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A COMPARISON OF CONFIRMATORY METHOD DEVELOPMENT AND VALIDATION OF ANTIBIOTIC: NITROFURAN METABOLITES (AMOZ, AOZ, AHD and SEM) IN FISH AND SHRIMP MATRIX AMONG SOME LC-MS/MS SYSTEMS (HPLC-QUATTRO MICRO API, UPLC-QUATTRO PREMIER XE AND UPLC-TQD)

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ABSTRACT

Confirmatory methods for determination of banned antibiotic: nitrofuran metabolites (AMOZ, AOZ AHD and SEM) were developed in HPLC-MS/MS (Quattro micro), AQUITY UPLC–TQD and AQUITY UPLC-Quattro Premier XE at the same time. The method were validated in Bagda shrimp (*Penaeus monodon*) and Tilapia (*Oreochromis niloticus*) fish. The liquid chromatographic separations were done with gradient elution by using XTerra® MS C18, 3.5µm, 2.1x150mm column in case of HPLC system whereas AQUITY UPLC BEH C18, 2.1x50mm, 1.7µm, Column in UPLC systems) same mobile phase 5mM ammonium acetate in water and methanol and same extraction protocol. Mass spectral acquisition was done using electrospray ionization in the positive ion mode applying MRM of two diagnostic transition reactions for AMOZ, AOZ AHD, SEM and AMOZ_D5 in derivatized condition. Shrimp and fish samples were extracted with ethylacetate and evaporated to dryness and finally reconstituted with 50% methanol. The method validation was carried out according to the criteria of Commission decision 2002/657/EC. The calibration curve showed a good linearity in the concentration range from 0.25 to 5.0 ng/g with the correlation coefficient of >0.997 in all cases. The decision limit (CCα) was in the range of 0.12-0.23ng/g whereas detection capability (CCβ) was in the range of 0.21-0.38 ng/g respectively. The precision of the method, expressed as

recovery values for the within laboratory reproducibility at the three levels of fortification (0.5, 1.00, 1.5 2.0) ng/g were less than 15%. The mean recoveries were in the range of 92–113%.

KEY WORDS: HPLC-QM, UPLC-TQD, QP-XE, Nitrofuran metabolites, Shrimp, Fish and Validation.

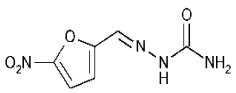
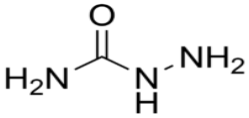
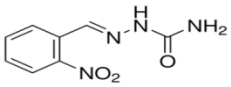
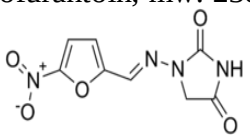
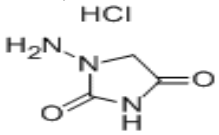
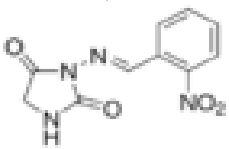
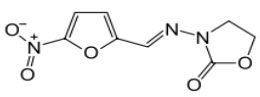
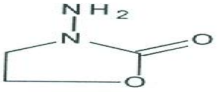
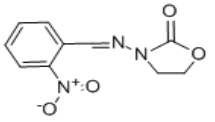
INTRODUCTION

Nitrofurans (**Figure1**), particularly furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT) and nitrofurazone (NFZ), belong to a class of synthetic broad spectrum antibiotics which all contain a characteristic 5-nitrofuran ring. Nitrofurans were commonly employed as feed additives for growth promotion, and mainly used for livestock (i.e. poultry, swine and cattle), aquaculture (i.e. fish and shrimp) and bee colonies in the prophylactic and therapeutic treatment of bacterial and protozoan infections such as gastrointestinal enteritis caused by *Escherichia coli* and *Salmonella spp.* fowl cholera and coccidiosis black heads.[1,2] In 1995, the use of nitrofurans for livestock production was completely prohibited in the EU[3] due to concerns about the carcinogenicity of the drug residues and their potential harmful effects on human health.[2,4,5] Under EU regulation, countries with products intended for the EU are bound by the same regulations as locally produced food,[6] therefore food imported into the EU should be free of nitrofurans. The use of nitrofurans for livestock has also been prohibited in countries such as Australia, USA, Philippines, Thailand and Brazil,[7]

Contrary to the complete ban of nitrofuran use in livestock production, the drugs are readily available for veterinary and human therapy: nitrofurazone is used for topical application on infected burns and skin infections;[8] furazolidone is available for the oral treatment of cholera,[9] bacterial diarrhoea, and giardiasis;[10] and nitrofurantoin is commonly used to treat infections of the urinary tract.[11] If nitrofuran remains in food, it causes mutagenesis, carcinogenicity and teratogenesis.[12,13] It has been reported that furazolidone overdose in mammals may not only cause muscle convulsions but also can lead to nerve breakage throughout the body.[14]. Nitrofurazone can also inhibit sperm production in mice.[15] The absorption and elimination half-lives of nitrofuran parent drugs in the serum are few minutes and few hours, respectively.[16] As a result of their rapid metabolism, nitrofuran parent substances are not suitable for monitoring and typically their metabolites are analyzed. For example semicarbazide (SEM) is the metabolites of nitrofurazone, which are usually derivatised to 2NP-SEM using *ortho*-nitrobenzaldehyde (*o*-NBA) in order to increase

molecular mass and improve the sensitivity of detection.[17] Prior to derivatisation, the release of bound metabolites from tissue is carried out under mildly acidic conditions (**Figure2**).

