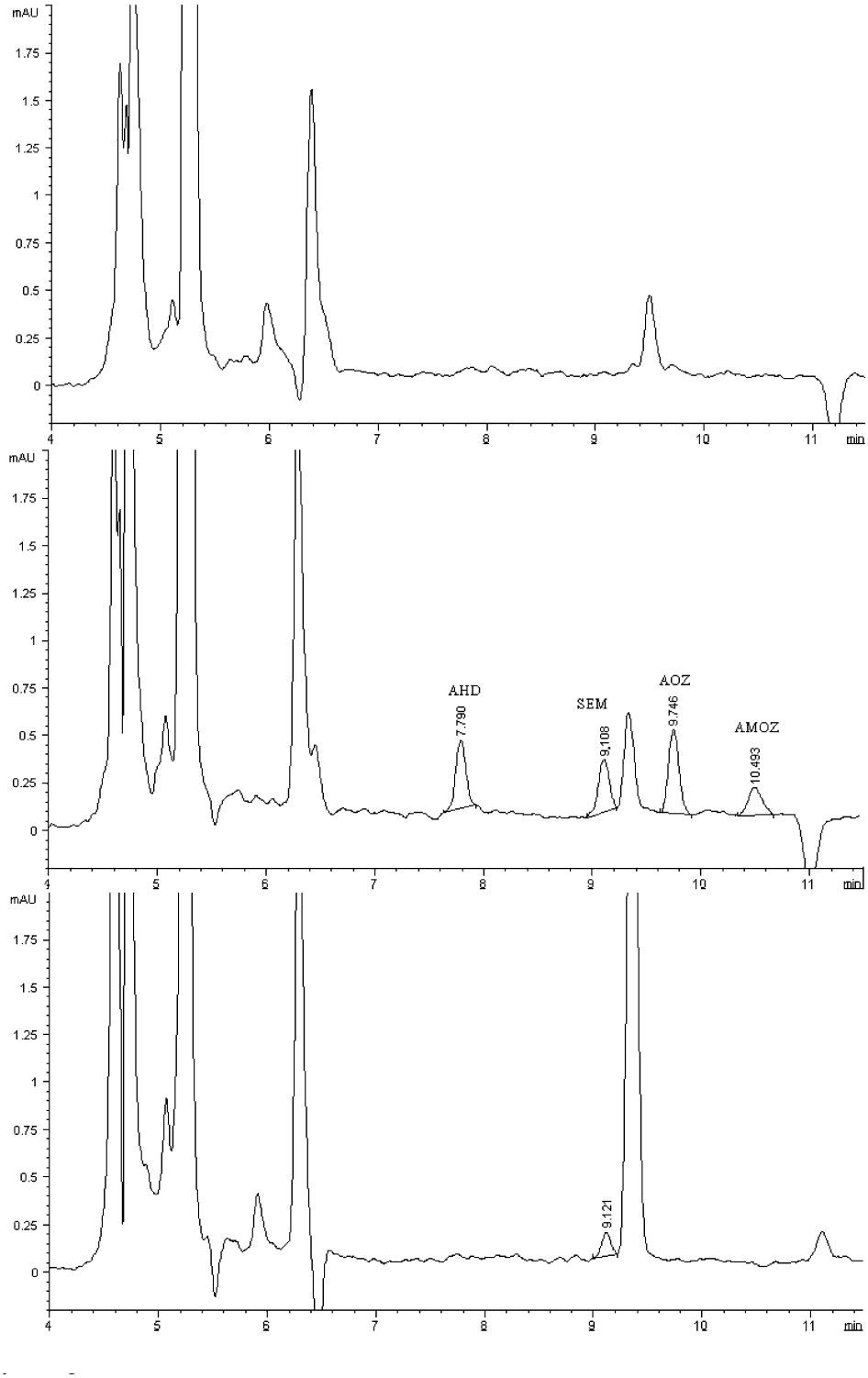


Figure 2. LC-DAD chromatograms of blank shrimp (top), shrimp fortified with a mixture of nitrofuran metabolites at 1 μg/kg (middle), and sample 5 (bottom).

