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Quantitative Determination of Four Nitrofurans and Corresponding Metabolites in the Fish Muscle by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry

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ABSTRACT

A method validated based on the European Union (EU) regulations for determining the presence of furazolidone, furaltadone, nitrofurazone, nitrofurantoin and their corresponding metabolites AOZ, AMOZ, SC and AH in fish muscle was developed. Samples were acid-hydrolyzed, treated with 2-nitrobenzaldehyde and extracted with ethyl acetate. The extracts were dried in a N_2 stream and redissolved in methanol:water (50:50, v/v). Analysis was performed by LC/ESI/MS/MS. This developed method carried limits of quantification lower than 10 μg/kg for the nitrofurans and 1.0 μg/kg for the metabolites. It was observed that the decision limit (CCα) ranged from 2.93 to 5.01 μg/kg for the nitrofurans and 0.19 to 0.43 μg/kg for the metabolites. The detection capability (CCβ) was between 3.62 and 6.20 μg/kg for the nitrofurans and between 0.23 and 0.54 μg/kg for the metabolites. The linear calibration curve parameters in the fortified fish muscle were between 1.0-100.0 μg/kg and 0.1-10.0 μg/kg for the nitrofurans and metabolites, respectively.

Key words: nitrofuran, metabolite, LC-ESI-MS/MS

INTRODUCTION

Nitrofuran is a group of broad spectrum synthetic antibacterial agent widely used as food preservative and food additive[(1)]. It is active in against bacterial enteritis caused by *Escherichia coli* and *Salmonella enterica*. Furazolidone, furaltadone, nitrofurazone and nitrofurantoin were the 4 nitrofurans generally used as additives in livestock and aquatic products (Figure 1). Since 1966, furazolidone has been used in aquatic products as an antiseptic in Japan. In 1993, the European Union (EU) attempted to start establishing a standard method and a maximum residual limit (MRL)[(2)]. The nitroreduction of various nitrofuran derivatives generates superoxide from the formation of nitro anion radicals and subsequent reaction with O_2[(3)]. In 1998, Taiwan banned nitrofuran for use in aquatic products as a feed additive due to the possible formation of DNA-adducts at guanine residues. In 1999, Taiwan passed an act detailing a standard method of nitrofuran use for the treatment of scale diseases of eels.

In 2003, a decision by the European Commission set a minimum required performance limit (MRPL) of 1.0 μg/kg for each nitrofuran metabolite, 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholino-methyl-2-oxazolidinone (AMOZ), scicarbazide (SC) and 1-aminohydantoin (AH), in poultry meat and aquaculture products[(4)]. Taiwan followed and set a MRPL of 1.0 μg/kg in 2007. However, traditional equipment is not capable of fulfilling the directives laid out in the recent statute. The high-performance liquid chromatographic-UV photodiode-array detection (HPLC/DAD) method was published in 1997 for the determination of nitrofuran residues in chicken eggs[(5)]. Diaz *et al.* attempted to use high-performance liquid chromatography with electrochemical detection to detect nitrofurantoin, furazolidone and furaltadone in milk[(6)]. Because nitrofurans metabolize rapidly *in vivo*[(7)], determination of their

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metabolites are more important and useful than that of the original nitrofuran. Increasing number of laboratories have analyzed residues of metabolites by LC/MS/MS in the past few years. For example, Szilard *et al*., Leitner *et al*. and Szilagyi *et al*. published methods for the determination of nitrofuran metabolites in animal tissue and animal feeds by high-performance liquid chromatography-tandem mass spectrometry[8-10]. Van Loco *et al*. subsequently attempted quantitative and qualitative determination of nitrofuran metabolites in animal tissue by LC/MS/MS[11,12]. In 2009, the Department of Health of Taiwan also promulgated a standard method for test of nitrofuran metabolites in foods by LC/MS/MS[13].