Because of the toxicity of nitrofuran, the European Union (EU) set the minimum required performance limit (MRPL: "minimum content of an analyte in a sample, which at least has to be detected and confirmed") for the detection of nitrofuran residues (metabolites) in food of animal origin at 1µg/kg.[6] Thus, a sensitive and reliable method for the determination of nitrofuran at residual levels is very important. In the past decade, several analytical methods have been developed for the screening and quantitation of nitrofuran metabolites in foods and biological samples. Some methods already reported for the determination of nitrofurans e.g. detection of (AOZ)[18] by enzyme immunoassay(ELISA) has been used for the detection of AOZ (3-amino-2-oxazolidone) in prawn tissue. Quantitative determination of four nitrofuran metabolites in chicken meat by HPLC-MSMS.[19] Analysis of matrix-bound nitrofuran residues in honey by HPLC-MSMS.[20] In our previous study we developed and validated a simultaneous confirmatory method for analysis of nitrofuran metabolites (AMOZ, AOZ, AHD AND SEM) in katla fish (*katla katla*) and bagda shrimp (*penious monodon*) by UPLC-MS/MS (QPXE)[21]. In present study we have focused on the development and validation of some confirmatory methods (following the EU decision 2002/657/EC) and their comparison for determination of antibiotic: nitrofuran metabolites (AMOZ, AOZ, AHD and SEM) in fish and shrimp matrix among some LC-MS/MS systems (HPLC-Quattro micro API, UPLC-Quattro premier XE and UPLC-TQD).

Parent Drug	Metabolite	Derivative
Nitrofurazone, mw: 198.14 	SEM, mw: 111.53 HCl 	2-NP-SCA, mw: 208.17 
Nitrofurantoin, mw: 238.16 	AHD, mw: 151.55 	2-NP-AHD, mw: 248.19 
Furazolidone, mw: 225.16 	AOZ, mw: 102.09 	2-NP-AOZ, mw: 235.20 
Furaltadone, mw: 324.29	AMOZ, mw: 201.22	2-NP-AMOZ, mw: 334.33

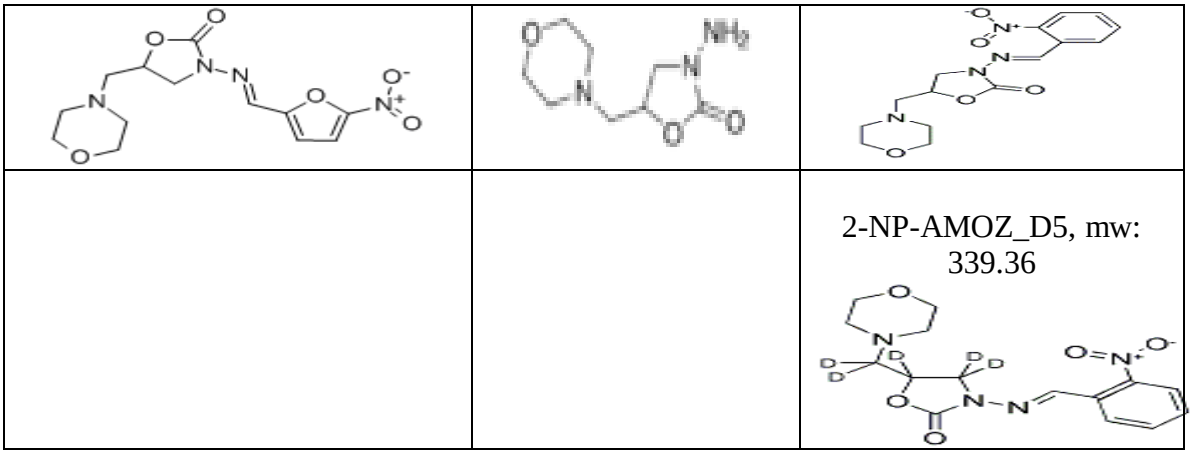


Figure1: Structures of nitrofuran parent compounds metabolites and nitrophenyl derivatives. nitrofurazone, SEM (semicarbazide), NPSEM 3[(2-nitrophenyl)methylene]-hydrazine carboxamide; Nitrofurantoin, AHD (1-aminohydantoin), NPAHD [3-(2-nitrobenzylidenamino)-2,4-imidazolidinedione]; Furazolidone, AOZ (3-amino-2-oxazolidinone), NPAOZ [3-(2-nitrobenzylidenamino)-2-oxazolidinone] and Furaltadone, AMOZ (3-amino-5-morpholinomethyl-1,3-oxazolidinone), NPAMOZ [5-(morpholinomethyl)-3-(2-nitrobenzylidenamino) -2-oxazolidinone], 2-NP-AMOZ_D5 [5-(morpholinomethyl)-3-(2-nitrobenzylidenamino)-2-oxazolidinone-d5].