Table 3. Regression Data and the LOD and LOQ from LC-DAD

analyte	R^2	LOD (μg/kg)	LOQ (μg/kg)
AHD	0.9980	0.2341	0.7803
AOZ	0.9979	0.2392	0.7973
SEM	0.9964	0.2092	0.6973
AMOZ	0.9972	0.2735	0.9118

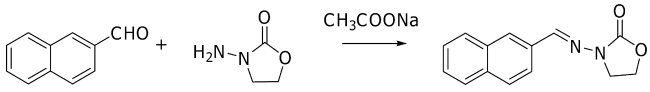
to 55% over 1 min, to 60% over 1 min, to 83% over 6.8 min, and to 100% over 0.1 min, after which it was held for 11.1 min. The column was equilibrated for 25 min prior to the next injection. A flow rate of 1 mL/min was used for the separation, and the injection volume was 20 μL. Mass spectrometric detection was carried out using an Applied Biosystem API 2000 triple-quadrupole instrument (Thornhill, Toronto, Canada) equipped with an APCI interface. Analyst software (1.1 version) from Applied Biosystems was used for system control and data acquisition. The mass analyzers Q1 and Q3 were operated at low resolution. The APCI source parameters, that is, ion source gas 1 (GS1), ion source gas 2 (GS2), temperature (TEM), curtain gas (CUR), discharge needle current (NC), and collision gas (CAD) in Q2, were

Table 4. Accuracy and Relative Standard Deviation of Performance Data of LC-DAD Analysis of Four Nitrofuran Metabolites in Blank Shrimp Samples at Three Fortification Levels

analyte	fortification level (μg/kg)	found (mean ± SD) (μg/kg, $n = 42$)	accuracy (%)	RSD (%)
AHD	1.00	1.10 ± 0.08	110	8
	1.50	1.46 ± 0.05	97	4
	2.00	1.93 ± 0.08	96	4
AOZ	1.00	1.12 ± 0.08	112	8
	1.50	1.49 ± 0.08	99	6
	2.00	1.95 ± 0.08	98	4
SEM	1.00	0.99 ± 0.13	99	13
	1.50	1.56 ± 0.17	104	11
	2.00	1.73 ± 0.08	86	5
AMOZ	1.00	1.06 ± 0.06	106	6
	1.50	1.51 ± 0.06	101	3
	2.00	2.01 ± 0.04	100	2

all optimized manually during the flow injection experiment until the highest signal achieved. The optimized MS/MS parameters and source parameters for the derivatives of nitrofuran metabolites are summarized in **Tables 1** and **2**, respectively.

Synthesis of 3-(2-Naphthaldehydeimino)-2-oxazolidinone (Assigned as NTAOZ).



A mixture of 3-amino-2-oxazolidinone (19.57 mg, 0.2 mmol) and sodium acetate (16.4 mg, 0.2 mmol) was dissolved in a mixture solution (2 mL) of water and ethanol (1:2). The pH of this solution was adjusted to 4 by 0.2 M HCl. To a mechanically stirred mixture of 3-amino-2-oxazolidinone was added a solution of 2-naphthaldehyde (NTA, 32.54 mg, 0.2 mmol) in 2 mL of ethanol dropwise (25−30 min). The resulting mixture was stirred for 7 h and left overnight. The white crystalline solid was recrystallized from ethanol, filtered off, washed well with absolute ethanol, and subsequently dried to yield naphthyl derivative of AOZ (33.84 mg; 70% yield), mp 251−252 °C; $R_f = 0.45$ (CH_2Cl_2/EtOAc, 1:1); IR (KBr) ν_{max} 3067, 3007, 2985, 2895, 1772, 1758, 1474, 1407, 1271, 1239, 1209, 1094, 1037, 979, 928, 863, 822, 747, 528, 477 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ_H 8.16 (1H, s, H-1), 7.98 (1H, s, H-1′), 7.96 (4H, m, Ar−H), 7.59 (2H, m, H-5, H-7), 4.52 (2H, dd, $J = 8.4$ and 7.3 Hz, H-5′), 3.99 (2H, dd, $J = 8.2$ and 7.5 Hz, H-4′); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 153.7 (qC, C-7′), 143.5 (CH, C-1′), 133.4 (qC, C-10), 132.7 (qC, C-9), 132.0 (qC, C-2), 128.3 (CH), 128.1 (2x, CH), 127.6 (CH), 126.8 (CH), 126.6 (CH), 122.7 (CH, C-3), 61.5 (CH_2, C-5′), 42.1 (CH_2, C-4′); APCITOFMS (positive ion mode) m/z 241.0972 $[M + 1]^+$ (calcd for $C_{14}H_{13}N_2O_2$, 241.0971). The high-resolution mass spectrum was obtained from a Bruker Daltonics microTOF with an APCI interface, scan range m/z 100−1200; capillary exit, 90.0 V; set reflector, 1300 V; set flight tube, 9000 V; and set detector, TOF 1950 V.