The aim of this study was to develop and validate a method for the simultaneous determination of nitrofurans and their metabolites in fish muscle.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

AOZ and AMOZ were provided by Riedel-de Haën (Berlin, Germany). 1-aminohydantoin hydrochloride (AH) and semicarbazide (SC) were purchased from Sigma-Aldrich (Steinheim, Switzerland). Furazolidone, furaltadone, nitrofurantoin and nitrofurazone were obtained from Sigma (Steinheim, Germany). 2-nitrobenzaldehyde (2-NBA) and ammonium acetate were obtained from Fluka (Berlin, Germany). Di-potassium hydrogen phosphate trihydrate was purchased from Merck (Darmstadt, Germany) and sodium hydroxide was purchased from Riedel-de Haën (Berlin, Germany). All reagents and solvents were of analytical grade or higher grade unless stated otherwise. Water was demineralised

Table 1. Optimized conditions for the analysis of nitrofurans and corresponding metabolites in fish tissue by LC/MS/MS

HPLC system	
Sample temperature	25 ± 2°C
Injection volume	25 μL
Column type	4.6 × 150 mm, 5 μm from Agilent, ZORBAX SB-C_{18}
Column temperature	25 ± 2°C
Flow rate	700 μL/min
Mobile phase solution	Mobile phase A 0.5 mM ammonium acetate
	Mobile phase B 100% methanol, HPLC grade
MS/MS detector	
Source temperature (at setpoint)	650°C
Electrospray capillary voltage	5500 V
CAD	5
Current Gas	10
CEM	2100 V
Multiple reaction monitoring (MRM) for selected ions	Shown in Table 2

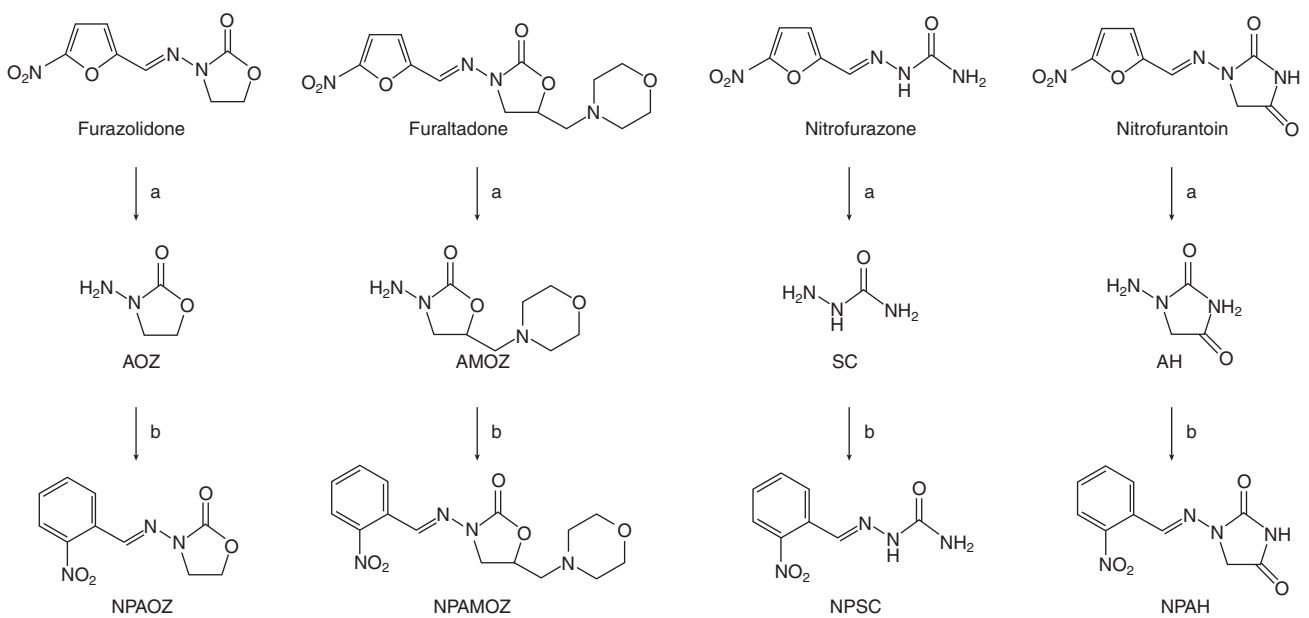

Figure 1. Molecular structures of nitrofurans (furazolidone, furaltadone, nitrofurazone and nitrofurantoin), metabolites (AOZ, AMOZ, SC and AH) , and nitrophenyl derivatives (NPAOZ, NPAMOZ, NPSC and NPAH). The process included (a) nitrofurans metabolized and (b) metabolite derivatized.

using a Millipore purification system (Millipore, Billerica, MA, USA). Individual stock standard solutions of AOZ, AMOZ, AH, SC, furazolidone, furaltadone, nitrofurantoin and nitrofurazone (1000 mg/L) were prepared by dissolving in methanol and stored in a refrigerator at 4°C. Working mixed standard solutions of these compounds were prepared with methanol and remained stable for at least two months.

