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Validation of a Rapid Quantitative Method for the Residues of Nitrofuran Metabolites in Loach by Accelerated Solvent Extraction and HPLC Triple Quadrupole Mass Spectrometry

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ABSTRACT - A rapid method using HPLC-MS/MS has been developed for quantitative determination of the metabolites of nitrofurans, namely 3-amino-2-oxazolidone (AOZ), 5-morpholinomethyl-3-amino-2-oxazolidinone (AMOZ), 1-ammino-hydantoin (AHD) and semicarbazide (SEM) in loach. The extraction procedure was founded on simultaneous acidic hydrolysis and derivatization using 2-nitrobenzaldehyde (2-NBA) for 1 hour at 50°C, followed by purification with liquid-liquid extraction. Recovery was evaluated by spiking standards into blank samples at three levels (0.5, 1.0 and 2.0 μg/kg), and the mean recovery was 75.1-108.1%. Precision values expressed as the relative standard deviation (%RSD) were ≤ 8.7% and ≤ 8.5% for intra-day and inter-day precision, respectively. Linearity was studied in the range of 0.2-20 μg/Kg for NBAOZ, 0.8-20 μg/Kg for NBAMOZ, 0.2-20 μg/Kg for NBAHD, and 0.1-20 μg/Kg for NBSEM, and the obtained coefficient correlations (r) were ≥ 0.99 for all compounds. Limits of detection (LODs) for the derivatized nitrofuran metabolites were established at 0.06 μg/Kg for NBAOZ, 0.24 μg/Kg for NBAMOZ, 0.06 μg/Kg for NBAHD, and 0.03 μg/Kg for NBSEM. Limits of quantification (LOQs) were established at 0.2 μg/Kg for NBAOZ, 0.8 μg/Kg for NBAMOZ, 0.2 μg/Kg for NBAHD, and 0.1 μg/Kg for NBSEM. This simplified rapid method for reducing the derivatization and hydrolysis times can be applied to the determination of nitrofuran residues in loach.

Key words : nitrofuran metabolites, loach, mass spectrometry, method validation, accelerated solvent extraction

Nitrofurans are widely used for the effective treatment of gastrointestinal infections that cause acute or chronic diarrhea, such as cholera and even for protozoal infections like Chagas disease (*Trypanosome cruzi*) and coccidiosis *(Isosprora, Eimeria*, *and Plasmodium*) in cattle, pigs and poultry. Their broad antimicrobial spectra cover *Escherichia coli, Staphylococcus saprophyticus, Enterococcus faecalis, Citrobacter ssp*., and *Vibrio cholera* effectively. The low cost makes nitrofurans suitable for application to industrial farm animals[4,23,25,28]. For modern beekeepers, nitrofurans are useful for eradicating troublesome foulbrood (Paenibacillus) in honeybee colonies[24]. In an intensive aquaculture environment, the need to enhance the productivity of fish yield and to control the outbreak of disease has resulted in the farm industry frequently employing various veterinary antibiotics including nitrofurans[28].

Despite these uses, some reports indicate that nitrofuran metabolites in bacterial systems act like most mutagenic compounds and cause genetic mutations and chromosomal damage in addition to acting as carcinogens (goitrogen triggers) and reproductive system toxins that cause congenital malformation, miscarriage, decreased sperm production and ovarian atrophy[20,21,30].

Substantial international attention has been focused on the potential human health risks of nitrofurans due to their role as carcinogens and mutagens. In Europe, nitrofurans were prohibited for use in all food producing animals (Council regulation (EEC) No 2909/93, 1993: Commission regulation (EC) No 1442/ 95, 1995, Commission (EU) Regulation No. 37/2010) in 1993 and 1995. A conclusive MRPL (Minimum Required Performance Limit) was set at 1 μg/Kg in the EU for the aforementioned major four nitrofurans in poultry and aquaculture products in 2003[12].

When nitrofuran metabolites were found in 2002-03 in

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poultry and aquaculture products from Thailand, Vietnam, India and Brazil, the news caused pandemodium. Similar cases were also reported in pork and poultry meat from Italy, Portugal and Greece[3,7]. Furaltadone, furazolidone, nitrofurazone and nitrofurantoin were the main nitrofurans banned in the EU for animals used as food due to their negative effects on food safety. International trade legislations on the use of chemotherapeutics vary among countries throughout the world and the guidelines vary in International organizations including the Food & Agriculture Organization (FAO), the World Health Organization (WHO), the International Office of Epizootics (OIE), the Committee for Veterinary Medicinal Products (CVMP) and national agencies[13]. However, nitrofurans are still typically used as veterinary drugs in several developing countries because of their low cost and high effectiveness[19]. Therefore, it is essential to develop rapid, sensitive, cost effective and reliable analytical methods for the detection of nitrofuran metabolite residues in edible animal tissues.