Other derivatives of nitrofuran metabolites were prepared as described above; all compounds were characterized by means of ^1H NMR, ^{13}C NMR, IR, and MS techniques. Their spectroscopic data and melting points are available in the Supporting Information.

Determination of Nitrofurans in Shrimp Using LC-DAD. The extraction was a modification of that developed by Horne et al. (*13*). An aliquot of blended shrimp tissue sample (20 ± 0.05 g) was placed in a conical flask. Thirty milliliters of 0.2 M HCl and 2 mL of 20 mM NTA in DMSO/MeOH (1:25) were added to the mixture, which was sonicated for 20 min before the addition of 0.12 g of NaOAc. One gram of KCl was added, and the mixture was sonicated for 5 min followed by incubation on the shaker overnight at 37 °C in the dark. After cooling, the mixture was adjusted to pH 7.4 ± 0.5 with 2.7 mL of a mixture solution of 2 M NaOH and 0.75 M K_2HPO_4 (1:1). The mixture was extracted twice with 10 mL of ethyl acetate on the shaker for 20 min. The combined extract was evaporated to dryness under the stream of nitrogen at 40 °C. The dry residue was redissolved in 0.4 mL of acetonitrile/H_2O (9:1). Two milliters of hexane was added to the extract to remove the matrix followed by vortex-mixing and centrifugation. The hexane layer was discarded, whereas the lower layer was filtered through a 0.2 μm nylon membrane filter prior to injection to LC-DAD.

Determination of Nitrofurans in Shrimp with LC-APCI-MS/MS. The above procedure was followed except that only a portion of 9 g of blended shrimp was used. Two hundred and seventy microliters of 50 ppb of an internal standard, AOZ-d_4·HCl, was spiked in the sample prior to the addition of 20 mM NTA and 0.2 M HCl, and the residue was redissolved in 0.15 mL of methanol/0.2 mM ammonium formate (9:1).

RESULTS AND DISCUSSION

The determination of protein-bound metabolites of nitrofuran in shrimp is based on the determination of product obtained after derivatization of released metabolites with a new reagent, 2-naphthaldehyde. Cleanup was performed in multiple steps by liquid−liquid extraction with ethyl acetate, evaporation of the extract to dryness, redissolvation of the residue in acetonitrile/water, and further matrix removal with hexane prior to separation on a ChromSpher 5 Pesticide column with diode array detection.

Two reversed phase columns, a ChromSpher 5 Pesticide column (250 × 4.6 mm, 5 μm) from Varian (Lake Forest, CA) and a LichroSpher 100 RP-18 column (250 × 4.6 mm, 5 μm) from Merck (Darmstadt, Germany), and one CN column, a Luna CN column (250 × 4.6 mm, 5 μm) from Phenomenex (Torrance, CA), were evaluated. The ChromSpher 5 Pesticide column gave the best performance. The pH control of ammonium acetate in the mobile phase proved to play an important role in attaining a proper separation of the analysis. The pH range from 5 and 7.7 was tested, and it was concluded that a pH value of 7.5 gave the best separation.

LC-DAD chromatograms of blank shrimp, fortified with a mixture of nitrofuran metabolites at 1 μg/kg, and shrimp sample 5 containing a detectable amount of SEM are shown in **Figure 2**. The order of elution is NTAHD < NTSEM < NTAOZ < NTAMOZ. No matrix peaks were observed around the retention time of all analytes in the blank shrimp sample.