II. *Sample Co-pretreatment for Nitrofurans and Metabolites*

A well-homogenized fish muscle (1.0 g) was accurately weighed into a 50-mL plastic tube, and hydrochloric acid (1.0 N, 0.5 mL), double-distilled water (5 mL) and 2-NBA (50 mM solution in dimethyl sulfoxide, 0.5 mL) were added. After vortexing, the mixture in the tube was incubated in a water bath at

Table 2. Transition reactions monitored by LC-ESI-MS/MS, retention time and peak area ratios

Analyte	Transition reactions (m/z)		Retention time (min)	Peak area ratio (%)
	Quantitation ion pair	Confirmation ion pair		
Furazolidone	226.2 → 67.3	226.2 → 78.6	4.79	8.8 ± 0.3
Furaltadone	325.3 → 100.0	325.3 → 85.5	2.66	15.9 ± 0.5
Nitrofurazone	199.2 → 78.6	199.2 → 107.2	4.88	46.0 ± 17.0
Nitrofurantoin	239.2 → 67.3	239.2 → 83.0	4.77	16.3 ± 4.0
NPAOZ	236.2 → 134.2	236.2 → 104.0	5.41	28.0 ± 1.0
NPAMOZ	335.2 → 291.2	335.2 → 262.2	4.36	34.0 ± 12.0
NPSC	209.2 → 192.2	209.2 → 134.0	5.47	87.0 ± 1.0
NPAH	249.3 → 134.2	249.3 → 178.0	5.37	82.0 ± 14.0

