



# Journal of Biological Sciences

ISSN 1727-3048

**science**  
alert

**ANSI***net*  
an open access publisher  
<http://ansinet.com>

## Method Development and Validation of Nitrofuran Metabolites in Shrimp by Liquid Chromatographic Mass Spectrometric System

<sup>1</sup>M.B. Hossain, <sup>1</sup>S. Ahmed, <sup>1</sup>M.F. Rahman, <sup>2</sup>B. Y. Kamaruzzaman,  
<sup>2</sup>K.C.A. Jalal and <sup>3</sup>S.M.N. Amin

<sup>1</sup>Department of Fisheries, Matshya Bhaban, Ramna, Dhaka, 1000, Bangladesh

<sup>2</sup>Institute of Oceanography and Maritime Studies (INOCEM), Kulliyah of Science,  
International Islamic University Malaysia, Jalan Istana Ahmad Shah,  
Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

<sup>3</sup>Department of Aquaculture, Faculty of Agriculture, 43400 Serdang, Selangor, Malaysia

**Abstract:** Liquid Chromatographic Mass Spectrometric System (LCMSMS) method was developed and validated in shrimp matrix for determination of nitrofuran metabolites in the laboratory of fish inspection and quality control, Department of Fisheries, Matshya Bhaban, Dhaka, Bangladesh. This confirmatory method was used for testing samples for residues of the metabolites of the following nitrofurans compounds, furazolidone (3-amino-2-oxazolidinone, AOZ); furaltadone (5-methyl-morpholino-3-amino-2-oxazolidinone, AMOZ); nitrofurazone (semicarbazide, SEM) and nitrofurantoin (1-aminohydantoin, AHD) in shrimp. The data were generated (3 levels and seven replicates per level) on each of three days for shrimp. The mean recoveries from the tissues were 88-110%, the decision limits ( $CC\alpha$ ) were 0.12-0.23 ppb and the detection capabilities ( $CC\alpha$ ) 0.21-0.38 ppb.  $CC\alpha$  and  $CC\beta$  were calculated using the procedure set out in ISO Guide 11843. Nevertheless,  $CC\alpha$  value for all nitrofurans metabolites can be investigated and meet the specified easily met the specified EU MRPL of  $1 \mu\text{g kg}^{-1}$  ranged from 0.12-0.23  $\mu\text{g kg}^{-1}$  and suitable for routine quality control operations.

**Key words:** Method validation, nitrofurans, shrimp, decision limit, detection capability

### INTRODUCTION

Bangladesh is blessed with both marine and freshwater fishery resources. The country has a vast inland water bodies (4703658 ha); open water (40, 24, 934 ha) and closed water (67, 8724 ha) from where we are getting fishes and shrimps and its products to meet our consumption and export requirement. Almost all produced shrimps are exported as processed frozen sea food and is the second largest export item in Bangladesh. The contribution of fisheries sub-sector to the total export earnings during 2010-11 was 2.73%. Presently 82 processing plants are in operation, all are HACCP certified and licensed by the Department of Fisheries for export of fish and fish products to European Union, USA, Japan, Russia, Korea, China and India. Food and Veterinary Office (FVO) of European Union (EU) audited Bangladesh shrimp sector with special reference to situation of residue contamination during 2007, 2008, 2010 and 2011. Use of the nitrofurans antibiotics in food-producing animals

(Fig. 1) was prohibited within the European Union (Commission Regulation, 1995) because of their potentially carcinogenic and mutagenic effects on human health. Previously, nitrofurans had been widely and effectively used for the prevention and treatment of gastrointestinal infections caused by *Escherichia coli*, *Salmonella* spp., *Mycoplasma* spp., *Coccidia* spp., coliforms and some other protozoa and as growth promoters in livestock. The government of Bangladesh declared the nitrofurans antibiotics as banned compound and changed the national regulation on the use of antibiotics in aquaculture production in order to comply with the EU food safety requirements (DOF, 2011).