Validation of the Method. Because the EU-MRPL for the nitrofuran metabolites is set at 1 μg/kg, the calibration range of the concentration of the residues was set from 0.5 to 5.0 μg/kg. The matrix-matched calibration curve for each analyte consists of a five-level blank shrimp fortified with a metabolite standard mixture at levels of 0.5, 1.0, 1.5, 2.0, and 5.0 μg/kg prior to acid hydrolysis. The matrix-matched calibration curve was performed in triplicate. Each prepared matrix-matched standard was measured once.

The matrix-matched calibration graphs were linear over the range of 0.5−5.0 μg/kg with correlation coefficients of 0.99 or better for all analytes. The limit of detection (LOD) and the limit of quantitation (LOQ) of each analyte were defined here as the concentrations corresponding to an analyte signal that gives 3 and 10 times the standard deviation obtained from peak areas of 10 independent blank shrimp samples fortified with metabolite standard mixture at a level of 0.5 μg/kg, respectively, as shown in **Table 3**.

Relative standard deviation (RSD, %) and accuracy for the LC-DAD method were assessed each day for 3 days by the analysis of seven replicates of blank shrimp and fortified shrimp containing 1.0, 1.5, and 2.0 μg/kg of mixture of standards. The whole process was performed in duplicate by different analysts.

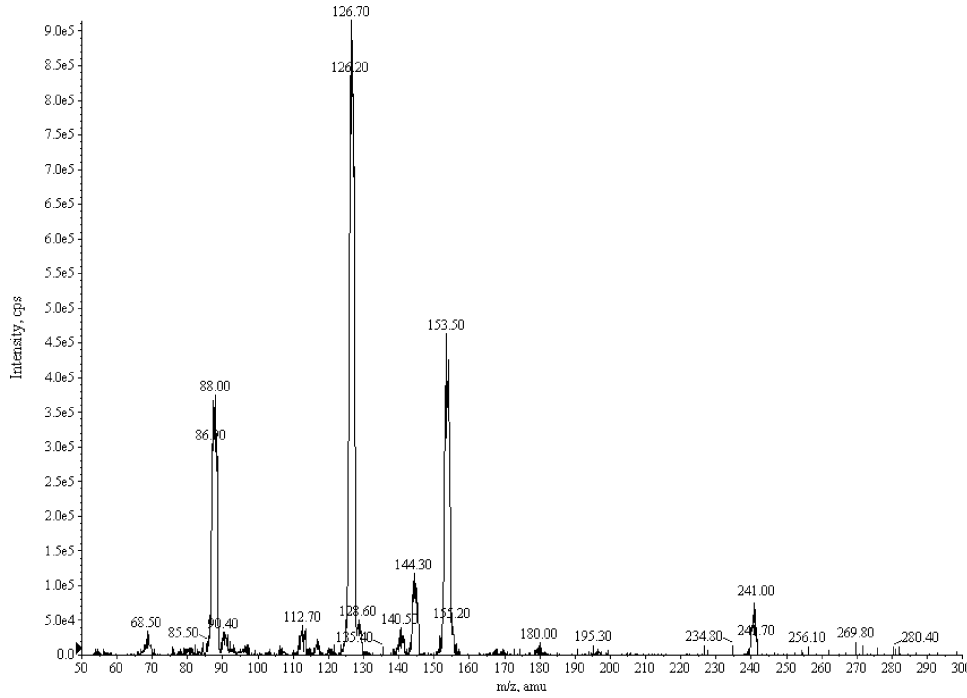


Figure 3. Typical product ion scan of the *m*/*z* 241.0 precursor ion of NTAOZ in positive ionization mode.

Table 5. Naphthyl Derivatives of Nitrofuran Metabolites, Their MS/MS Transitions, and Peak Ratios