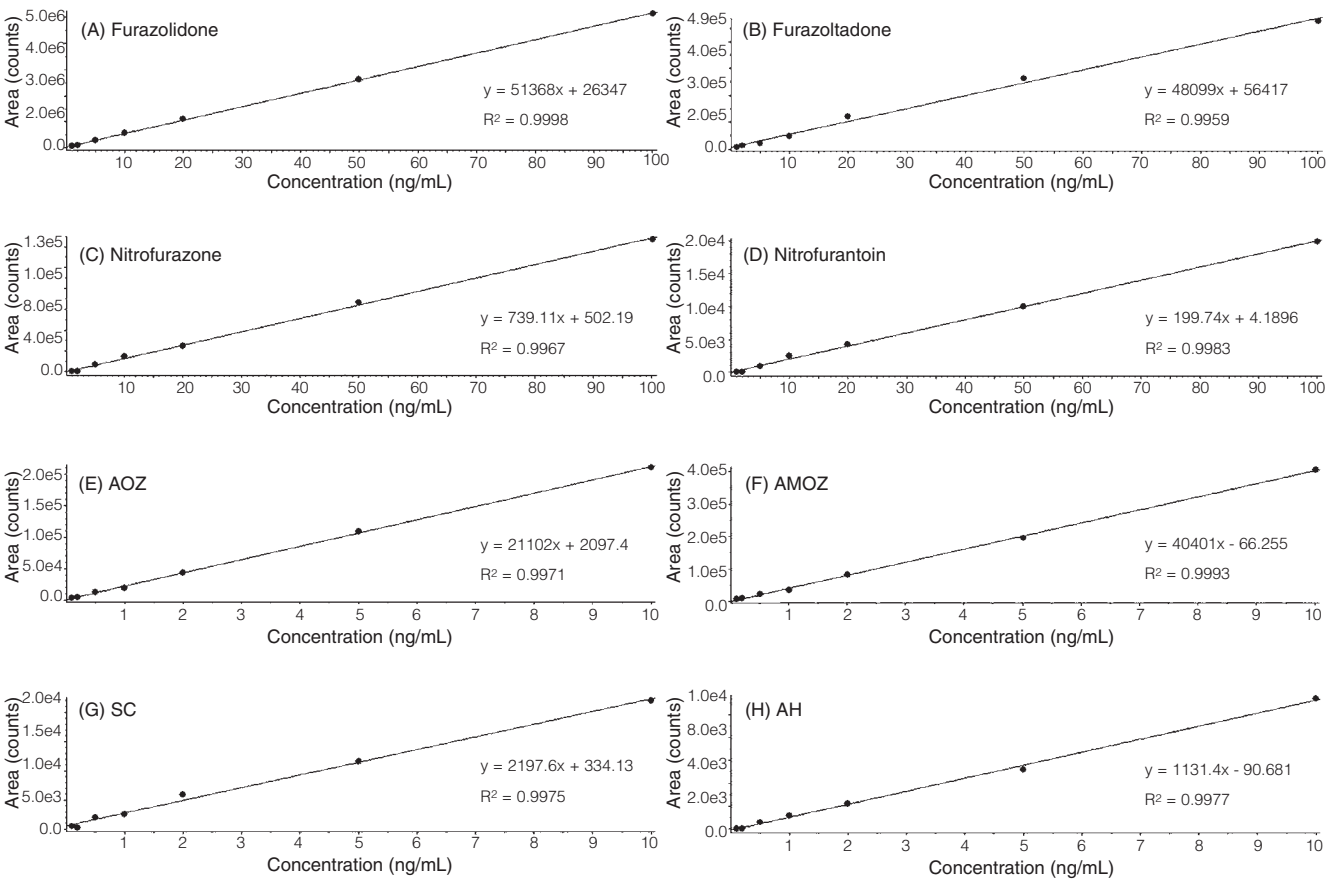

Figure 2. Calibration curves of nitrofurans and corresponding metabolites prepared with spiked concentration levels in fish muscle.

37°C overnight. Sodium hydroxide (1.0 M, 0.4 mL) and dipotassium hydrogen phosphate (0.1 M, 5 mL) were added to neutralize the mixture at pH = 7.0 ± 0.3. Ethyl acetate (HPLC grade, 7 mL) was added to the plastic tube containing the mixture and extracted twice, and the organic phase was combined into a 15-mL glass tube. After drying in a nitrogen stream, the residue was reconstituted with methanol/double-distilled water (50:50, v/v, 1 mL) and the lipid component in the samples was removed with 2 mL *n*-hexane. The final solution was filtered using a 0.2-μm filter and the sample was transferred into a HPLC vial.

III. *LC/ESI/MS/MS Spectrometry*

The LC/ESI/MS/MS system consisted of an Agilent Series 1100 HPLC system (Agilent Technologies, Waldborm, Germany) connected to a Sciex API 4000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). HPLC analysis was performed on a C_{18} reversed-phase column (4.6 × 150 mm), 5 μm; Agilent, ZORBAX SB-C_{18}) at room temperature. The mobile phase was composed of a mixture of solvents (0.5 mM ammonium acetate and methanol of HPLC grade) in gradient (Table 1). The running time for each sample was set for 9 min and the injection volume was 20 μL.

The MS detection system included an electrospray ionization source. The ion source block temperature was set at 650°C and the electrospray capillary voltage was set at 5500 V. Nitrogen was used as the collision gas at a pressure of 5 m Torr (Table 2).

IV. *Quantitation*

The nitrofurans and metabolites were quantified by calibration curves, which were spiked at 7 concentration levels from 1.0 to 100 μg/kg for nitrofurans and 0.1 to 10.0 μg/kg for metabolites in fish muscle. The calibration curves are shown in Figure 2.

V. *Method Validation*

Identification of the nitrofurans and metabolites in the samples was based on the Commission Decision 2002/657/EC criteria[4]. The stability of the stored standard solution of mixed nitrofurans was tested under four different conditions: storage in the light for 8 hr per day at 25°C; in darkness at 25°C; in darkness at 4°C; and in darkness at -20°C. The storage time was extended to 45 days, until degradation phenomena were observable during quantification. Calculation of the concentration of each residual analyte was performed using a solution of the analyte freshly prepared at the time of analysis by the following formula: Analyte Remaining (%) = C_t /C_{fresh} × 100%**,** where Ct = concentration at time point, C_{fresh} = concentration of fresh solution. The maximum storage time and the optimum storing conditions were

Table 3. Performance data of LC/ESI/MS/MS analysis of nitrofurans and metabolites in fish muscle