Various studies have demonstrated that the nitrofurans are rapidly metabolised by animals *in vivo* but that persistent, tissue-bound metabolites are formed which may be released by acid hydrolysis in the stomach, giving rise to potentially toxic chemicals. As a result of their rapid metabolism, nitrofurans parent substances are not suitable for monitoring and typically their metabolites

**Corresponding Author:** K.C.A. Jalal, Institute of Oceanography and Maritime Studies (INOCEM), Kulliyah of Science, International Islamic University Malaysia, Jalan Istana Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

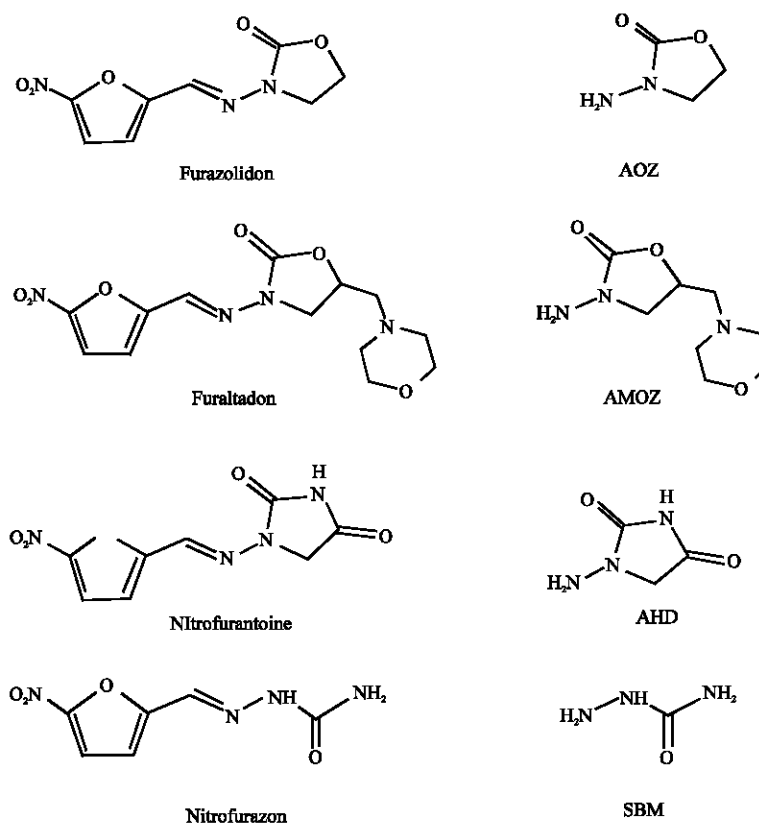


Fig. 1: Chemical structures of 4 Nitrofurans and their correspond metabolites

are analyzed. The LC-MS/MS method is used for the qualitative and quantitative analysis of four derivatized nitrofuran metabolites (AOZ, AMOZ, AHD and SEM) as their 2-nitrophenyl (NP) derivatives (NPAOZ, NPAMOZ, NPAHD and NPSEM) found in shrimp and fish. According to the decision of EU the MRPL is  $1.0 \mu\text{g kg}^{-1}$  for all of the nitrofuran metabolites. A “zero” tolerance level has been established by the EU for this antibiotic.

These experiments were conducted as to develop mass spectrometry reference (confirmatory) method for bound residues of nitrofurans in shrimp, to validate the developed methods according to the standards required by the European Commission; to test the pre-export shrimp sample as a part of food safety requirement of European Commission and to provide the validated methods to testing laboratories.

#### MATERIALS AND METHODS

The experiment was conducted in the laboratory of fish inspection and quality control, Department of Fisheries, Matshya Bhaban, Dhaka, Bangladesh for a period of six months from October 2010-March 2011. The analysis was done by using the following standards

which were prepared in laboratory following the guide line given by European Union for Chemical Analysis of Antibiotic Residues in Food (Stephany and van Ginkel, 1996).