analyte	MS/MS transition (*m*/*z*)	peak[a] ratio
NTAHD	(a) 251.7 $[M - H]^- \rightarrow$ 99.1 $[M - H - C_{10}H_7CN]^-$ (b) 251.7 $[M - H]^- \rightarrow$ 153.3 $[M - H - C_3H_3N_2O_2]^-$	0.11
NTAOZ	(a) 241.0 $[M + H]^+ \rightarrow$ 126.7 $[M + H - C_4H_6N_2O_2]^+$ (b) 241.0 $[M + H]^+ \rightarrow$ 88.0 $[M + H - C_{10}H_7CN]^+$	0.53
NTAOZ-d_4	(a) 244.9 $[M + H]^+ \rightarrow$ 126.8 $[M + H - C_4H_6N_2O_2]^+$ (b) 244.9 $[M + H]^+ \rightarrow$ 91.9 $[M + H - C_{10}H_7CN]^+$	0.55
NTSEM	(a) 214.1 $[M + H]^+ \rightarrow$ 127.1 $[M + H - CH_2N_2CONH_2]^+$ (b) 214.1 $[M + H]^+ \rightarrow$ 154.4 $[M + H - NH_2CONH_2]^+$	0.57
NTAMOZ	(a) 340.2 $[M + H]^+ \rightarrow$ 112.1 $[M + H - CO_2 - C_{10}H_7CH_2N_2CH_3]^+$ (b) 340.2 $[M + H]^+ \rightarrow$ 267.1 $[M + H - CO_2 - H_2C{=}NH]^+$	0.47

[a] (a) = quantifier transition (b) = qualifier transition, [a]ratio of qualifier to quantifier.

Thus, the total number of replicates at each fortification level was 42. As shown in **Table 4**, high accuracy and good RSD ranging from 86 to 112% and from 2 to 13%, respectively, were obtained, indicating the effective efficiency of the derivatization process, neutralization, extraction using ethyl acetate, and cleanup steps using hexane.

Because no certified reference material exists yet for nitrofuran metabolite analysis, accuracy was further checked by comparing the LC-DAD method to the LC-APCI-MS/MS method.

In the LC-APCI-MS/MS method, identification of the presence of nitrofuran metabolites in a sample was based on the retention time of naphthyl derivatives of nitrofuran metabolites during chromatographic separation, two MS/MS transitions, and a proper ratio of these two transitions according to Commission Decision 2002/657/EC criteria (*20*). In selecting MS/MS transitions for naphthyl derivatives of nitrofuran metabolites, the precursor ions that were most abundant and relatively stable were first chosen for Q1 scan mode. Then the precursor ion parameters (DP, FP, EP, and CEP) were all adjusted to optimize the signal intensity.

A product ion scan was performed for each selected precursor ion to identify appropriate fragment ions. Two of the most abundant and stable product ions were chosen, and CE and CXP were then manually optimized for each of the two MS/MS transitions. **Figure 3** shows a typical product ion scan of the *m*/*z* 241.0 precursor ion of NTAOZ. The ions at the *m*/*z* 153.5, 126.7, and 88.0 correspond to $[M + H - CO_2 - NHCHCH_3]^+$, $[M + H - CO_2 - NHCHCH_3 - HCN]^+$, and $[M + H - C_{10}H_7CN]^+$, respectively.

Two MS/MS transitions, 241.0 → 126.7 and 241.0 → 88.0, were chosen for confirmatory analysis of NTAOZ, and the former was used for quantifier and the latter for qualifier. Naphthyl derivatives of nitrofuran metabolites, MS/MS transitions, and peak ratios are presented in **Table 5**.

In the LC-APCI-MS/MS method less sample was required due to the higher selectivity and sensitivity of the method compared to LC-DAD. Data were acquired in time-scheduled mode so that enough points were obtained to accurately define the chromatographic peaks and therefore to increase the sensitivity of the method (*21*). Period 1 (duration of 7 min) is not shown in **Table 1** because the elution time of the first peak is at 7.34 min.

Typical ion chromatograms of blank shrimp and samples fortified with nitrofuran metabolites at 1 μg/kg are shown in **Figure 4**. Compared to LC-DAD chromatograms, LC-MS/MS chromatograms are obviously cleaner, arising from the inherent selective advantage of this technique.

Matrix-matched calibration was performed by internal standard calibration using AOZ-d_4·HCl as an internal standard. The ratio of the peak area of naphthyl derivative of nitrofuran metabolites to the peak area of naphthyl derivative of AOZ-d_4 was plotted against the concentration of nitrofuran metabolite fortified to blank shrimp. As shown in **Table 6** the linearity is high for all compounds, with correlation coefficients better than 0.99. The LOD and LOQ of each analyte were defined here as those described in the LC-DAD method except that the peak area ratio of the