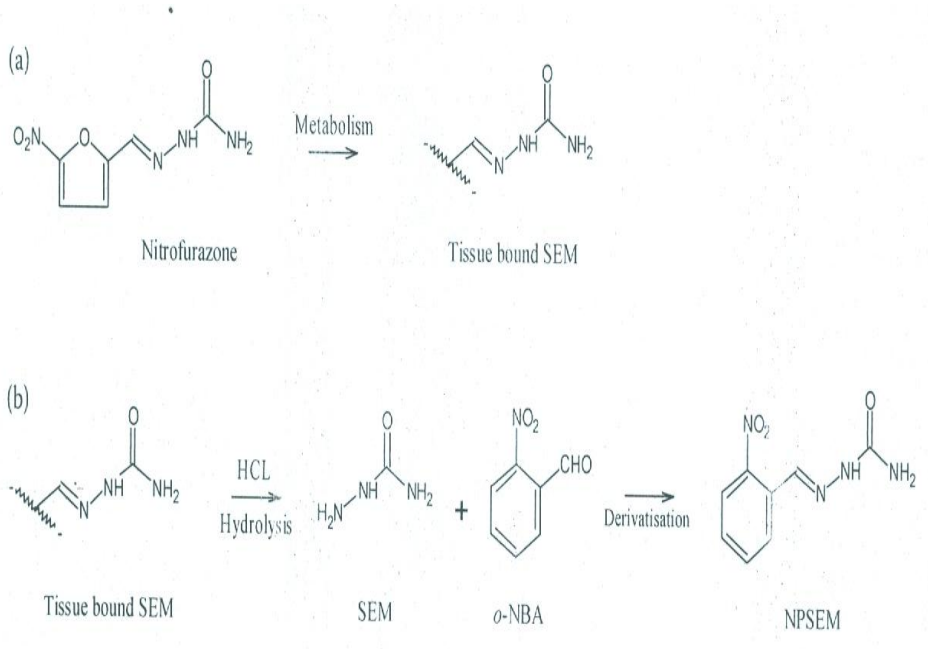


Figure2: (a) Metabolism of parent drugs (Nitrofurazone to tissue-bonded SEM). (b) Hydrolysis of tissue-bonded metabolites (Tissue-bonded SEM to SEM) and derivatization with o-NBA (2-nitrobenzaldehyde) to produce target analyte (SEM to derivative NPSEM).

MATERIAL AND METHODS

Apparatus and Chemicals

Micro pipette: eppendorf; Analytical balance(4 dp): Shimadzu AUY 220; Syringe filter: 4mm PTFE 0.2µm (Waters, USA); Incubator with shaker: KullanmaKilavuzunu, ST 402; Nitrogen Evaporator (Organomation Associates Jnc.); Vortex mixer: Barnstead Thermolyne, M 16710-33;Test tubes: IWAKI TE32 Pyrex, Asahi, Indonesia; Ethylacetate& n-Hexane (HPLC grade); Acetonitrile and Methanol (HPLC and MS grade), Sigma Aldrich, Germany; Sodium hydroxide (NaOH): Marks, Germany, 2-nitrobenzaldehyde (2-NBA): Marks, Germany; Dimethyl sulfoxide(DMSO): Merck Germany; Hydrochloric acid (HCl): 37% HCl (specific gravity 1.19), Marks, Germany;KH_2PO_4 (anhydrous); NaCl: RANKEM, S0160; Phosphate buffered saline(PBS); Purified Water (deionized) from Milli-Q apparatus (Millipore, Bedford, USA),Nitrofuran standards (AMOZ, AOZ, SEM, AHD), assay >99%, Sigma Fluka, Germany.

General reagents: 0.5 mM ammonium acetate solution: Take 0.0192 gm ammonium acetate was dissolved into 500 ml deionized water.

2-NBA Solution: Weigh 151.2 mg 2-nitrobenzaldehyde (Merck) into a 10 ml volumetric flask, dissolve and make up to the mark with methanol. Prepare fresh every day.
HCl (0.2M): Take 8.3 ml of 37% HCl (specific gravity 1.19) and dilute to 0.5 litre withwater in a volumetric flask.

KH_2PO_4 (0.3M): Take 20.403 g of anhydrous potassium dihydrogen phosphate and dissolve in 500 ml water in a volumetric flask.
NaOH (1.0M): Take 20 g sodium hydroxide and dissolve in 500 ml water in a volumetric flask.

Standards Solutions

Stock standard AMOZ, d5-AMOZ and AOZ, 1000 ppm: 10 mg of each standard (AMOZ, d5-AMOZ and AOZ) was dissolved separately in three separate 10 ml volumetric flask and made upto marked with methanol and stored in a refrigerator for 12 months.

Stock standard AHD (as HCl salt), 1000ppm: 13.16 mg of standard was dissolved in a 10 ml volumetric flask made upto marked with methanol and stored in a refrigerator for 12 months.

Stock standard SEM (as HCl salt), 1000 ppm: 14.85 mg of standard was dissolved in a 10 ml volumetric flask using DMSO and made upto marked with methanol and stored in a refrigerator stored in a refrigerator for 12 months.

Mixed standard (AMOZ, AOZ, AHD, SEM), 10 ppm: Pipette 100 µl of each stock standard (1000ppm) of SEM, AHD, AOZ and AMOZ into a 10 ml volumetric flask and make up to the mark with methanol and stored in a refrigerator for a maximum of 03 months.

10 ppm d5-AMOZ internal standard: Pipette 100 µl of stock standard d5-AMOZ (1000 ppm)into a 10 ml volumetric flask and make up to the mark with methanol.

10 ng/ml mixed working standards (AMOZ, AOZ, AHD, and SEM): freshly prepared from 10ppm mixed standard by diluting with methanol.

10 ng/ml d5-AMOZ working standard: freshly prepared from 10ppm d5-AMOZby diluting with methanol.

Solution of NP derivatives was prepared in same way.

Negative samples Collection: Shrimp (Bagda) samples were collected from deep sea and check internally to ensure free from contamination of nitrofuran metabolites. Fish samples were cultured and collected in standard condition without adding any antibiotic. It was completely free from nitrofuran contamination.

Samples to be analyzed

(i) Reagent Blank: two sample containing 1 ± 0.02g of Type-I water and carry out through the procedure to check for process contamination.

(ii) Matrix Blank: two matrix samples (usually collected from deep sea) are carried out through the procedure to check matrix interference and contamination.

(iii)Matrix Blank_IS: two matrix samples spiked with internal standard (AMOZ_d5) only and carry out through process to check any nitrofuran contamination in internal standard.

(iv) Spiked Recovery (QC) Samples: two matrix blank samples are spiked with the nitrofuran metabolites at the 1.0 ppb and are carried out through the procedure to check the recovery of the method.

(v) Calibration Curve Samples: Matrix based calibration curve (0.25 to 5.00ppb) was prepared by spiking nitrofuran (metabolite) standards and internal standard.

(i) Analyte samples: sample to be tested for nitrofuran metabolites.

All these samples were weight 1 ± 0.02g into 50 ml screw capped polypropylene centrifuge tube separately and then subjected for chemical treatment and LC-MS/MS analysis.