	Furazolidone					Furaltadone					Nitrofurazone					Nitrofurantoin				
Conc.[a]	5	10	20	50	100	5	10	20	50	100	5	10	20	50	100	5	10	20	50	100
Overall Mean (n = 36)	4.9	10.3	20.0	50.0	99.0	4.7	11.0	19.4	51.6	99.3	5.3	10.9	20.6	51.5	99.0	4.7	11.0	19.4	51.6	99.3
Trueness (%)	98	103	100	100	99	95	110	97	103	99	106	109	103	103	99	95	110	97	103	99
Within-day Precision (%)	2.0	1.6	1.6	1.9	0.8	7.0	1.1	4.0	3.3	1.0	5.7	4.3	3.9	2.3	0.3	8.7	5.4	1.1	1.1	1.2
Between-day Precision (%)	3.6	2.6	2.5	2.4	1.4	19.9	14.0	12.9	7.7	1.8	9.0	5.7	4.6	4.3	1.1	18.2	8.7	7.6	6.3	1.6
	AOZ					AMOZ					SC					AH				
Conc.[a]	0.5	1.0	2.0	5.0	10.0	0.5	1.0	2.0	5.0	10.0	0.5	1.0	2.0	5.0	10.0	0.5	1.0	2.0	5.0	10.0
Overall Mean (n = 36)	0.5	1.0	2.0	5.0	10.0	0.4	1.0	1.9	5.29	9.9	0.5	1.0	2.0	5.0	9.9	0.5	1.0	2.0	5.0	10.0
Trueness (%)	99	97	101	100	100	82	97	94	104	99	102	102	99	100	99	96	95	102	100	100
Within-day Precision (%)	5.0	1.1	4.0	1.3	1.0	9.6	9.0	7.7	9.5	1.8	15.8	12.7	4.1	0.8	0.3	15.4	11.4	4.8	0.8	0.1
Between-day Precision (%)	6.5	8.2	8.2	1.8	1.8	16.3	13.2	11.7	11.6	3.2	23.1	19.3	5.0	1.0	3.3	26.8	19.3	12.8	2.3	1.9

[a]Fortification concentration levels (μg/kg).

recorded. Because there is no certified reference material (CRM) available**,** the trueness of measurements was gauged through the recovery of additions of seven fortified concentration levels of the analytes to blank fish muscle. Recovery was calculated using the following formula: Trueness (%) = (C_m/C_f) × 100% (C_m = mean of measurements, C_f = addition of fortified concentration levels). Recovery data were considered acceptable when the trueness was within ± 10% of the target value. Precision (within- and between-day) was calculated by analysis of blank fish muscle fortified with each nitrofuran and derived metabolite at five fortification levels (5.0, 10.0, 20.0, 50.0, 100.0 μg/kg and 0.5, 1.0, 2.0, 5.0, 10.0 μg/kg for nitrofurans and metabolites, respectively), and the experiments were performed by the same operator in triplicate at the same day and on twelve separate occasions in a month. The individual fortification levels converted to nitrofurans and metabolites are given in Table 3. The values of decision limit (CCα) and detection capability (CCβ) were calculated following the Commission Decision 2002/657/EC guidelines. CCα was calculated as the corresponding concentration of the y-intercept of a calibration curve constructed with blank fish tissue, which was spiked fortified concentrations of nitrofuran and metabolite standard solutions at concentration levels above the minimum required performance concentration levels in equidistant steps, plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept. CCβ was calculated as the corresponding concentration at the decision limit plus 1.64

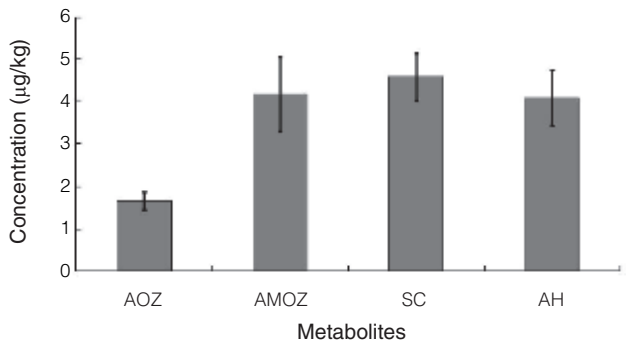

Figure 3. Metabolites (AOZ, AMOZ, SC and AH) hydrolyzed with 0.5 mL of 1 M hydrochloric acid from spiked 100 μg/kg furazolidone, furaltadone, nitrofurazone and nitrofurantoin in the fish muscle. Vertical bars indicate standard deviation of the average value (n = 24).