**Standard preparation procedure:** The 0.5 mM ammonium acetate solution was prepared by taking 0.0192 g ammonium acetate and dissolved into 500 mL deionized water. Then weighed 151.2 mg 2-nitrobenzaldehyde (Merck) into a 10 mL volumetric flask, dissolve and make up to the mark with methanol. Then, 8.3 mL of 37% HCl (specific gravity 1.19) was diluted to 0.5 liter with water in a volumetric flask to prepare HCl (0.2 M). 20.403 g of anhydrous potassium dihydrogen phosphate was taken and dissolved in 500 mL water in a volumetric flask to prepare  $\text{KH}_2\text{PO}_4$  (0.3 M). NaOH (1.0 M) was prepared by mixing 20 g sodium hydroxide dissolved in 500 mL water in a volumetric flask.

**Preparation nitrofuran metabolite standards:** Stock standard AMOZ,  $1000 \mu\text{g mL}^{-1}$  in methanol was prepared by adding 10 mg vial from Fluka dissolved with methanol in a 10 mL volumetric flask and store in a refrigerator. Then, Standard AHD (as HCl salt),  $1000 \mu\text{g mL}^{-1}$  in

methanol was prepared by taking 13.16 mg vial from Fluka dissolved with methanol in a 10 mL volumetric flask and store in a refrigerator and stored it for 12 months. Stock standard SEM (as HCl salt), 1000  $\mu\text{g mL}^{-1}$  in methanol was prepared by adding 14.85 mg vial from Fluka dissolved with methanol in a 10 mL volumetric flask and store in a refrigerator and stored it for 12 months.

**Intermediate standards:** The 10  $\mu\text{g mL}^{-1}$  mixed standard (AMOX, AOZ, AHD, SEM) in methanol was prepared by taking 1 mL of each stock standard 1000  $\mu\text{g mL}^{-1}$  of SEM, AHD, AOZ and AMOX into a 100 mL volumetric flask and make up to the mark with methanol. Then, 1  $\mu\text{g mL}^{-1}$  mixed standard (AMOX, AOZ, AHD, SEM) in methanol was prepared by adding 1 mL of mixed standard into 10 mL volumetric flask and make up to the mark with methanol. 100  $\mu\text{g mL}^{-1}$  mixed standards (AMOX, AOZ, AHD and SEM) in methanol was produced by mixing 1 mL of mixed standard into a 10 mL volumetric flask.

**Internal standard:** Stock internal d5-AMOX standard 1000  $\mu\text{g mL}^{-1}$  was prepared by dissolving the contents of 10 mg vial from Fluka with methanol in a 10 mL volumetric flask and stored in a refrigerator.

**Intermediate internal standard:** The 10  $\mu\text{g mL}^{-1}$  d5-AMOX internal standard was prepared by mixing 1 mL of stock standard 1000  $\mu\text{g mL}^{-1}$  of AMOX d5 into a 100 mL volumetric flask and make up to the mark with methanol. 4.5.2 1  $\mu\text{g mL}^{-1}$  d5-AMOX internal standard was prepared by adding 1 mL of 10  $\mu\text{g mL}^{-1}$  of AMOX d5 into a 10 mL volumetric flask and make up to the mark with methanol. Then, 4.5.3 100  $\mu\text{g mL}^{-1}$  d5-AMOX internal standard was prepared by adding 1 mL of 1  $\mu\text{g mL}^{-1}$  of AMOX d5 into a 10 mL volumetric flask and make up to the mark with methanol. After that 10 ng  $\text{mL}^{-1}$  d5-AMOX working standard was prepared by mixing 1 mL of 1  $\mu\text{g mL}^{-1}$  of AMOX d5 into a 10 mL volumetric flask and make up to the mark with methanol.

