Original Scientific Article

DEVELOPMENT AND VALIDATION OF CONFIRMATORY METHOD FOR ANALYSIS OF NITROFURAN METABOLITES IN MILK, HONEY, POULTRY MEAT AND FISH BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Fatih Alkan[1], Arzu Kotan[1], Nurullah Ozdemir[2]

[1]Pendik Veterinary Control Institute, Department of Pharmacology,
Laboratory of Residue. 34890 Pendik, Istanbul, Turkey
[2]Namik Kemal University, Veterinary Faculty, Department of Pharmacology and
Toxicology, Degirmenalti Mevkii, 59030 Tekirdag, Turkey

ABSTRACT

In this study we have devoloped and validated a confirmatory analysis method for nitrofuran metabolites, which is in accordance with European Commission Decision 2002/657/EC requirements. Nitrofuran metabolites in honey, milk, poultry meat and fish samples were acidic hydrolised followed by derivatisation with nitrobenzaldehyde and liquid-liquid extracted with ethylacetate. The quantitative and confirmative determination of nitrofuran metbolites was performed by liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS) in the positive ion mode. In-house method validation was performed and reported data of validation (specificity, linearity, recovery, CC_α and CC_β). The advantage of this method is that it avoids the use of clean-up by Solid-Phase Extraction (SPE). Furthermore, low levels of nitrofuran metabolites are detectable and quantitatively confirmed at a rapid rate in all samples.

Key words: fish, honey, LC-MS/MS, milk, nitrofuran metabolites, poultry meat

INTRODUCTION

Nitrofurans are broad spectrum antibacterial agents known as Schiff's bases, which are derivates of nitrofuraldehyde. In veterinary medicine, it were used in the treatment of gastrointestinal and dermatological infections in beef, pork, poultry, fish and shrimp, and also applied as a contribution to the systemic and feed as growth promoters. In addition, nitrofurans were used in the treatment of bacterial infections in bee colony health (1).

Nitrofurans have been prohibited from use in food-producing animals in the European Union and most countries due to public health and safety concerns, particularly in relation to the carcinogenic potential of either the parent compounds or their metabolites (2). The use of nitrofurans in food-producing animal was prohibited in Turkey (3).

A minimum required performance limit (MRPL) for nitrofurans is set in European Union for the metabolites in poultry meat and aquaculture products at the level of 1 µg kg^{-1} for all metabolites (4).

Analytically, residues are checked only for marker metabolites of the 4 nitrofuran chemicals, in particular: 3-amino-2-oxazolidinone (AOZ) for furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) for furaltadone, 1-aminohydantoin (AHD) for nitrofurantoin and semicarbazide (SEM) for nitrofurazone (5).

Several methods have been reported in the analysis of nitrofuran metabolite in food samples. These include thin-layer chromatography (TLC) (6), high performance liquid chromatography diode-array detector (HPLC DAD) (7) and UV detector (8, 9), liquid chromatography-mass

Corresponding author: Assist. Prof. Nurullah Ozdemir, PhD
E-mail address: nozdemir@nku.edu.tr
Present address: University of Namik Kemal, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology 59030 Tekirdag, Turkey
Phone: +902822504719, Fax: +902822509960

spectrometry (LC-MS/MS) (10, 11, 12). LC-MS/MS analyses were considered very sensitive and commonly used the confirmatory analysis.

In the current study, a method was developed and validated for quick confirmatory analysis of nitrofuran metabolites (AOZ, AMOZ, AHD and SEM). All samples were acidic hydrolised followed by derivatisation with nitrobenzaldehyde and liquid-liquid extracted with ethylacetate. The quantitative and confirmatory determination of nitrofuran metabolites was performed by liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS) in the positive ion mode.