Nitrofurans are quickly broken down in the animal metabolic system and metabolized to protein-bound metabolites. Consequently methods aimed at the detection of the parent drug are not practical for use with most food products. Therefore, methods for the determination of nitrofurans should be founded upon the detection of their metabolites such as 1-aminohydantoin (AHD), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidinone (AOZ) and semicarbazide (SEM) shown in Fig. 1[15]. These four sta-

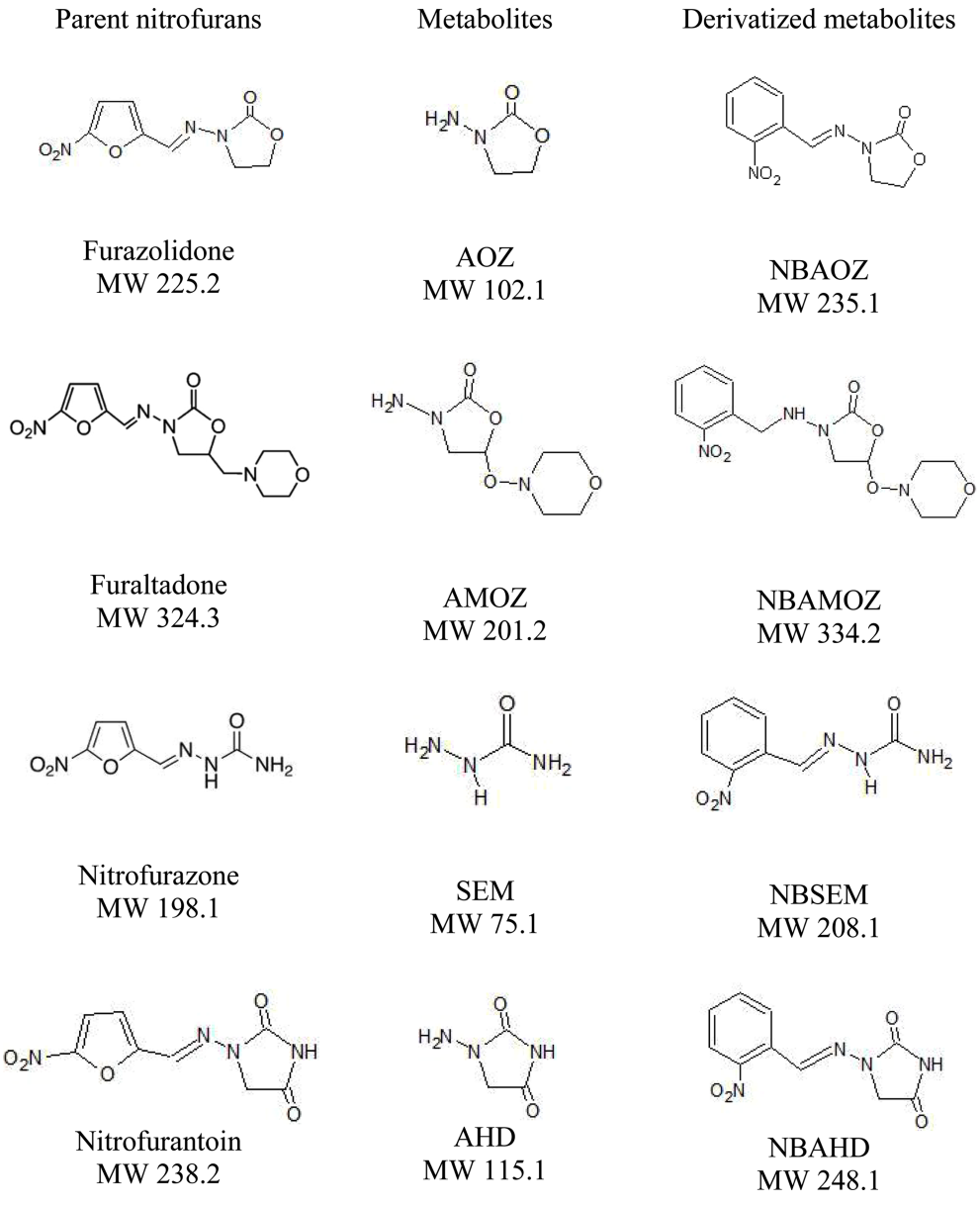

Fig. 1. Chemical structure of nitrofurans, their metabolites and derivatized compounds with 2-nitrobenzaldehyde.

ble and extant tissue metabolites can be released from proteins under acidic conditions. A derivatization process can then be carried out using a 2-nitrobenzaldehyde reagent, enabling detection in diverse sample matrices such as honey, meat, eggs, milk, poultry muscle and shrimp[16].