Extraction Procedure

- Add 8 ml cold methanol to each tube & vortex for 1 minute and centrifuge at 4000 rpm for 4 minutes.
- Discard the methanol and repeat using 4 ml methanol.
- Add 5 ml of 0.2 M HCl and 50 µl of 2-nitrobenzaldehyde
- Add 200 µl of 10 ng/ml of d5-AMOZ to all tubes for equivalent concentration of 2.00ppb and required volume of nitrofuran (4 mixed) standards (10 ng/ml) was added to the standard curve tubes to make equivalent concentration 0.25 to 5 ppb and that of QC samples 1.00 ppb (the EU MRPL)except reagent blank and matrix blank.
- Incubate all tubes in a water bath at 37 ±2 °C for 16± 2 hours (overnight). Avoid exposure of samples to light.
- Neutralize the samples by adding 500 µl of 0.3 M KH_2PO_4 to each tube and adjust to pH 7.0 ±0.5 with 1M NaOH solution.
- Add 4 ml ethyl acetate to each tube and vortex for 1 minute.
- Centrifuge for 8 minutes at 4000 rpm and collect the organic layer (upper layer) into a clean tube.
- Repeat the extraction using 4 ml ethyl acetate, centrifuge and combine the organic layers.
- Evaporate to near dryness under nitrogen gas at 50°C. Add 1 ml 50% methanol and vortex for 1minute to reconstitute the sample. Pass the sample through a 0.2 µm syringe filter and collect in a vial for subsequent LC-MS/MS analysis.

LC-MS/MS Analysis

Instruments

System-1: LC: Alliance Waters 2695 separation module, Detector: Quattro micro API (QAB 1485), Waters, USA

System-2: LC: AQUITY UPLC, Waters, Detector: Quattro Premier XE, Waters, USA

System-3: LC: AQUITY UPLC, Waters, Detector: TQ Detector, ACQ-TQD#QBB933, Waters, USA

Software: Masslynx v4.1

Columns for liquid chromatographic (LC) separation

System-1: XTerra® MS C18, 3.5µm, 2.1x150mm, Waters, USA (Made in Ireland), PN: 186000408

System-2 and System-3: AQUITY UPLC BEH C18, 1.7µm, 2.1x100(mm), Waters, USA.

Inlet Parameters

System-1: Run Time: 6 minutes, Injection volume: 50 µl, column temperature: 20^0C, sample temperature: 10^0C.

System-2 and System-3: Run Time: 6 minutes, Injection volume: 10 µl, column temperature: 35^0C, sample temperature: 10^0C.

Mobile Phase: Solvent A: 0.5 mM ammonium acetate buffer solution, Solvent B: Methanol,

LC Separation Method: Gradient.

MS Method Parameters

System-1: Number of function: 1, Function 1: MRM of 9 mass pairs, Type: MRM, Ion Mode: ES+, Inter Channel Delay (sec): 0.020, Inter Scan Time (sec): 0.100, Start Time: (min): 0.0, End Time (min): 12.0

System-2: API Probe Delay Temp: 20^0C, Number of function: 1, Function 1: MRM of 9 mass pairs, Type: MRM, Ion Mode: ES+, Inter Channel Delay (sec): -1.000, Inter Scan Time (sec): -1.000, Span (Da): 0.0, Start Time: (min): 0.0, End Time (min): 6.0

System-3: Number of function: 1, Function 1: MRM of 9 mass pairs, Type: MRM, Ion Mode: ES+, Inter Channel Delay (sec): -1.000, Inter Scan Time (sec): -1.000, Span (Da): 0.0, Start Time: (min): 0.0, End Time (min): 6

Tune Parameters

System-1: Capillary (kV): 3.50, Cone (V): 35.00, Extractor (V): 2.00, RF (V): 0.5, Source temperature (^{0}C): 130, Desolvation Temperature (^{0}C): 350, Cone gas flow (L/Hr): 10, Desolvation gas flow (L/Hr): 400, Collision gas flow (mL/min): 0.13 to maintain $3.75x10^{-3}$ pressure (mbar), Multiplier (V): 650

System-2: Capillary (kV): 3.00, Cone (V): 35.00, Extractor (V): 3.00, RF (V): 0.2, Source temperature (^{0}C): 120, Desolvation Temperature (^{0}C): 350, Cone gas flow (L/Hr): 20, Desolvation gas flow (L/Hr): 800, Collision gas flow (mL/min): 0.10 to maintain $3.75x10^{-3}$ pressure (mbar), Multiplier (V): 650

System-3: Capillary (kV): 3.80, Cone (V): 30.00, Extractor (V): 3.00, RF (V): 0.1, Source temperature (^{0}C): 150, Desolvation Temperature (^{0}C): 375, Cone gas flow (L/Hr): 50,

Desolvation gas flow (L/Hr): 900, Collision gas flow (mL/min): 0.10 to maintain $3.75x10^{-3}$ pressure (mbar), Multiplier (V): 650

Analyzer Settings

System-1: LM1 Resolution 1: 14.00, HM1 Resolution: 14.00, Ion Energy 0:7, MS Mode Entrance: 50.00, MS Mode Collision Energy: 3.00, MS Mode Exit: 50.00, MSMS Mode Entrance: 2.00, MSMS Mode Collision Energy: 20.00, MSMS Mode Exit: 2, LM2 Resolution: 14.00, HM2 Resolution: 12.00, Ion Energy2: 1.20.

System-2: LM1 Resolution: 14.00, HM1 Resolution: 15.00, Ion Energy1: 0.80, MS Mode Entrance: 50.00, MS Mode Collision Energy: 2.00, MS Mode Exit: 50.00, MSMS Mode Entrance: 1.00, MSMS Mode Collision Energy: 20.00, MSMS Mode Exit: 0.50, LM2 Resolution: 15.00, HR2 Resolution: 15.00, Ion Energy2: 1.00.