MATERIAL AND METHODS

Reagents and standards

3-amino-2-oxazolidinone (AOZ), 1-aminohydantoin (AHD), AMOZ-d5, $(C^{13})_3$-AHD, $C^{13}N^{15}$-N^{15}-SEM, 2NP-AOZ, 2NP-AMOZ, 2NP-AHD and 2-nitrobenzaldehyde (2-NBA), 3-amino-5-morpholinomethyl-2-oxazolidinon (AMOZ), semicarbazide hydrochloride (SEM), AOZ-d4, 2NP-AOZ-D4, 2NP-AMOZ-D5, 2NP-$(C^{13})_3$–AHD and 2NP-$C^{13}N^{15}$-N^{15}-SEM were obtained from Dr. Ehrenstorfer and Witega. The purity of all compounds was greater than 99%.

Methanol (MeOH) and ethylacetate (LC grade), hydrochloric acid (HCl), dimethylsulphoxide (DMSO), n-hexane and potassium hydrogen phosphate were supplied by Merck. The water was purified with a from a Milli-Q purifying system (Elga PureLab Prima).

Individual standart stock solutions of 1 mg/mL were prepared in methanol, but SEM was prepared in DMSO. Working solutions of 10 ng/mL were diluted by methanol. All standard stock soutions were stored -20 ºC, and the working solutions were stored in refrigerator.

The concentration and content of mix standard solution were used to spiked samples with AMOZ, AOZ, AHD and SEM at a 8, 16, 20 and 20 ng/mL respectively. The concentration and content of internal mix standard solutions were used AOZ-d4, AMOZ-d5, $(C^{13})_3$-AHD and $C^{13}N^{15}$-N^{15}-SEM at a 40, 40, 100 and 100 ng/mL, respectively.

Sample preparation

Collected samples for validation were known to be negative in the screening analysis within the national program for residues control in Turkey. Only, the milk samples were centrifuged at 3500 g, +4ºC, 15 min and upper the fat layer was removed before extraction.

Two grams or 2 mL homogenised samples (milk, honey, poultry meat and fish) were weighed into 50 mL polypropylene centrifuge tubes. Standard spiking solution mix (50, 100, 150 and 200 µL), internal standard solution mix (100 µL) and 5 mL of 0.1 M HCl were added. The extraxtion tube was shaken for 2 min by vortex. 2-nitrobenzaldehyde (2-NBA) (50 mM, 300 µL) were added and the mixture shaken for 2 min by vortex. The tube were capped and incubated overnight (16 h) in the 37ºC temperature. After derivatization, the samples were cooled in room temperature and neutralized by addition 1 mL of 1 M K_2PO_4 and mixed for 2 min by vortex. Ethylacetate (5 mL) and n-hexane (3 mL) was added and mixed for 15 min by vortex and centrifuged at 4000 g for 15 min. The organic phase (6 mL) was collected into a 15 mL graduated glass tubes. The organic fraction was evaporated to dryness under a stream of nitrogen in a water bath at 42°C.

The dry residue was reconstituted with n-hexane (2 mL) and mixed for 2 min by vortex and methanol/water (5/95) (0.7 mL) was added and mixed for 2 min by vortex. 0.5 mL samples were taken from the lower phase with the help of syringe or automatic pipette and filtered using a 0.2 µm syringe filter into an autosampler vial.

Instrumentation

Chromatographic analyses were performed on a LC-MS/MS equipment consisted of a Thermo Electron TSQ Quantum Access Max, mass spectrometer controlled by the Xcalibur (2.2 SP1) software.

Chromatographic separations were achieved on Phenomenex Synergy Hydro RP (150x2.00 mm 80Å 4 µm), protected with a C18 guard column. The mobile phase was deionised water/methanol (80/20) (A) and methanol acidified with 0.1% acetic acid. The linear gradient was: 0-2 min 100% A, 2-9 min 10% A and 9-15 min 100% A and flow rate of 0.25 mL/min. Injection volume was 50 µL. The column was thermostated at 40 ºC. The analysis of samples were carried in the positive ESI-MS-MS ion mode.

Mass spectrometry

MS/MS parameters and precursor-product ions of each compound were tuned by direct infusion in the SRM mode and 0.25 mL/min flow rate of the mobile phase A and B) (50:50).