**Sample extraction procedure:** Aliquots (1.00 $\pm$ 0.05 g) of homogenized tissue from shrimp is taken into test tubes and washed with 8 mL cooled methanol and centrifuged by 4000 rpm for 4 min. This washing step is repeated with 4 mL methanol. The sample is then mixed with 5 mL of 0.2 M HCl and 50  $\mu\text{L}$  of 2 nitrobenzaldehyde (2 NBA, 100 mM in methanol) and incubated overnight at 37°C. Then, neutralized and adjusted pH with 1 M NaOH. After the addition of 4 mL ethyl acetate, the sample was centrifuged and organic layer was transferred to a clean tube. The sample was further extracted with 4 mL ethyl acetate, centrifuged and the organic layer added to the

Table 1: Gradient condition for LC

Time	Buffer solution (mM)	Methanol(mL)
0.00	95	5
6.00	10	90
7.00	10	90
7.50	95	5

Table 2: Selected ions, collision energy and cone voltage

Ions monitored	Collision energy (V)	Cone voltage (V)
m/z 209.18-166.10 (SEM transition)	9	32
m/z 209.18-192.10 (SEM transition)	11	32
m/z 236.17-103.95 (AOZ transition)	21	35
m/z 236.17-133.79 (AOZ transition)	12	35
m/z 248.96-134.07 (AHD transition)	11	33
m/z 249.20-178.09 (AHD transition)	15	35
m/z 335.09-262.2 (AMOX transition)	25	35
m/z 335.09-291.2 (AMOX transition)	11	35
m/z 340.36- 296.32 (AMOX-D5 transition)	10	42

first extract. After evaporation to near dryness the sample was reconstituted in 50% methanol and passes through 0.45  $\mu\text{m}$  syringe filter. The sample was analyzed by reversed phase chromatography with detection by LC/MS/MS in positive mode. AMOX-d5 was carried through the analytical procedure to compensate for any analyte loss and for ion suppression during the MS stage.

**LC-MS-MS analysis procedure:** For liquid chromatography separation the following gradient condition was applied. The run time was about 12 min. The four nitrofurantol metabolites eluted from the LC about 7.5 min. Gradient condition for LC are as in Table 1. During mass spectrum analysis, the following parents and daughter ions were selected in MRM (Multiple Reaction Monitoring) by using the respective collision energy and cone voltage. Under our condition, m/z 166.10 for SEM, m/z 133.79 for AOZ, m/z 134.07 for AHD and m/z 291.2 for AMOX products were the base peak and were used for quantization. The selected ions, collision energy and cone voltage are as in Table 2.

## RESULTS

**Quantification:** Nitrofurantol metabolites in shrimp muscle were quantified by means of a calibration curve at six calibration levels ranging from 0.5-5 ppb. Solvent blank, matrix blank, negative and positive control samples are used each analytical batch as an internal quality control measures. At MRPL (Minimum Required Performances Limit) level the response of individual transition of nitrofurantol metabolites in shrimp matrix are in Fig. 2.

**Overall summaries of validation data:** The recovery of each analyte in shrimp was satisfactory. The mean recovery of AMOX, AOZ, AHD, SEM in shrimp matrix were 96.66, 95.66, 95.33 and 103.66%, respectively. Details