In the present research, our aim is to establish a time-saving analysis method to detect four nitrofuran metabolites by modifying a recently developed analysis method. There are several reliable methods for the determination of nitrofuran metabolites including the registered analysis method for the Food Code in Korea. However, most of these methods require long hydrolysis reaction times exceeding 16 hours[2,5,6,8,10,11,14,17,22,26,27,29]. A rapid method using accelerated solvent extraction (ASE) was developed to reduce the extraction time and shorten the derivatization time to 1 hour instead of 16 hours[26]. To date, there is no known validated analysis method for nitrofuran metabolites on a loach matrix. Therefore, here, we develop and validate a novel fast quantitative method by elevating the reaction temperature for hydrolysis and the derivatization time. A higher temperature facilitates the release of nitrofuran metabolites from tissue proteins with HCl. This novel fast quantitative method is validated according to the FDA guidelines.

Materials and Methods

Reagents, Chemicals and Samples

Reference standards of AOZ, AMOZ, SEM hydrochloride and AHD were purchased from Sigma-Aldrich (Steinhein, Germany). Isotopically-labelled AOZ-d4 and AMOZ-d5, used as internal standards, were also bought from Sigma-Aldrich (Steinhein, Germany). All reference standard purities were over 99%. 2-Nitrobenzaldehyde (2-NBA), used as a derivatization reagent, was purchased from Sigma-Aldrich (Steinhein, Germany). Ethyl acetate and dimethyl sulfoxide (DMSO) were purchased from Burdick & Jackson (Mashegon, MI, USA) as HPLC grade. MS analysis grade methanol was obtained from Sigma-Aldrich (Steinhein, Germany). MS analysis grade ammonium formate and formic acid were purchased from Merck (Darmstadt, Germany). HPLC grade hexane was purchased from Merck (Darmstadt, Germany). Reagent grade sodium hydroxide was purchased from Duksan pure chemical Co. Ltd. (Seoul, Korea). Analytical reagent grade 37% hydrochloric acid was purchased from Sigma-Aldrich (Steinhein, Germany).

Stock and Working Standard Solution

Standard solutions and internal standard solutions were prepared by diluting individual stock solutions containing 100 mg/L of the analytes dissolved in methanol, and were stored at −20ºC immediately. The final mixed working standard solutions were diluted with methanol to the concentrations of 0.5, 1, 2, 4, 5, 10, 20, 40, 50 and 100 μg/L. Mixed internal standard solutions of 100 μg/L were prepared by dissolving the stock solution in methanol.

Extraction procedure

Loach (Misgurnus mizolepsis) was selected as the sample for the fish matrices. The blank matrix sample was obtained from a sample confirmed to be in a non-analyte residue state. 5 g (± 0.05 g) blender-homogenized tissue was weighed in a 50 mL polypropylene centrifuge tube and fortied with 200 μL internal standard solution (100 μg/L) to obtain a concentration of 20 μg/Kg. Fortication of the samples for validation was carried out with the addition of 200 μL mixed working standard solutions at different concentrations: 0.5, 1, 2, 4, 5, 10, 20, 40, 50, 100 μg/L. The samples were allowed to stand for 15 minutes at room temperature. 1 M dibasic potassium phosphate was prepared from 175.6 g analysis grade dibasic potassium phosphate (K_2HPO_4 from Sigma-Aldrich, Steinhein, Germany) dissolved in 1 L distilled water. 0.76 g analysis grade 2-NBA was dissolved in 100 mL DMSO as a derivatization reagent. This solution was prepared immediately before use. Sample tubes were vortexed vigorously for roughly 10 seconds following the addition of 10 mL 0.125 M hydrochloric acid and 200 μL 0.05 M 2-NBA solution. The mixture was shaken vigorously for 60 minutes at 50ºC instead of the conventional hydrolysis and derivatization conditions of 16 hours and 37ºC[2,5,6,10,11,17,27,29]. The samples were left to cool to room temperature, then 1 mL 0.1 M dibasic potassium phosphate and 1 M sodium hydroxide were added to each centrifuge tube to adjust the pH to roughly 7.0. The samples were centrifuged at 2,000 G and 30ºC for 10 minutes and each supernatant was transferred to a new centrifuge tube. 10 mL hexane was added to the sample tubes and fat was extracted by vortexing for 30 seconds. Following centrifugation for 5 minutes at 2,000 G and 30ºC, each underlayer was transferred to a new centrifuge tube. 8 mL ethyl acetate was added and the mixture was agitated for 10 minutes using an SR-2 DW strong shaker, followed by centrifugation at 2,500 G for 10 minutes. The entire ethyl acetate layer was transferred to a glass tube. This liquid-liquid extraction (LLE) process was conducted once again with 8 mL ethyl acetate and the extract was transferred to each glass tube. The extracts were evaporated and condensed under the gentle stream of nitrogen gas at 50ºC. The analytes were eluted with 1 mL methanol: DW (1:1, v/v). Each meltage was transferred to a 1.5 mL Eppendorf tube and centrifuged at 7,500 G for 10 minutes. The supernatant was carefully collected and filtered through a 0.2 μm syringe lter before HPLC-MS/MS analysis.