The concentration and content of mix standards derivatizes and internal standards used for control of the MS-detector, were 25 µg kg^{-1} for 2-NP-SEM-$C^{13}N^{15}$-N^{15} and 2-NP-$(C^{13})_3$-AHD and other standards at 10 µg kg^{-1} .

MS/MS detector parameters are given in Table 1. The precursor-product ions of each compound and their collision energy are given in Table 2.

Table 1. MS detector parameters

Ionization Mode	ESI +	
Spray Voltage	3000	
Vaporizer Temperature	300 °C	
Capillary Temperature	300 °C	
Sheath Gas	35 psi	
Ion Sweep Gas	0,5 psi	
Aux Gas	15 psi	
Skimmer Offset	3	
Q2 CID Gas	1,3 psi	
Data Proses	5	
Mass peak width in amu	Q1=0,7	Q3=0,7

RESULTS

Specificity/Selectivity

Specificity/selectivity were evaluated via analysis of blank matrix samples fortified with mixed benzimidazole and nitroimidazole standards (concentration of 1 µg kg^{-1} each) and with standards of nitrofuran metabolites. According to analysis no significant peaks with an S/N (signal to noise) ratios of 3 or more and chromatographic interference were being observed at the retention times of the targeted nitrofuran metabolites.

Linearity

The matrix calibration curves were at four levels: 0.4, 0.8, 1.2 and 1.6 µg kg^{-1} for AOZ, AMOZ, AHD and SEM, which is in accordance with the MRPL levels. For each compound we made three matrix calibration curves, using blank samples, over three days with 6 replicates at four concentration levels. No significant differences were found between the different matrix curves (r^2>0.9941) shown in Table 3.

Table 2. LC–MS/MS confirmation parameters for the analytes

| Analyte	MS MH+ (m/z)	MS-MS (m/z)	Collision Energy	Width	Tube Lens	Dwell Time
2-NP-SEM	209.0	166.0*	11	0,05	98	0,1
		192.0	13	0,05	98	0,1
2-NP-SEM-C^{13}N^{15}-N^{15}	212.0	168.0	10	0,05	115	0,1
2-NP-AHD	249.0	134.0	12	0,05	71	0,1
		104.1*	22	0,05	71	0,1
2-NP-(C^{13})$_3$-AHD	252.0	134.0	12	0,05	71	0,1
2-NP-AOZ	236.0	134.0	13	0,05	64	0,1
		104.0*	22	0,05	64	0,1
2-NP-AOZ-D4	240.0	133.9	12	0,05	65	0,1
2-NP-AMOZ	335.0	291.0	12	0,05	70	0,1
		261.9*	17	0,05	70	0,1
2-NP-AMOZ-D5	340.0	296.0	12	0,05	71	0,1

*Confirmation ion
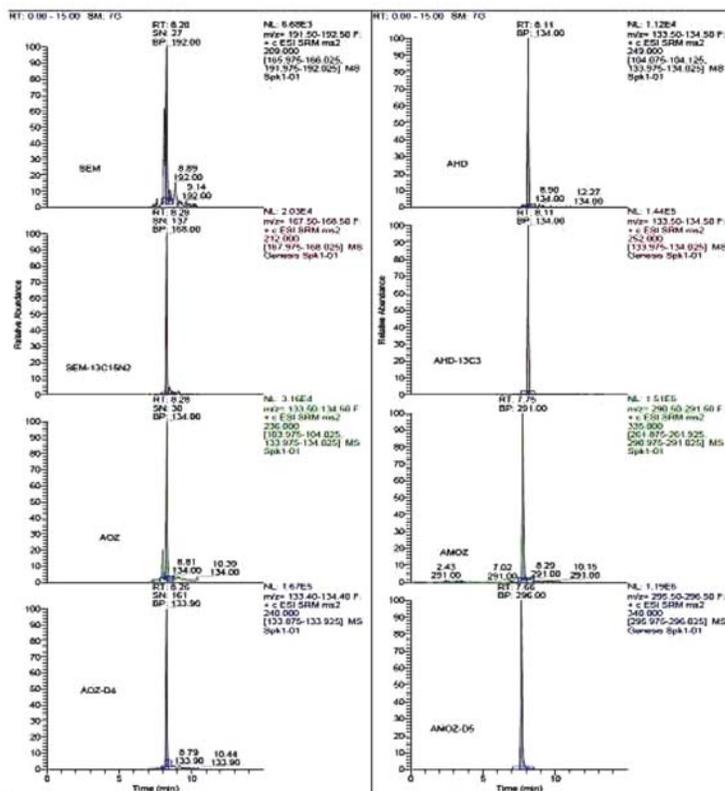
Figure 1. Chromatograms of blank milk samples fortified at 0.2 μg kg^{-1} for AMOZ, 0.3 μg kg^{-1} for AOZ and AHD, 0.4 μg kg^{-1} for SEM