System-3: LM1 Resolution: 8.70, HM1 Resolution: 14.83, Ion Energy1: 0.21, MS Mode Entrance: 50.00, MS Mode Collision Energy: 2.00, MS Mode Exit: 50.00, MSMS Mode Entrance: 2.00, MSMS Mode Collision Energy: 18.00, MSMS Mode Exit: 2.0, LM2 Resolution: 8.34, HR2 Resolution: 14.86, Ion Energy2: 0.86.

CALCULATION OF RESULTS

The nitrofuran metabolites quantified by means of a calibration prepared using pre-formed 2-nitrophenylderivatives curve at six calibration levels ranging from 0.25 to 5.00 µg/kg of the underivatised metabolites.

$$\text{Ion Ratio, R} = \frac{\text{Peak area of primary ion (PAPI)}}{\text{Peak area of secondary ion (PASI)}}$$

Response factor (RF)

$$\text{RF} = \frac{\text{PAPI of interested substance x ISC}}{\text{Peak area of internal standard ion}}$$

CAP concentration, X = (RF-b)/a

Where, ISC= Internal Standard Concentration

a=Slope of calibration curve, b= intercept of calibration curve.

Confirmation criteria

The selectivity of this method is judged by the use of two transitions for each analyte which count for 4 identification points (IPs), as defined by the EU criteria set out in Commission Decision 2002/657/EC. The nitrofurans are listed in Table II of Commission Regulation

(EC) No 37/2010 (unauthorized compounds with no MRL). This means that the minimum number of IPs to consider for their identification is four. Consequently our method fulfils this requirement. Nitrofuran metabolites were considered as positively identified in the samples when the peak area ratio of the various transitions were within the tolerance set by Commission Decision 2002/657/EC. In addition, the relative retention time of the analyte must be equal to that of the calibration standard to within ± 2.5%.

RESULTS

The detection method for confirmation of nitrofuran metabolites was firstly developed in LC-MS/MS systems using standard and internal standard. The detectors of systems (Quattro micro, Quattro Premier XE and TQD) were firstly operated in positive ES+ MS mode to select characteristic ions as the precursors of standards (2NP-AMOZ, 2NP-AOZ, 2NP-AHD and 2NP-SEM) and deuterated internal standard (2NP-AMOZ_D5). Both standards and internal standards were then analyzed LC-MS/MS systems in a positive ionization product ion scan mode by selecting precursor ion. The collision- induced dissociation (CID) experiments of these ions, giving rise to daughter ions for standards and internal standard (Figure3). The selected transitions and the optimal MS–MS conditions are given in **Table1** for all systems. The developed gradient methods with in acetonitrile and in water for systems are shown the **Table2**.

Analytical performance

Method validation was carried out according to criteria described in Decision 2002/657/EC. The parameters taken into account were: response, linearity, decision limit (CCα), detection capability (CCβ), reliability and accuracy. The calibration curve showed a good linearity in the concentration range of 0.25-5.00 µg/kg with the correlation coefficient >0.996. The analytical performance for validation samples are shown in Table3-Table6 and Figure3-F for shrimp samples and that of shown in Table5 and Figure4 for fish samples. The Limit of decision (CCα) and detection capability (CCβ) were calculated using the procedure set out in ISO Guide 11843, as described in Commission Decision 2002/657/EC. The validation data were generated (3 levels and seven replicates per level) on each of two days for shrimp and one day for fish matrices separately for all systems and CCα (µg/kg) and CCβ(µg/kg) values were calculated by using three different days (2+1) data shown in Table7 and figure8. The trueness was expressed in terms of recovery rates. The mean recoveries of spiked samples were in the range of 88-109%.