Instrumentation and Apparatus

The Applied Biosystems triple quadrupole mass spectrometer API 4000 (Framingham, Massachusetts, USA) was used in the present study. The liquid chromatograph was equipped with an Agilent (Santa Clara, CA, USA) 1200 system with a G1322A degasser, a G1312A binary pump 1313A autosampler, and a G1316A column oven. HPLC separation was performed on a Capcell Pak UG C_{18} (2.0 × 150 mm, 5 μm, Shiseido, Tokyo, Japan). A Polytron homogenizer from Brinkmann instruments (Santa Monica, California, USA) was used to blend all loach samples. A vortex Genie-2 mixer from Scientific Industries Co. (Springfield, Massachusetts, USA) and an SR-2 DW strong sample shaker from TAITEC (Saitama, Japan) were used for sample homogenization. A 5424R Eppendorf (Hamburg, Germany) was used for sample centrifugation. The pH was measured using a pH meter from Sartorius AG (Gottingen, Germany). Samples were incubated for the hydrolysis reaction in a reciprocating shaking water bath from Kukje Engineering (Seoul, South Korea). Nitrogen evaporation was performed with an EvaT-0200 total concentration system from Goojung engineering (Seoul, South Korea). A model BL 2105 analytical balance from Sartorius (Goettingen, Germany) was used to weigh the reagents, standards and samples. The standards and reagents were carefully pipetted using an electronic Transferpette™ from Brand Tech Scientific Inc. (Essex, Connecticut, USA). A Pall Corporation (Ann Arbor, MI, USA) syringe lter (PVDF, 13 mm diameter, pore size 0.2 μm) was used for sample purification. An Eppendorf centrifuge tube (Hamburg, Germany) was used for the final layer separation to defat. Distilled deionized water was generated using an Aqua max 310 Milli-Q Plus water system from Millipore (Bedford, MA, USA).

Chromatographic Separation Parameters

LC analyses were performed on an Agilent high-performance liquid chromatography 1200 system (Santa Clara, CA, USA). The column oven temperature was maintained at 30°C using a Capcell Pak UG C_{18} (2.0 × 150 mm, 5 μm). The mobile phase constituted of solvent A (0.1% formic acid in distilled water with 10 mM ammonium formate) and solvent B (methanol). The flow rate was set to 300 μL/min and the separation of nitrofuran metabolites was achieved using the following linear gradient: 0-0.5 min, 100% A: 0.5-10 min, 100% to 0% A: 10-12 min, 0% A: 12.1-17 min, 100% A. The injection volume was 5 μL. Mass spectrometry was performed on an AB SCIEX API 4000 mass spectrometer (Framingham, Massachusetts, USA) fitted with an electrospray ionization source. The ion source gas 1 and ion source gas 2 were set to 10 L/h and the curtain gas was set to 25 L/h at a capillary temperature of 600°C. The ESI source was operated in positive mode. The data was acquired in multiple reaction monitoring (MRM) mode and the corresponding cone voltage and collision energy are shown in Table 1.

Method Validation

The developed method was validated according to the US FDA guidelines for selectivity, specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and recovery. This method was validated on a multi-residue scale with simultaneous analysis of AOZ, AMOZ, SEM and AHD, including their derivatized nitrophenyl counterparts. It is difficult to detect nitrofuran metabolites by themselves in loach, therefore, derivatized metabolites with 2-nitrobenzaldehyde were used throughout the validation study. Fortification and treatment were performed on the day of analysis and the samples were analyzed. A response was signified by the peak of the analyte. The linearity of the method was evaluated by creating matrix-matched calibration curves using blank loach samples fortified before sample preparation.