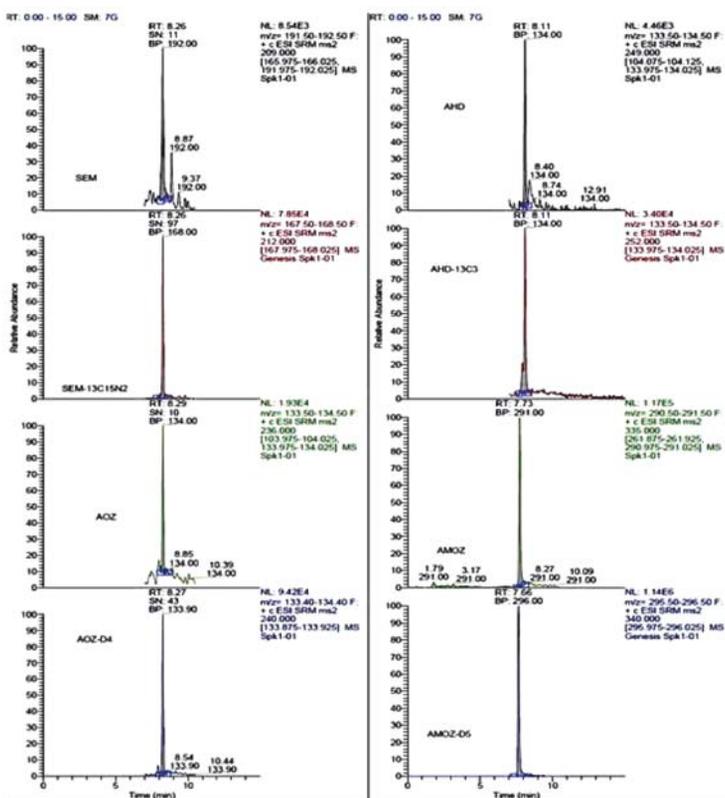**Figure 2.** Chromatograms of blank honey samples fortified at 0.2 μg kg^{-1} for AMOZ, 0.4 μg kg^{-1} for AOZ and SEM, 0.5 μg kg^{-1} for AHD