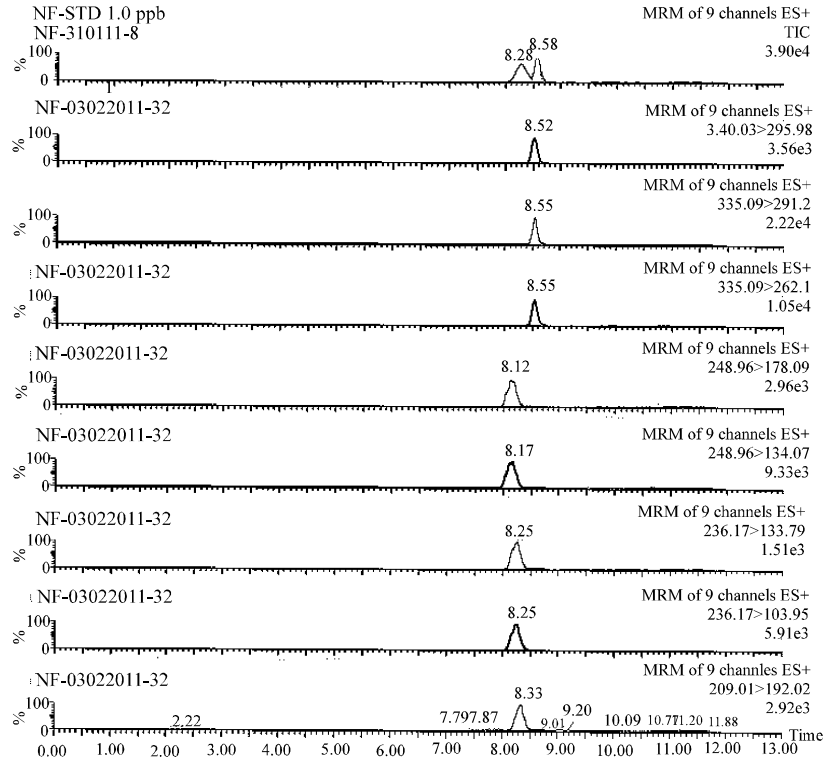


Fig. 2: Chromatograms of NF metabolites showing the RT (retention time) and response of different analyte

Table 3: Validation data of AMOZ, AOZ, AHD and SEM in shrimp at 3 different concentrations

Analyte	Fortification level	Overall mean ( $\mu\text{g kg}^{-1}$ )	Overall recovery (%)	Within day CV	Between day CV	Intermediate precision CV
AMOZ	0.5	0.47	95	6.7	2.7	7.2
	1.0	0.97	97	2.5	8.4	8.8
	1.5	1.47	98	2.2	5.0	5.5
AOZ	0.5	0.44	88	7.4	7.7	10.7
	1.0	0.97	97	5.8	4.4	7.3
	1.5	1.53	102	2.5	2.2	3.3
AHD	0.5	0.44	88	9.5	2.3	9.7
	1.0	0.97	97	5.3	6.2	8.1
	1.5	1.51	101	2.6	2.4	3.5
SEM	0.5	0.48	96	10.5	9.7	14.3
	1.0	1.05	105	6.1	8.9	10.8
	1.5	1.64	110	2.6	9.6	9.9

Table 4:  $CC\alpha$  and  $CC\beta$  values of different analyte in shrimp

Analyte	$CC\alpha$ ( $\mu\text{g kg}^{-1}$ )	$CC\beta$ ( $\mu\text{g kg}^{-1}$ )
AMOZ	0.15	0.26
AOZ	0.12	0.21
AHD	0.13	0.22
SEM	0.23	0.38

validation data of AMOZ, AOZ, AHD and SEM in shrimp at 3 different concentration areas in Table 3.

**Decision limit and detection capability ( $CC\alpha$  and  $CC\beta$ ):**  $CC\alpha$  and  $CC\beta$  were calculated using the procedure set out in ISO Guide 11843, as described in Commission Decision (2002b). The data were generated validation data (3 levels and 7 replicates per level) on each of 3 days for

shrimp. Values and  $CC\alpha$  and  $CC\beta$  of different analyte in shrimp are as in Table 4.

## DISCUSSION

The selectivity of this method was assessed 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 (2002a). This means that the minimum number of IPs to consider for their identification is four. Consequently, our method fulfils this requirement. Nitrofurans 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 (2002b). In addition, the relative retention time of the analyte must be equal to that of the calibration standard to within  $\pm 2.5\%$ .

The validation of the method in shrimp and fish meat was conducted following the European Union (EU) criteria for the analysis of veterinary drug residues in foods (Mottier *et al.*, 2005). The overall mean recovery of the analyte from the tissues was 96.41% and ranging from 88-110%. The decision limits ( $CC\alpha$ ) were 0.12-0.23 ppb and the detection capabilities ( $CC\beta$ ) 0.21-0.38 ppb. The method is robust and suitable for routine quality control operations.