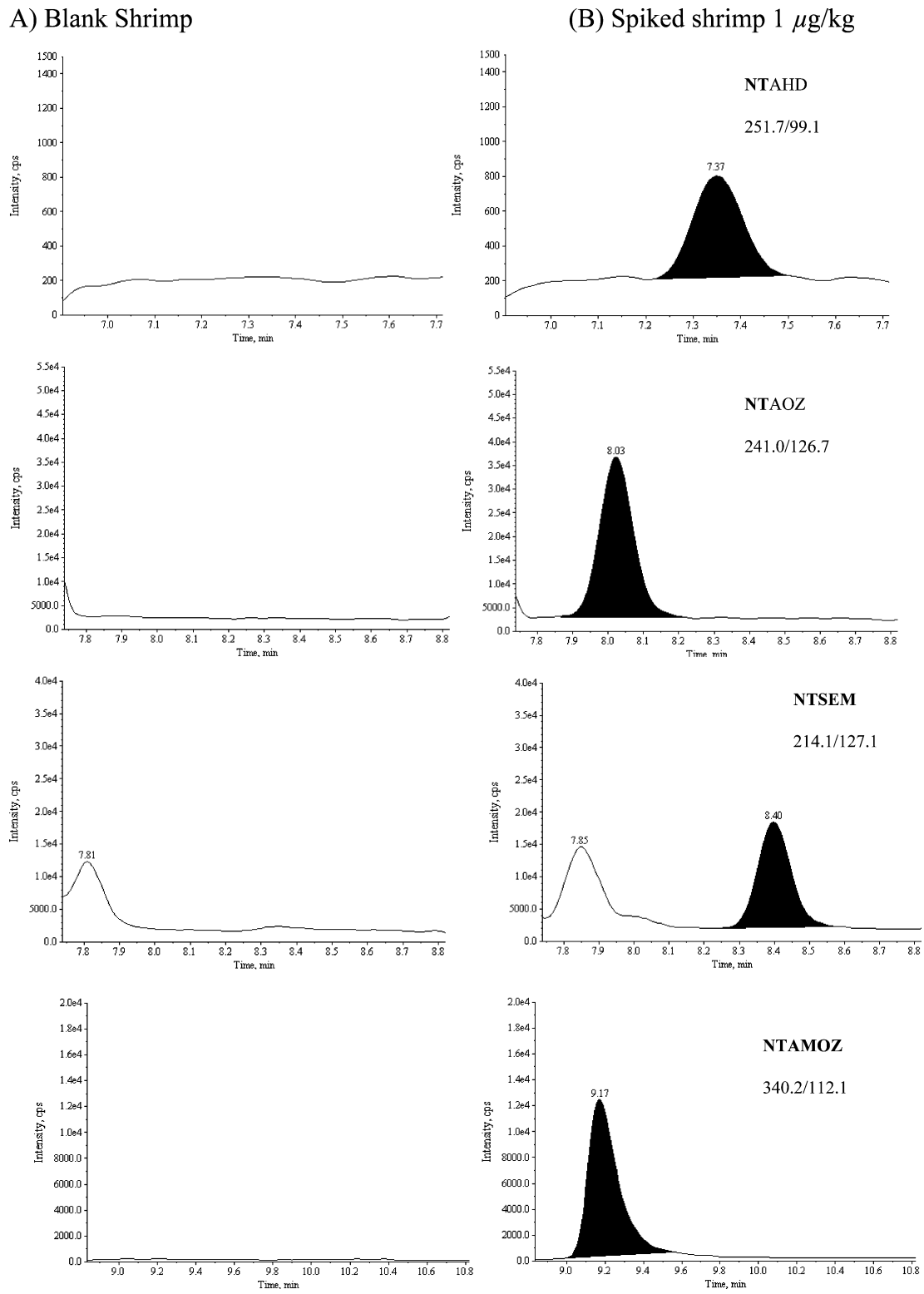


Figure 4. MRM chromatograms obtained by LC-APCI/MS/MS of a blank shrimp sample (**A**) and a blank shrimp sample fortified at a 1 μg/kg level of each derivative of nitrofuran metabolites (**B**).

naphthyl derivative of nitrofuran metabolites to the naphthyl derivative of AOZ-d_4 was used for analyte signal. As shown in **Table 6**, the LOD and LOQ for all analytes are below 1.0 μg/kg. The data represented in **Table 7** show high accuracy and good RSD at 1, 1.5, and 2 μg/kg levels for all analytes, again indicating the effective efficiency of the derivatization process, neutralization, extraction using ethyl acetate, and cleanup using hexane.