Selectivity and Specificity

The twenty-four blank samples from six different sources

Table 1. Mass spectrometry parameters for the analysis of derivatized nitrofuran metabolites

Analyte	Retention Time (min)	Precursor Ion(m/z)	Product 1 ion (m/z)	DP*/CE** /CXP*** (eV)	Product 2 ion (m/z)	DP/CE /CXP (eV)	Product 3 ion (m/z)	DP/CE /CXP (eV)	Ion Ratio (%)
NBAHD	7.92	248.9 $[M+H]^+$	134.0	76/19/6	178.1	76/21/8	104.0	76/31/16	100/47/38
NBAOZ	7.93	236.0 $[M+H]^+$	134.1	71/19/12	149.0	71/21/12	104.0	71/33/16	100/38/29
NBAMOZ	7.82	334.7 $[M+H]^+$	291.2	61/17/16	262.1	61/25/14	156.0	61/23/12	100/44/24
NBSEM	7.96	209.0 $[M+H]^+$	192.0	17/16/56	166.1	15/8/56	134.0	17/10/56	100/69/43
NBAOZ-d4	7.93	240.1 $[M+H]^+$	134.0	71/30/12	−	−	−	−	−
NBAMOZ-d5	7.79	340.1 $[M+H]^+$	296.2	61/17/17	−	−	−	−	−

*DP: Declustering Potential
**CE: Collision Energy
***CXP: Collision cell exit potential

were used to confirm the absence of possible interference near the retention time for the target compounds and compared with a standard solution containing all derivatives at a concentration of LOQ.

Linearity

For the calibration procedure, the internal standard calibration methods were adapted to the present study in order to overcome the matrix effect. Linearity was studied in the fortified blank samples at ten different concentrations for the standard solution, with the assistance of correction using the internal standard concentrations. Linearity was studied in the range of 0.2-20 μg/Kg for NBAOZ, 0.8-20 μg/Kg for NBAMOZ, 0.2-20 μg/Kg for NBAHD, and 0.1-20 μg/Kg for NBSEM. The corresponding correlation coefficients (*r*) were also evaluated.

The linearity of the method was tested on five consecutive days. Calibration curves were constructed from the area ratios and the concentration ratios of the target compounds and the internal standards.

Sensitivity (LOD, LOQ, CCα and CCβ)

The LOD was determined using the signal-to-noise ratio, with the signal at least 3 times higher than the baseline noise. For this experiment, decreasing concentrations of the standard solution were fortified in blank loach samples.

LOQ was obtained using 10 signal-to-noise ratios (S/N). The decision limit (CCα) and detection capability (CCβ) were calculated by plotting all data obtained from the accuracy study and applying calibration curves as described in the European Commission Decision 2002/657/EC[12].

Precision, Accuracy and Recovery

Precision was determined from intra-day (repeatability) and inter-day (reproducibility) measurements using five replicates of standard spiked samples at three different concentration levels, which were prepared on ve consecutive days. Blank samples were fortified with the standards for the inter-day series recovery test. Accuracy was expressed using % relative standard deviation (%RSD) and presented as recovery rate results. The recovery was determined by comparing the measured concentration with the spiked concentrations.

Recovery tests were conducted using fortified loach samples (n = 5) at three levels, with the goal of assessing the trueness of this method. Intra-day series accuracy was assessed at the same concentration levels evaluated in the recovery study (from 0.2 to 2.0 μg/Kg), with five replicates analyzed for each level. Inter-day series accuracy was determined with five fortified samples at three different levels (0.5, 1.0 and 2.0 μg/Kg) on five different days.

Results

The method described in this paper aimed to provide fast determination of marker residues for nitrofuran metabolites. It is essential for agencies to have a fast and robust analysis method to protect people from hazardous residues in food. Moreover, our efforts focused on shortening the time required to obtain clear results and the method has never been attempted before at a food safety agency. The side chains of nitrofuran metabolites that bind to tissue protein are released during the acid hydrolysis procedure. The derivative reagent 2-NBA reacts with the free side chains of the metabolites, forming 2-nitrobenzaldehide derivatives.

Extraction Optimization

To determine the optimum fast sample preparation procedure, it is crucial to conduct a test with fully-blended samples to obtain good recovery results. 10 mL 0.125 M HCl was added for 1 hour for acid hydrolysis, and 200 μL 0.05 M derivatization reagent solution was sufficient to initiate the derivative reactions. With all analytes, it was essential to use enough HCl throughout the hydrolysis reaction to maintain acidic conditions. The reaction temperatures tested for acid hydrolysis and derivatization were 37, 50, and 60°C, using a loach matrix to shorten the preparation time, and the results were compared with the recovery results from 1 hour at 37°C[2,5,6,10,11,17,25,27,29]. Table 2 shows that the 1 hour hydrolysis reaction at 50°C gave the best recovery results and was similar to the results of recovery at 37°C for 16 hours. The addition of K_2HPO_4 helped to neutralize the analytes and increase the solubility. Sodium hydroxide (roughly 1 mL)

Table 2. Comparison of recovery test results for the changing acid hydrolysis reaction temperature and time conditions in loach matrix (n = 5)