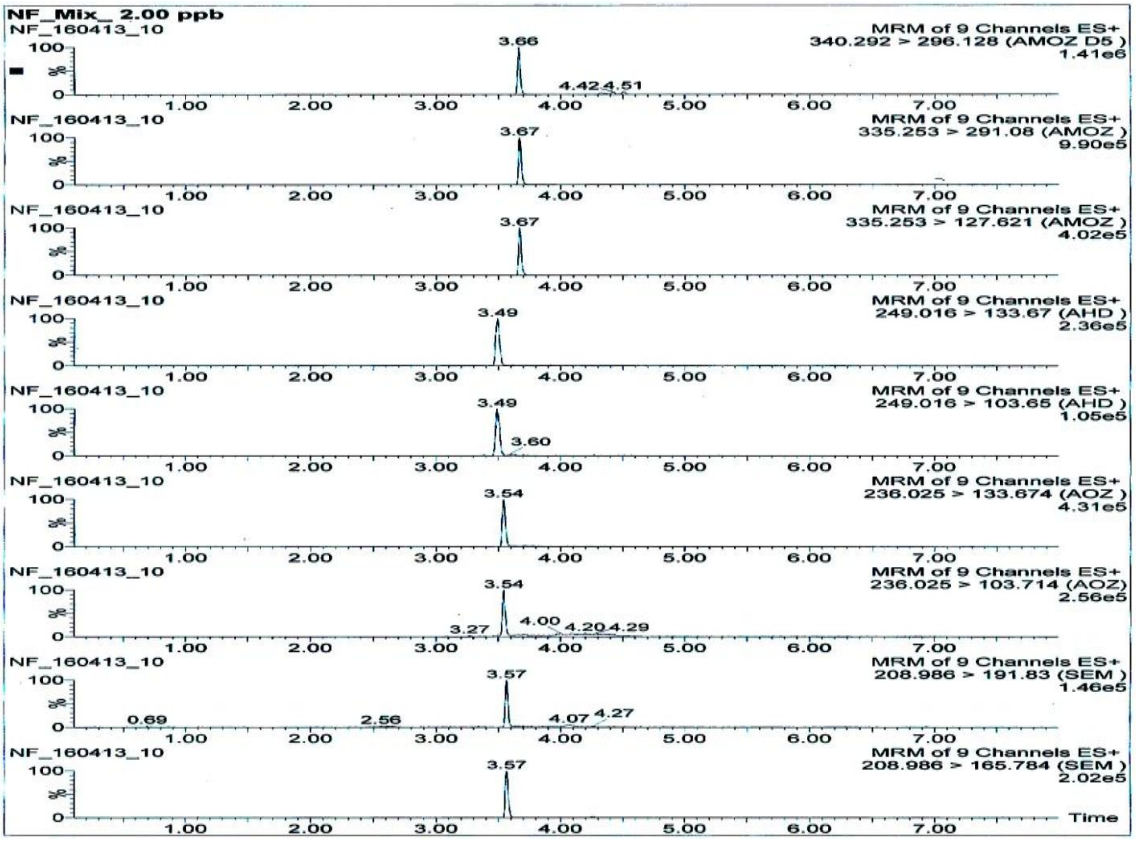


Figure3: Chromatogram of nitrofuran metabolites (2NP-AMOZ, 2NP-AOZ, 2NP-AHD, 2NP-SEM and 2NP-AMOZ_D5).

Table1: Ion monitored and optimal MS-MS condition for nitrofuran metabolites

LC-MS/MS	Prnt (Da)	Dau (Da)	Dwell (s)	Cone (V)	Coll (eV)	Delay (s)	Compound	Formula (Da)
System-1	335.09	291.20	0.150	30.00	11.00	0.020	2NP-AMOZ	334.33
	335.09	262.10	0.150	25.00	14.00	0.020		
	236.17	133.79	0.150	30.00	12.00	0.020	2NP-AOZ	235.20
	236.17	103.95	0.150	30.00	15.00	0.020		
	248.96	134.07	0.150	30.00	12.00	0.020	2NP-AHD	248.19
	248.96	178.09	0.150	30.00	14.00	0.020		
	209.01	166.16	0.150	30.00	10.00	0.020	2NP-SEM	208.17
	209.01	192.02	0.150	30.00	11.00	0.020		
	340.03	295.98	0.150	30.00	13.00	0.100	2NP-AMOZ_D5	339.36
System-2	335.51	291.34	0.025	23.00	11.00	0.020	2NP-AMOZ	334.33
	335.51	99.81	0.025	23.00	27.00	0.020		
	236.36	133.85	0.025	29.00	11.00	0.020	2NP-AOZ	235.20
	236.36	77.80	0.025	29.00	21.00	0.020		
	249.38	133.83	0.025	29.00	11.00	0.020	2NP-AHD	248.19
	249.38	103.79	0.025	29.00	21.00	0.020		
	209.25	165.85	0.025	25.00	09.00	0.020	2NP-SEM	208.17
	209.25	191.97	0.025	25.00	13.00	0.020		
	340.61	296.38	0.025	23.00	11.00	0.100	2NP-AMOZ_D5	339.36
System-3	335.25	291.08	0.025	27.00	11.00	-1.000	2NP-AMOZ	334.33

	335.25	127.62	0.025	27.00	21.00	-1.000		
	236.03	133.67	0.025	29.00	13.00	-1.000	2NP-AOZ	235.20
	236.03	103.65	0.025	29.00	23.00	-1.000		
	249.02	133.67	0.025	29.00	11.00	-1.000	2NP-AHD	248.19
	249.02	103.65	0.025	29.00	23.00	-1.000		
	208.99	165.78	0.025	24.00	9.00	-1.000	2NP-SEM	208.17
	208.99	191.83	0.025	24.00	13.00	-1.000		
	340.29	296.13	0.025	24.00	11.00	-1.000	2NP-AMOZ_D5	339.36

Table2: Gradient Time-Table: Solvent A: 0.5mM ammonium acetate in water and Solvent-B: Methanol

System-1					System-2 and system-3				
SL No	**Time**	**Flow Rate (µl/min)**	**%A**	**%B**	**SL No**	**Time**	**Flow Rate (µl/min)**	**%A**	**%B**
1	Initial	0.200	95	5	1	0.00	0.250	95	5
2	2.5	0.200	95	5	2	1.00	0.250	95	5
3	3.0	0.200	10	90	3	3.25	0.250	1.0	99
4	8.0	0.200	10	90	4	4.00	0.250	1.0	99
5	10	0.200	95	5	5	5.25	0.250	95	5
6	12	0.200	95	5	6	8.00	0.250	95	5

Table3: Summary of AMOZ validation in shrimp and fish matrix using system1, 2 and 3.

Overall Summary of AMOZ Validation in shrimp and fish matrices						
	Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
System1	0.5	0.54121	108.240	10.3	8.3	5.8
	1.00	1.03670	103.670	8.6	6.5	6.6
	1.50	1.59330	106.220	6.4	7.6	8.0
System2	0.5	0.54335	108.670	6.9	5.6	7.5
	1.00	1.07000	107.000	4.6	6.7	5.3
	1.50	1.59330	106.220	5.5	5.3	5.4
System3	0.5	0.55000	110.000	6.9	4.6	5.5
	1.00	1.03330	103.330	4.8	4.7	4.3
	1.50	1.56165	104.110	4.4	3.3	3.4