Table 6. Regression Data, Limit of Detection (LOD), and Limit of Quantitation (LOQ) from LC-APCI-MS/MS

analyte	R^2	LOD (μg/kg)	LOQ (μg/kg)
AHD	0.9928	0.2039	0.6798
AOZ	0.9994	0.1610	0.5368
SEM	0.9968	0.2032	0.6772
AMOZ	0.9960	0.2697	0.8991

Table 7. Accuracy and Relative Standard Deviation of Performance Data of LC-APCI-MS/MS of Four Nitrofuran Metabolites in Blank Shrimp Samples at Three Fortification Levels

analyte	fortification level (μg/kg)	found (mean ± SD) (μg/kg, $n = 7$)	accuracy (%)	RSD (%)
AHD	1.00	0.78 ± 0.03	78	9
	1.50	1.44 ± 0.06	96	11
	2.00	2.10 ± 0.07	105	10
SEM	1.00	1.04 ± 0.03	104	6
	1.50	1.40 ± 0.02	93	7
	2.00	1.94 ± 0.02	97	7
AOZ	1.00	1.02 ± 0.03	102	5
	1.50	1.51 ± 0.03	101	3
	2.00	2.11 ± 0.03	106	6
AMOZ	1.00	1.03 ± 0.05	103	9
	1.50	1.49 ± 0.27	99	6
	2.00	2.06 ± 0.26	103	8

Table 8. Comparison of Analysis of Nitrofuran Metbolites in Shrimp Using LC-DAD versus LC-APCI-MS/MS ($n = 6$)

	SEM	
shrimp	LC-DAD	LC-MS/MS
1	nd[a]	<LOQ[b]
2	nd	nd
3	<LOQ	<LOQ
4	<LOQ	<LOQ
5	<LOQ	<LOQ
6	<LOQ	<LOQ
7	nd	nd

[a] Not detectable. [b] Limit of quantitation.

The methods have been applied to the analysis of three kinds of shrimp, namely, giant freshwater shrimp (*Macrobrachium resenbergii*), white leg shrimp (*Litopenaeus vannamei*), and giant tiger prawn (*Penaeus monodon*), purchased from seven supermarkets in Bangkok as shown in **Table 8**. Of seven samples, SEM was detected in only four samples using LC-DAD and five samples using LC-APCI-MS/MS. This is due to the slightly lower detection limit of the latter as shown in **Table 5**. However, SEM could not be quantified because the quantity present in all five samples was lower than the LOQ of both methods. Considering the LOQ of the two methods and the opinion of the scientific panel of the EFSA on food additives, flavoring, processing aids, and materials in contact with food (*4*), the quantity of SEM found in the five samples at <1 μg/kg may not be critical as far as carcinogenicity is concerned.

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Supporting Information Available: IR, ^{1}H NMR, ^{13}C NMR, and MS data of NTA derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Cooper, K. M.; Samsonova, J. V.; Plumpton, L.; Elliott, T. C.; Kennedy, D. G. Enzyme immunoassay for semicarbazide—the nitrofuran metabolite and food contaminant. *Anal. Chim. Acta* **2007**, *592*, 64–71.

(2) Hoogenboom, L. A. P.; Berghmans, M. C. J.; Polman, T. H. G.; Parker, R.; Shaw, I. C. Depletion of protein-bound furazolidone metabolites containing the 3-amino-2-oxzazolidinone side chain from liver, kidney and muscle tissue from pigs. *Food Addit. Contam.* **1992**, *9*, 623–630.

(3) Cooper, K. M.; Mulder, P. P. J.; van Rhijn, J. A.; Kovacsics, L.; McCracken, R. J.; Young, P. B.; Kennedy, D. G. 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**, *22*, 406–414.

(4) Szilagyi, S.; Beatriz de la Calle, M. Semicarbazide in baby food: a European survey. *Eur. Food Res. Technol.* **2006**, *224*, 141–146.

(5) Leitner, A.; Zöllner, P; Lindner, W. Determination of the metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography—tandem mass spectrometry. *J. Chromatogr., A* **2001**, *939*, 49–58.