Matrix	Temperature and time conditions	Mean compound recovery (%)			
		NBSEM (RSD%)	NBAOZ (RSD%)	NBAHD (RSD%)	NBAMOZ (RSD%)
Loach	16 hours, 37°C	100.7 (0.6)	99.0 (2.7)	101.7 (35.0)	100.0 (7.5)
	1 hour, 37°C	107.0 (48.9)	84.0 (2.4)	64.3 (6.3)	93.0 (9.7)
	1 hour, 50°C	119.7 (2.1)	96.7 (2.6)	104.0 (6.3)	100.7 (3.2)
	1 hour, 60°C	117.3 (4.9)	98.0 (0.0)	117.3 (5.0)	99.7 (2.5)

was added to adjust the pH to approximately 7.0 for optimum solubility in ethyl acetate. In order to obtain a clear supernatant, the sample tube was centrifuged for 10 minutes at 2,000 G. Hydrolysis was carried out to release nitrofuran metabolites, which were bound to tissue, followed by the addition of ethyl acetate for LLE. The samples were diluted with 1 mL 50% methanol following concentration under a stable stream of nitrogen at 50°C.

Optimization of LC-MS/MS Conditions

The LC-MS/MS parameters were optimized for 5 µl injections of each analyte using the ABsiex Analyst™ (version 1.6.2) software. MRM modes were applied to evaluate the performance of this method with positive electrospray ionization. Each analyte was evaluated in the positive ionization (ESI^+) mode by monitoring the selected reaction to compose the multiple-selected ion reaction monitoring (MRM) method.

The quantitative ion transitions were *m/z* 334.7 → *m/z* 291.2 for NBAMOZ, *m/z* 209.0 → *m/z* 192.0 for NBSEM, *m/z* 248.9 → *m/z* 134.0 for NBAHD and *m/z* 236.0 → *m/z* 134.1 for NBAOZ, *m/z* 340.1 → *m/z* 296.2 for NBAMOZ-d5, *m/z* 240.1 → *m/z* 134.0 for NBAOZ-d4. The qualifying ion transitions *m/z* 334.7 → *m/z* 262.1 and *m/z* 156.0 for NBAMOZ, *m/z* 209.0 → *m/z* 166.1 and *m/z* 134.0 for NBSEM, *m/z* 248.9 → *m/z* 178.1 and *m/z* 104.0 for NBAHD, *m/z* 236.0 → *m/z* 134.1 and *m/z* 149.0 and *m/z* 104.0 for NBAOZ were used to confirm the decision. The most abundant and stable product ions were chosen to identify the target analytes. Table 1 presents the MRM transitions, individual declustering potentials and collision energy voltages applied to the analysis. The retention times for the analytes were 7.92, 7.93, 7.82, 7.96, 7.93 and 7.79 minutes for NBAHD, NBAOZ, NBAMOZ, NBSEM, NBAOZ-d4 and NBAMOZ-d5, respectively, with a 300 µL/min flow rate, as shown in Fig. 2. MS parameter tests were performed for all analytes at an ion source temperature (TEMP) of 600°C, an ionization spray voltage (ISV) of 5500 eKv, a curtain gas (CUR) of 25 kPa, a collision gas of 8 kPa, an ion Source gas 1 (GS 1, nebulizer gas) of 40 kPa, and an ion Source gas 2 (GS 2, turbo gas) of 45 kPa. The MS/MS transitions and transition ratios for derivatives of the nitrofuran metabolites are presented in Table 1. The ratios of transition are in agreement with the criteria outlined in the FDA Guidance[9].

Method Validation

Method validation was conducted according to the US FDA validation guidelines[9]. Optimization of the MS parameters was carried out by performing a series of syringe injections containing diluted standard solutions of the derivatized nitrofuran metabolites (100 µg/L) with 50% mobile phase B, using ESI+ as an ionization source. The optimization process was assisted by the Analyst™ software, which automatically determined the precursor ion and the optimal fragment voltage for performing syringe injections of the solution. Subsequently, the optimized fragment voltage was chosen, the precursor ion was fragmented and collected according to the change in collision energy, and the product ions were selected by the software. The ion with the highest abundance was selected and used for quantification purposes. The other two transition ions were chosen for identification.