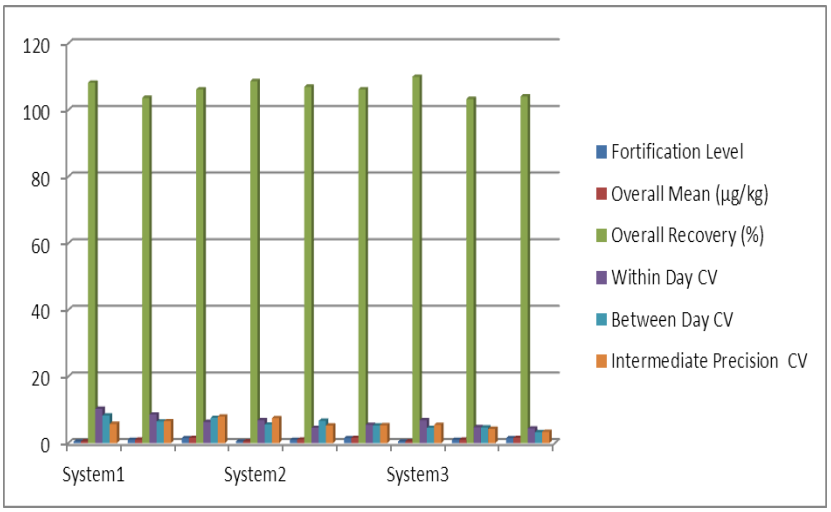


Figure4: Summary of AMOZ validation in shrimp and fish matrices using LC-MS/MS systems

Table4: Summary of AOZ validation in shrimp and fish matrix using system1, 2 and 3.

Overall Summary of AOZ Validation in shrimp and fish matrices						
	Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
System1	0.5	0.5352	107.030	12.3	9.3	5.8
	1.00	1.0660	106.600	7.6	6.5	6.6
	1.50	1.5633	104.220	4.4	5.6	8.0
System2	0.5	0.5435	108.700	5.9	6.6	7.5
	1.00	1.0712	107.120	4.6	5.7	6.3
	1.50	1.5783	105.220	3.5	4.3	5.6
System3	0.5	0.5400	108.000	9.9	5.6	5.5
	1.00	1.0533	105.330	5.8	4.7	4.5
	1.50	1.5467	103.110	4.2	3.3	3.6

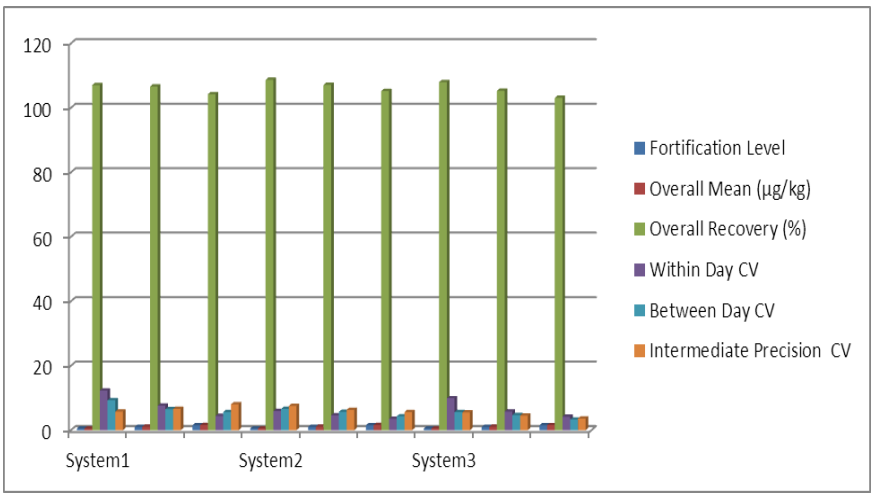


Figure5: Summary of AOZ validation in shrimp and fish matrices using LC-MS/MS systems

Table5: Summary of AHD validation in shrimp and fish matrix using system1, 2 and 3.

Overall Summary of AHD Validation in shrimp and fish matrices						
	Fortification Level	Overall Mean (µg/kg)	Overall Recovery (%)	Within Day CV	Between Day CV	Intermediate Precision CV
System1	0.5	0.4933	98.660	9.3	10.3	9.8
	1	1.0660	106.600	8.6	7.5	5.6
	1.5	1.5412	102.750	6.4	8.6	7.0
System2	0.5	0.5035	100.700	5.9	6.6	7.1
	1	1.0712	107.120	4.6	7.7	5.6
	1.5	1.5183	101.220	5.5	3.3	7.6
System3	0.5	0.52	104.000	9.9	5.6	5.5
	1	1.0833	108.330	5.8	3.7	4.5
	1.5	1.5665	104.433	3.2	5.3	1.6

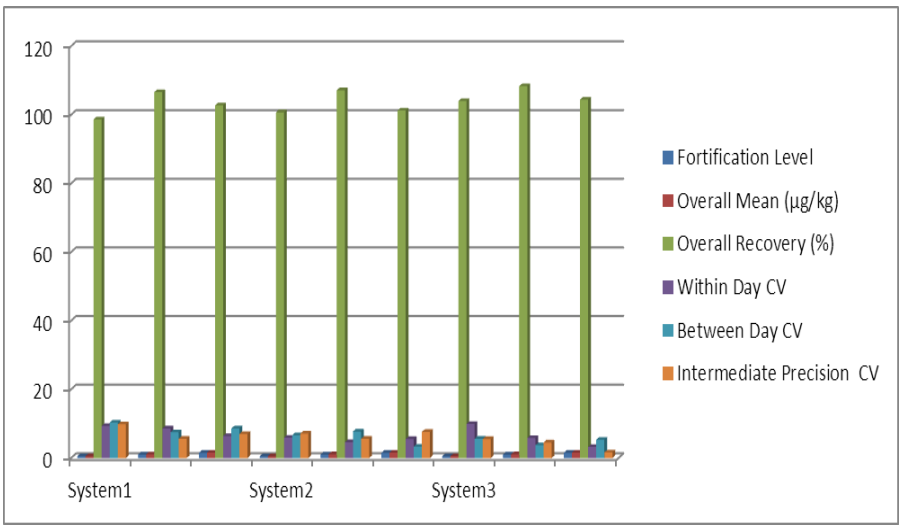


Figure6: Summary of AHD validation in shrimp and fish matrices using LC-MS/MS systems.