(6) Lopez, M. I.; Feldaufer, M. F.; Williams, A. D.; Chu, P.-S. Determination and confirmation of nitrofuran residues in honey using LC-MS/MS. *J. Agric. Food Chem.* **2007**, *55*, 1103–1108.

(7) Chu, P.-S.; Lopez, M. I. Determination of nitrofuran residues in milk of diary cows using liquid chromatography—tandem mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 2129–2135.

(8) Chu, P.-S.; Lopez, M. I. Liquid chromatography—tandem mass spectrometry for the determination of protein bound residues in shrimp dosed with nitrofurans. *J. Agric. Food Chem.* **2005**, *53*, 8934–8939.

(9) Bock, C.; Gowick, P.; Stachel, C. Matrix-comprehensive in-house validation and robustness check of a confirmatory method for the determination of four nitrofuran metabolites in poultry muscle and shrimp by LC-MS/MS. *J. Chromatogr., B* **2007**, *856*, 178–189.

(10) Mottier, P.; Khong, S.-P.; Gremaud, E.; Richoz, J.; Delatour, T.; Goldmann, T.; Guy, P. A. Quantitative determination of four nitrofuran metabolites in meat by isotope dilution liquid chromatography—electrospray ionization—tandem mass spectrometry. *J. Chromatogr., A* **2005**, *1067*, 85–91.

(11) Hu, X.-Z.; Xu, Y.; Yediler, A. Determination of residual furazolidone and its metabolite, 3-amino-2-oxazoldinone (AOZ), in fish feeds by HPLC-UV and LC-MS/MS, respectively. *J. Agric. Food Chem.* **2007**, *55*, 1144–1149.

(12) Commission Decision 181/2003/EC, March 13, 2003, amending decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *Off. J. Eur. Communities* **2003**, *L 71*, 17—18.

(13) Horne, E.; Cadogan, A.; O'Keeffe, M.; Hoogenboom, L. A. P. Analysis of protein-bound metabolites of furazolidone and furaltadone in pig liver by high performance liquid chromatography and liquid chromatography—mass spectrometry. *Analyst* **1996**, *121*, 1463–1468.

(14) Hormazábal, V.; Asp, T. N. Determination of the metabolites of nitrofuran antibiotics in meat by liquid chromatography—mass spectrometry. *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 2759–2770.

(15) Conneely, A.; Nugent, A.; O'Keeffe, M. Use of solid phase extraction for the isolation and clean-up of a derivatized furazolidone metabolite from animal tissues. *Analyst* **2002**, *127*, 705–709.

(16) Vass, M.; Diblikova, I.; Cernoch, I.; Franek, M. ELISA for semicarbazide and its application for screening in food contamination. *Anal. Chim. Acta* **2008**, *608*, 86–94.

(17) Cooper, K. M.; Caddell, A.; Elliott, C. T.; Kennedy, D. G. Production and characterization of polyclonal antibodies to a derivative of 3-amino-2-oxazolidinone, a metabolite of the nitrofuran furazolidone. *Anal. Chim. Acta.* **2004**, *520*, 79–86.

(18) Diblikova, I.; Cooper, K. M.; Kennedy, D. G.; Franek, M. Monoclonal antibody-based ELISA for the quantification of nitrofuran metabolite 3-amino-2-oxazolidinone in tissues using a simplified sample preparation. *Anal. Chim. Acta* **2005**, *540*, 285–292.

(19) Wickramanayake, P. U.; Tran, T. C.; Hughes, J. G.; Macka, M.; Simpson, N.; Marriott, P. J. Simultaneous separation of nitrofuran antibiotics and their metabolites by using micellar electrokinetic capillary chromatography. *Electrophoresis* **2006**, *27*, 4069–4077.
(20) Commission Decision 2002/657/EC, August 12, 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Communities* **2002**, *L 221*, 8−36.
(21) Hernádez, F.; Sancho, J. V.; Pazo, O.; Pitarch, E. Rapid direct determination of pesticides and metabolites in environmental water samples at sub-μg/L level by on-line solid phase extraction-liquid chromatography−electrospray tandem mass spectrometry. *J. Chromatogr., A* **2001**, *939*, 1–11.

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