Wunsch *et al.* (2004) reported that the recovery rate of analytes were 66.8-88.9%, the decision limits ( $CC\alpha$ ) were 0.049-0.20 ppb and the detection capabilities ( $CC\beta$ ) were 0.098-0.416 ppb in the honey analysis.

Rodziewicz and Zawadzka (2007) observed that the recovery rate of analytes were 84.5-109%, the value of decision limits ( $CC\alpha$ ) were 0.25-0.57 ppb and the detection capabilities were ( $CC\beta$ ) 0.32-0.77 ppb during determination of nitrofurans metabolites in animal tissues by LC-MS/MS method. These results were quite consistent with the findings in these studies. So, the results of this study agree with the findings revealed by above authors.

For veterinary drug residues with no permitted limit (like the nitrofurans) the EU allows an assessment of Measurement Uncertainty to be made by reference to  $CC\alpha$  (Sanco/2004/2726-rev 4 December 2008). Under Commission Decision 2002a, a non-compliant result is one defined as containing the identified analyte with at least 99% certainty. In a sample with reported analyte content in excess of  $CC\alpha$ , the probability that the analyte is present is greater than 99%.

### CONCLUSION

The method was developed and validated for qualitative and quantitative measurement of nitrofurans metabolites in shrimp muscle in Fish Inspection and Quality Control Laboratory, Dhaka by LCMSMS system. The  $CC\alpha$  value for all nitrofurans metabolites investigated easily met the specified EU MRPL of  $1 \mu\text{g kg}^{-1}$  ranged from 0.12-0.23  $\mu\text{g kg}^{-1}$  and suitable for routine quality control operations.

### ACKNOWLEDGMENT

We would like to acknowledge the officers and staffs of Fish Inspection and Quality Control (FIQC), Department of Fisheries, Dhaka, Bangladesh for assisting in sample preparation. We also acknowledge

Dr. Glenn Kennedy, Chemical surveillance Branch, Agrifood and Bioscience Institute, Northern Ireland, UK, for his sincere guidance during the experimental period.

### REFERENCES

- Commission Decision, 2002a. Commission decision of 12 August 2002 implementing council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official J. Eur. Commun., L221: 8-36.
- Commission Decision, 2002b. Commission decision of 27 March 2002 concerning certain protective measures with regard to poultrymeat and certain fishery and aquaculture products intended for human consumption and imported from Thailand. Official J. Eur. Commun., L84: 77-78.
- Commission Regulation, 1995. Commission Regulation (EC) No 1442/95 of 26 June 1995 amending Annexes I, II, III and IV of Council Regulation (EEC) No 2377/90 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official J. Eur. Commun., L143: 26-30.
- DOF, 2011. Fish week compendium. Department of Fisheries, Ministry of Fisheries and Livestock, Dhaka, Bangladesh.
- Mottier, P., S.P. Khong, E. Gremaud, J. Richo, T. Delatour, T. Goldmann and P.A. Guy, 2005. Quantitative determination of four nitrofurans metabolites in meat by isotope dilution liquid chromatography-electrospray ionisation-tandem mass spectrometry. *J. Chromatogr. A*, 1067: 85-91.
- Rodziewicz, L. and I. Zawadzka, 2007. Determination of nitrofurans metabolites in animal tissues by LC-MS/MS method. *Rocz Panstw Zakl Hig*, 58: 625-632.
- Stephany, R.W. and L.A. van Ginkel, 1996. European Union Regulatory Residue Analysis of Veterinary Drugs: A Strategic Approach. In: *Veterinary Drug Residues: Food Safety*, Moats, W.A. and M.B. Medina (Eds.). Chapter 4, American Chemical Society, USA., ISBN-13: 9780841234192, pp: 22-30.
- Wunsch, H., M. Walter, E. Jantzen and J. Kuballa, 2004. Determination of chloramphenicol and nitrofurans metabolites in honey and beeswax. [http://www.galab.de/laboratories/lebensmittel/pdf/galab\\_apimondia\\_2004.pdf](http://www.galab.de/laboratories/lebensmittel/pdf/galab_apimondia_2004.pdf)