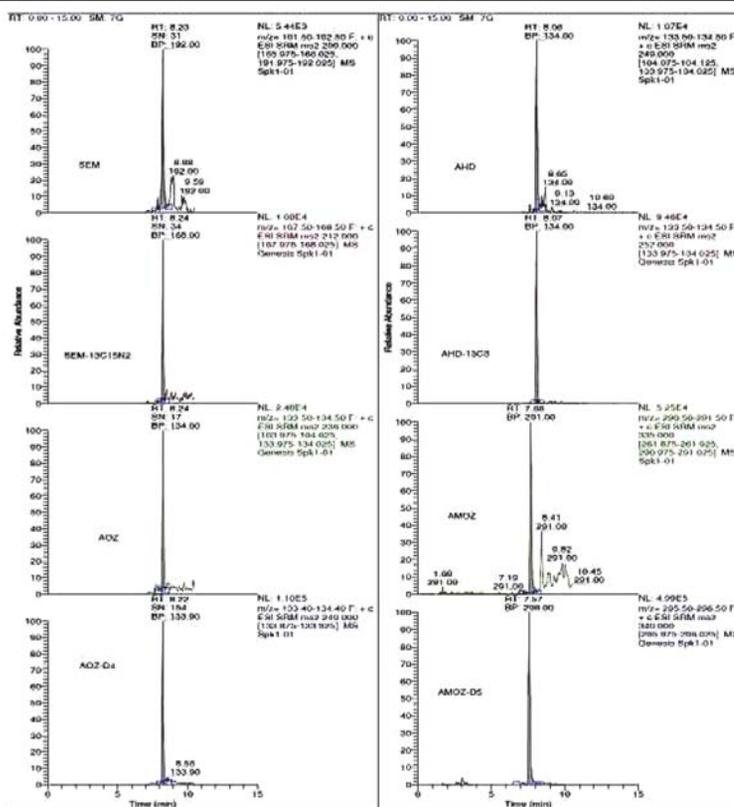**Figure 3.** Chromatograms of blank poultry meat samples fortified at 0.2 μg kg^{-1} for AMOZ, 0.4 μg kg^{-1} for AOZ, 0.5 μg kg^{-1} for SEM and AHD

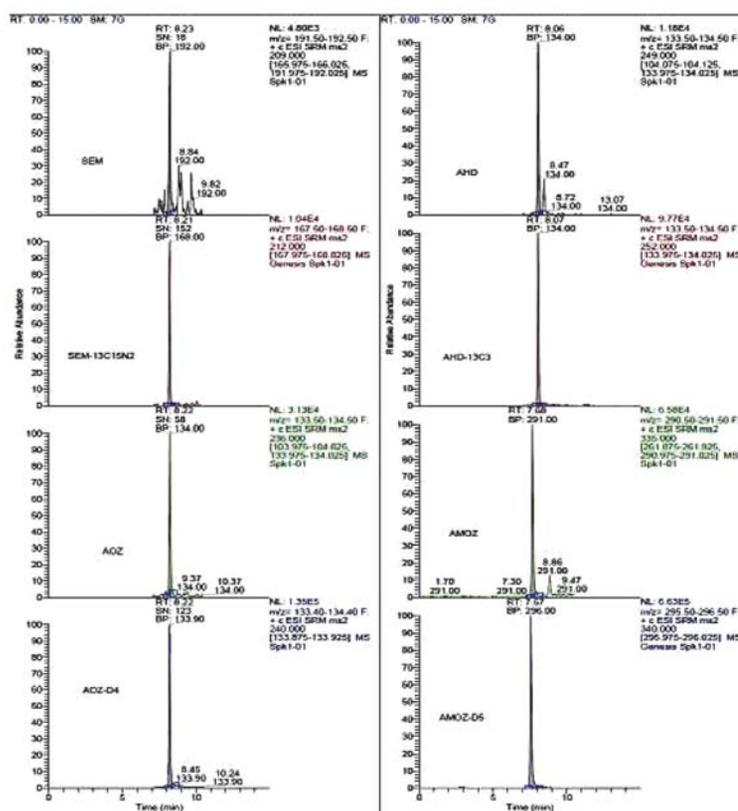**Figure 4.** Chromatograms of blank fish samples fortified at 0.2 μg kg^{-1} for AMOZ, 0.4 μg kg^{-1} for AOZ, 0.5 μg kg^{-1} for SEM and AHD

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Decision limit (CC$_\alpha$) and detection capability (CC$_\beta$)

The CC$_\alpha$ and CC$_\beta$ for banned substances were calculated with the application of the following formula;

CCα = C$_1$ + 2.33 x SD$_{wIR}$

where in C$_1$ is lowest concentration level of the validation study (MRPL) and SD$_{wIR}$ is the standard deviation from within-laboratory reproducibility.