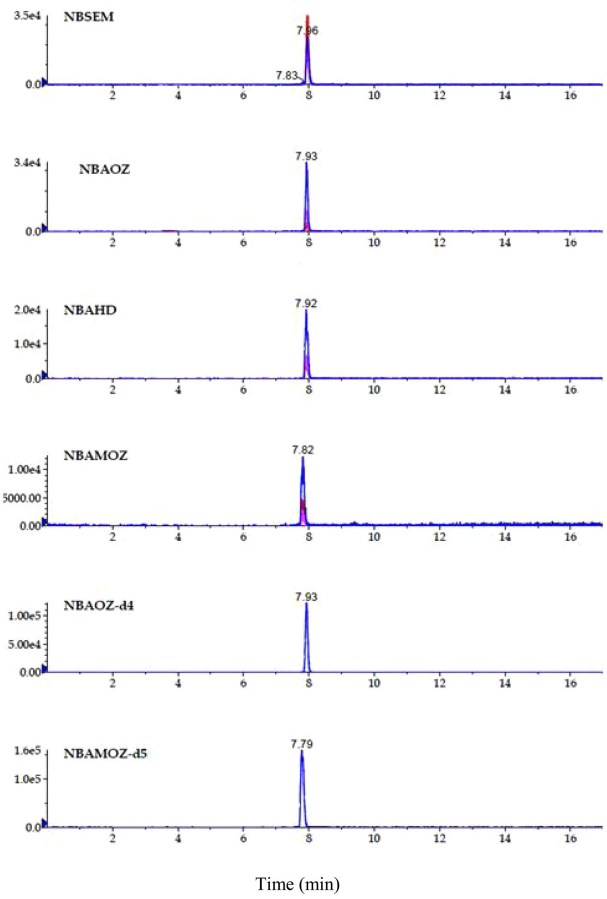

Fig. 2. Chromatogram of loach spiked with AOZ, AMOZ, AHD and SEM at a concentration of 1 µg/Kg.

Selectivity and Specificity

The selectivity and specificity of the method were evaluated for the validation test using 24 blank samples from six different sources. Confirmation was performed to determine whether there was any interference around the relative retention time of the target analytes.

Linearity

Linearity (n = 5) was evaluated using calibration curves that were constructed using a blank sample fortified with target nitrofuran metabolites. The NBAOZ curve was at a con-

centration range of 0.2-20 μg/Kg, the NBAMOZ curve was at a concentration range of 0.8-20 μg/Kg, the NBSEM curve was at a concentration range of 0.1-20 μg/Kg and the NBAHD curve was at a concentration range of 0.2-20 μg/Kg. As summarized in Table 3, all analytes appeared to have excellent linearity in the studied concentration range, with correlation coefficients (*r*) higher than 0.99 for MS detections. Calibration curves included the reference point of 1 μg/L for the decision action.

Sensitivity (LOD, LOQ, CCα and CCβ)

Signal to noise ratio (S/N) was used to determine the LOD if the method exhibited baseline noise. S/N was found by comparison with the signals from samples with known analyte concentrations and blank samples. LOD was determined by establishing the minimum concentration at which S/N was over 3. In addition, LOQ was evaluated by establishing the minimum concentration at which S/N was over 10. The LOD and LOQ for this method are summarized in Table 4.

In the 2002/657/EU European Union Decision, CCα is the smallest quantity of matter that can be detected, identified and quantified in a sample with a β error probability. These α and β error probabilities were adopted, and in quantitative methods, should be equal to 0.05 for a substance with a maximum residue limit of 0.01 for prohibited veterinary antimicrobials. The obtained CCα and CCβ are shown in Table 3.

CCα can be defined as $CC\alpha = X + k \times Sr$

where X is the intercept of the calibration graph, k is 1.64 and Sr is the between-day reproducibility of blank samples. CCβ can be calculated as $CC\beta = CC\alpha + k \times Sr$,

where k and Sr are the same as for the CCα calculations[18].

Precision, Accuracy and Recovery

Accuracy and precision were verified for each nitrofuran metabolite as the intra-day and inter-day variability, following the validation methodology from the US FDA guidelines[9]. Precision was identified as the RSD (%) and determined in terms of peak area at three concentration levels (0.5, 1.0 and 2.0 μg/Kg). The obtained results are summarized in Table 4. The RSD (%) values were between 2.4 and 8.5 for inter-day precision (n = 5) when MS detection was used. The accuracy of the methods was evaluated by the intra-day precision. These levels were equal to or slightly above or below the legislated Minimum Required Performance Limit (MRPL) for nitrofuran metabolites[12].

The compound recovery range was 96.7-119.7% at 50ºC

Table 3. Calibration curves and sensitivity of metabolites in loach (n = 5)

Analyte	Linearity range (μg/Kg)	Calibration curve Equation (*r*)	CCα (μg/Kg)	CCβ (μg/Kg)
NBAOZ	0.2-20	$y = 0.1391\ x - 0.0453$ (0.9967)	2.2	4.4
NBAMOZ	0.8-20	$y = 0.0408\ x - 0.0024$ (0.9991)	1.1	2.2
NBSEM	0.1-20	$y = 0.2930\ x + 0.0338$ (0.9995)	1.7	3.3
NBAHD	0.2-20	$y = 0.2528\ x - 0.0453$ (0.9967)	2.2	4.4

Table 4. Corrected intra-day and inter-day accuracy as well as precision results in loach samples spiked with working standard solution (n = 5)