Table6: Summary of SEM validation in shrimp and fish matrix using system1, 2 and 3.

Overall Summary of SEM Validation in shrimp and fish matrices						
	Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
System1	0.5	0.540	108.000	12.3	9.3	5.8
	1.00	1.130	113.000	7.6	6.5	6.6
	1.50	1.520	101.347	4.4	5.6	8.0
System2	0.5	0.524	104.700	5.9	6.6	7.5
	1.00	1.071	107.120	4.6	5.7	6.3
	1.50	1.389	92.600	3.5	4.3	5.6
System3	0.5	0.501	100.240	9.9	5.6	5.5
	1.00	1.063	106.330	5.8	4.7	4.8
	1.50	1.507	100.493	4.2	3.3	3.6

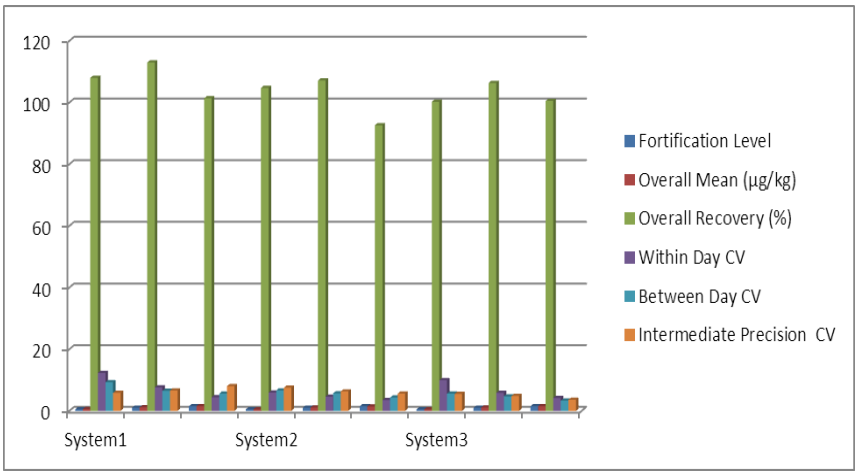


Figure7: Summary of SEM validation in shrimp and fish matrices using LC-MS/MS systems

Table7: CCα (µg/kg) and CCβ(µg/kg) of nitrofuran metabolites in shrimp and fish matrices using LC-MS/MS systems

	Parameter	CCα (µg/kg)	CCβ(µg/kg)
System-1	AMOZ	0.186	0.312
	AOZ	0.217	0.365
	AHD	0.192	0.322
	SEM	0.189	0.318
System-2	AMOZ	0.196	0.329
	AOZ	0.231	0.388
	AHD	0.195	0.328
	SEM	0.198	0.333
System-3	AMOZ	0.154	0.262
	AOZ	0.124	0.211
	AHD	0.129	0.220
	SEM	0.226	0.384

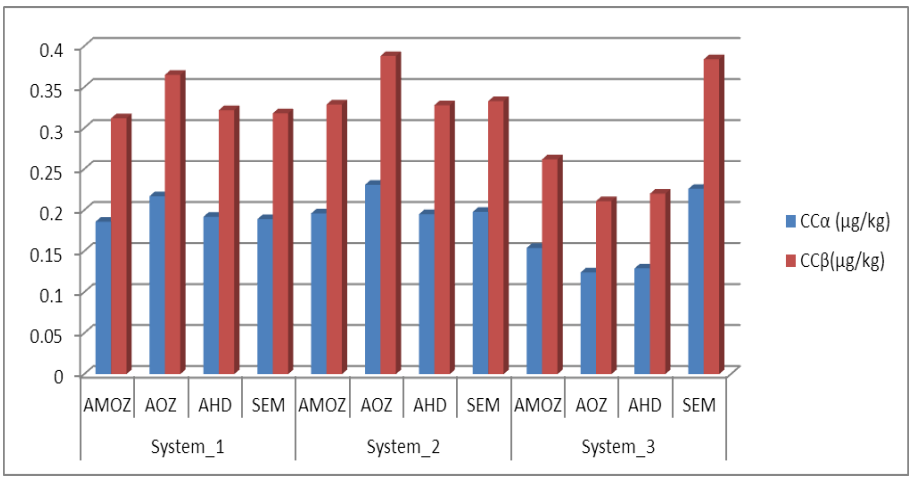


Figure8: CCα (µg/kg) and CCβ(µg/kg) of nitrofuran metabolites in shrimp and fish matrices using LC-MS/MS systems

DISCUSSION

The selectivity of these method were judged by the use of two transitions for each analyte which count for 4 identification points (IPs), as defined by the EU criteria set out in Commission Decision 2002/657/EC. Consequently our methods fulfil this requirement. The precursor and daughter ions obtained in the result have a good agreement with previous findings, [19-21] which indicate the compounds were identified accurately. The instruments were in well and good condition by which our previous findings on chloramphenicol,[22-24] dyes[25] were done properly. The sharp peak shape of transitions without any contamination were self explanatory for developed gradient method in compound separation in all cases. There were no peak in chromatogram of a solvent blank, reagent blank, matrix blank (negative sample) and a single chromatogram in case of matrix with internal standard (IS) which indicated that there were no contamination in sample, extraction process and analytical systems. The performance characteristics of the method presented in this paper indicate that it may be preferably used to test nitrofuran metabolites in food control by all systems. CCα and CCβ in all cases for all systems were less than MRPL (minimum required performance limit) of nitrofuran metabolites(1.0 ng/g), which indicates all systems were fit for analysis of nitrofuran metabolites.

CONCLUSION

The methods were developed and validated as per guideline and commission decision 2002/657/EC. The developed confirmatory methods for nitrofuran metabolites analysis in fish and shrimp matrices were good enough for analysis in all systems.

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