CCβ = CCα + 1.64 x SD$_{wIR,CC\alpha}$

where in SD$_{wIR,CC\alpha}$ is standard deviation at CC$_\alpha$ concentration.

For each compound of CC$_\alpha$ and CC$_\beta$ were calculated from the linearity study. The mean value CCα and CCβ were presented in Table 3.

ESI-MS conditions. Figure 1, 2, 3 and 4 show LC/MS/MS extracted-ion chromatograms obtained from analysis of a spiked samples.

This method has been developed and in-house validated in four different matrices (milk, honey, poultry meat and fish) according to the European Commission Decision 2002/657/EC requirements (13). Also, all four matrices have been accredited according to ISO 17025 by TURKAK (Agency of Accreditation in Turkey).

The majority of the method for nitrofuran metabolites in food then employ a solid phase extraction (SPE) step in order clean-up. Barbosa et al. (7), determinated of nitrofurans in animal feeds by liquid chromatography-UV photodiode

Table 3. Summary of Linearity, CC$_\alpha$, and CC$_\beta$

Analyte	Calibration range ($\mu g\ kg^{-1}$)	Linearity (r²)				CC$_\alpha$ ($\mu g\ kg^{-1}$)				CC$_\beta$ ($\mu g\ kg^{-1}$)			
		Honey	Milk	Poultry Meat	Fish	Honey	Milk	Poultry Meat	Fish	Honey	Milk	Poultry Meat	Fish
AOZ	0.4-1.6	0.999	0.998	0.994	0.997	0.44	0.33	0.45	0.45	0.46	0.35	0.48	0.48
AMOZ	0.2-0.8	0.999	0.998	0.998	0.996	0.21	0.22	0.22	0.23	0.22	0.23	0.23	0.24
AHD	0.5-2.0	0.998	0.999	0.995	0.996	0.54	0.34	0.56	0.59	0.57	0.37	0.60	0.65
SEM	0.4-0.6	0.996	0.995	0.994	0.995	0.44	0.47	0.59	0.61	0.47	0.51	0.65	0.69

Recovery

The method recoveries and RSDs were determined from 6 replicates at four concentration levels spiking blank samples over three days. The recovery results were observed in acceptable range of 70-110%. All the data relating to method recovery and precision were summarisedin Table 4; mean recoveries ranging and CV% values were satisfactory, required by Decision 2002/657/EC.

Evaluation

In order to evaluate this method, it eventually participating in the Food Analysis Performance Assessment Scheme (FAPAS), the test of "Nitrofuran Metabolites in Prawns" (FAPAS 02229, 12/05/2014, Lab No:75). AOZ total assigned value were 0.934 $\mu g\ kg^{-1}$, the results of our laboratory 0.8 $\mu g\ kg^{-1}$. Z-score were -0.7. The result is good and satisfactory.

DISCUSSION

To measure nitrofuran metabolites using the selective reaction-monitoring (SRM) mode, full scan and product ion spectra of the analytes were investigated under the LC conditions described in mass Spectrometry section. Nitrofuran metabolites could be detected under positive ionization mode

array detection and liquid chromatography-ionspray tandem mass spectrometry. Following ethyl acetate extraction at mild alkaline conditions and purification on NH$_2$ column (SPE), the nitrofurans are determined using liquid chromatography with photodiode-array detection (LC-DAD).

Mottier et al. (11) quantitative determinated of four nitrofuran metabolites in meat by isotope dilution liquid chromatography–electrospray ionisation–tandem mass spectrometry. This study, was used a method liquid–liquid extraction and clean-up on a polymeric solid phase extraction cartridge (SPE) are then performed before LC–MS/MS analysis by positive electrospray ionisation (ESI).