Analyte	Intra-day series		Inter-day series		LOD (μg/Kg)	LOQ (μg/Kg)
	Spiked level (μg/Kg)	Accuracy (Precision%)	Spiked level (μg/Kg)	Accuracy (Precision%)		
NBAOZ	0.2	116.6 (7.1)	0.5	102.4 (6.0)	0.06	0.2
	0.4	102.1 (2.1)	1.0	108.1 (3.8)		
	0.8	103.3 (3.0)	2.0	92.3 (2.4)		
NBAMOZ	0.8	100.6 (4.6)	0.5	99.3 (3.1)	0.24	0.8
	1.6	99.1 (6.2)	1.0	98.4 (4.4)		
	2.0	99.9 (3.8)	2.0	98.1 (3.4)		
NBSEM	0.1	105.4 (3.6)	0.5	87.4 (7.0)	0.03	0.1
	0.2	101.8 (5.4)	1.0	86.3 (8.1)		
	0.4	103.1 (3.0)	2.0	85.1 (6.4)		
NBAHD	0.2	115.7 (4.7)	0.5	78.2 (8.5)	0.06	0.2
	0.4	105.5 (8.7)	1.0	75.1 (7.3)		
	0.8	104.6 (6.0)	2.0	81.3 (5.4)		

for 1 hour in a loach matrix. This method can be used as a fast quantitative method as summarized in Table 2. Based on these results, the accuracy and precision of 4 nitrofuran metabolites were tested using the aforementioned method. Mean values were calculated from five individual sample measurements at each concentration level for loach. Intra-day accuracy was 99.1-116.6% and inter-day accuracy was 75.1-108.1%. Inter-day mean recovery was 101% for NBAOZ, 98% for NBAMOZ, 86% for NBSEM, and 78% for NBAHD. The acceptability of the accuracy and precision results depend on the fortification levels of the analytes under the AOAC manual for Peer-Verified Methods[1]. In this regard, the results obtained using the developed methodology would be acceptable. This method provides reliable sensitivity and fast results as an LC-MS/MS analysis method, compared with published methods that take over 16 hours from sample preparation to confirmation of the decision action. This method also meets FDA validation guidelines[9].

Discussion

An LC-MS/MS method for the simultaneous determination of nitrofuran metabolites in loach was developed and validated. In general, nitrofuran metabolite analysis research deals with matrix problems. However, existing analysis methods have some disadvantages due to having a long preparation time, with a sample process lasting over 16 hours.

The development of a faster and robust analysis method is very important for food safety. It is worth mentioning that nitrofurans are still frequently used for the production of seafood despite government regulations. The present study has demonstrated that this is a cost effective, sensitive, and rapid method for the determination of nitrofuran metabolites in loach. In conclusion, the methodology can be considered a standard quantitative method for reporting nitrofuran marker residue levels in loach. All the limits validated using the method were below the recommended concentrations established by the Commission Decision 2003/181/EC.

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국문 요약

미꾸라지에서의 Nitrofuran계 대사물질인3-amino-2-oxazolidone (AOZ), 5-morpholinomethyl-3-amino-2-oxazolidinone (AMOZ), 1-ammino-hydantoin (AHD)와 semicarbazide (SEM)의 잔류량을 검사하기 위해HPLC-MS/MS를 이용한 신속한 정량법이 개발되었다. 2-nitrobenzaldehyde (2-NBA)를 이용해 50°C에서 1시간 동안 산 가수분해와 유도체화 과정을 거친 뒤에, 액-액 분배로 정제와 추출을 하였다. 회수율은 음성시료에 3가지 농도 0.5, 1.0, 2.0 μg/kg의 표준액을 첨가하여 평가하였고 평균 회수율은 75.1-108.1% 이었다. 정밀성(%RSD)은 일내 8.7% 이하, 일간 8.5% 이하였다. 직선성은 NBAOZ는 0.2-20 μg/Kg, NBAMOZ는 0.8-20 μg/Kg, NBAHD는 0.2-20 μg/Kg, NBSEM 는 0.1-20 μg/Kg 범위에서 모두 상관계수 0.99이상이었다. 검출한계(LOD)는 NBAOZ 0.06 μg/Kg, NBAMOZ 0.24 μg/Kg, NBAHD 0.06 μg/Kg, NBSEM 0.03 μg/Kg이었고, 정량한계(LOQ)는 NBAOZ 0.2 μg/Kg, NBAMOZ 0.8 μg/Kg, NBAHD 0.2 μg/Kg, NBSEM 0.1 μg/Kg 이었다. 가수분해 및 유도체화 소요시간을 1시간으로 줄여 만든 신속 간편한 이 시험법이 미꾸라지 중 nitrofuran metabolites잔류량 분석에 적합함을 확인할 수 있었다.

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