Consequently this LC–ESI–MS–MS method allows the simultaneous determination of nitrofuran metabolites in four matrix. The method avoids the use of clean-up by SPE and could be performed quickly. The obtained validation results indicate the accordance of the method with Decision 2002/657/EC (13). The repeatability and within-laboratory reproducibility (precision) of the method are less than 9.86 % for all analytes. The CC$_\alpha$ and CC$_\beta$ are below the MRPL of 1 $\mu g\ kg^{-1}$. This method has been used for confirmatory analysis of nitrofuran metabolites in honey, milk, poultry meat and fish sample.

Table 4. Recovery of nitrofuran metabolites: between-day repeat measures

	Honey				Milk				Poultry Meat				Fish			
	Added Amount µg kg⁻¹	Mean Amount Calculated µg kg⁻¹	Rec %	CV %	Added Amount µg kg⁻¹	Mean Amount Calculated µg kg⁻¹	Rec %	CV %	Added Amount µg kg⁻¹	Mean Amount Calculated µg kg⁻¹	Rec %	CV %	Added Amount µg kg⁻¹	Mean Amount Calculated µg kg⁻¹	Rec %	CV %
AOZ	0.4	0.40	100.50	5.27	0.3	0.30	98.96	4.69	0.4	0.40	99.75	3.98	0.4	0.41	102.08	1.23
	0.8	0.80	100.25	1.42	0.6	0.60	99.67	2.13	0.8	0.78	97.54	4.88	0.8	0.79	98.13	4.22
	1.2	1.19	99.22	0.82	0.9	0.91	101.81	0.64	1.2	1.24	103.61	1.57	1.2	1.21	100.42	2.33
	1.6	1.61	100.35	0.67	1.2	1.19	99.14	0.62	1.6	1.58	98.60	0.40	1.6	1.60	100.10	1.46
AMOZ	0.2	0.20	101.00	3.09	0.2	0.20	100.50	1.99	0.2	0.20	99.33	3.64	0.2	0.20	100.00	1.80
	0.4	0.39	98.58	1.30	0.4	0.40	100.75	1.55	0.4	0.40	99.58	1.82	0.4	0.40	100.33	1.70
	0.6	0.61	100.83	0.29	0.6	0.59	98.33	1.48	0.6	0.61	101.28	1.53	0.6	0.60	99.44	1.37
	0.8	0.80	99.79	1.25	0.8	0.81	100.71	1.26	0.8	0.80	99.46	0.62	0.8	0.80	100.25	1.07
AHD	0.5	0,50	99.33	4.42	0.3	0.31	102.00	3.32	0.5	0.52	103.93	2.90	0.5	0.48	96.40	5.24
	1.0	1,00	99.70	3.12	0.6	0.59	98.56	2.02	1.0	0.98	97.70	4.88	1.0	1.01	101.13	4.81
	1.5	1.52	101.00	1.44	0.9	0.90	99.93	1.01	1.5	1.49	99.07	4.04	1.5	1.53	102.11	1.15
	2.0	1.99	99.53	0.98	1.2	1.20	100.31	0.43	2.0	2.02	100.85	1.76	2.0	1.97	98.73	0.82
SEM	0.4	0.40	100.00	2.50	0.4	0.39	97.67	6.84	0.5	0.49	97.73	6.29	0.5	0.49	98.73	9.86
	0.8	0.80	100.29	3.25	0.8	0.80	99.67	3.93	1.0	1.00	99.97	3.88	1.0	0.99	98.57	0.06
	1.2	1.20	99.64	4.00	1.2	1.23	102.67	1.25	1.5	1.54	102.33	3.94	1.5	1.55	103.11	1.83
	1.6	1.60	100.13	1.58	1.6	1.58	98.73	1.84	2.0	1.98	98.83	2.40	2.0	1.97	98.68	1.89

CONCLUSION

A rapid and sensitive method described in this paper provides reliable, simultaneous quantitative analysis for nitrofuran metabolites residues in milk, honey, poultry meat and fish samples. The optimized procedure provides significant advantages including simplicity, low operation cost, avoids the use of clean-up by SPE.

Thus should be performed quickly confirmative analysis for nitrofuran metabolites residues in milk, honey, poultry meat and fish samples and used as a routine analysis.

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