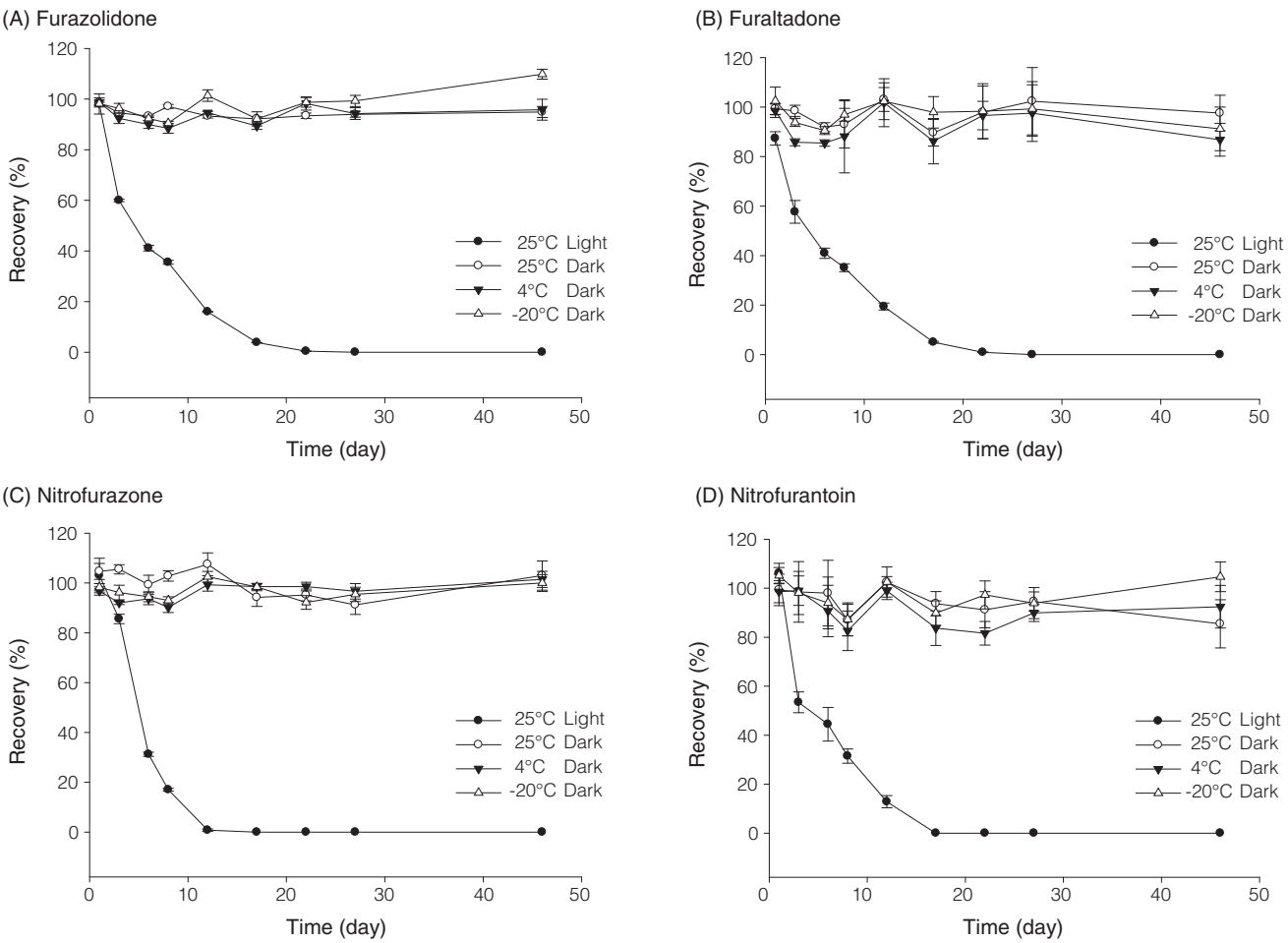

Figure 4. Stability of the mixed standard nitrofurans solution at the 100 μg/L concentration level under different conditions in 46 days.

times the standard deviation of the within-laboratory reproducibility, which was the mean of the decision limit.

RESULTS AND DISCUSSION

I. *Development of Nitrofurans and Metabolites Co-pretreatment Method*

The extraction procedure of nitrofuran followed a modified method of McCracken and Kennedy[14]. The main difference was that 1.0 g of sample and a total of 10 mL of extraction solvent (ethyl acetate) were used in the present study, because less extraction solvent and smaller sample size were more convenient for the extraction procedure. In our sample pretreatment procedure, 2-5% nitrofurans were acid-hydrolyzed into their corresponding metabolites (Figure 3). In the clean-up step, 2 mL of *n*-hexane was added to extract the lipid component in the sample. After centrifugation at high speed, the *n*-hexane and methanol/double-distilled water (50:50, v/v, 1.0 mL) layer was very clearly delineated, and hence it was easy to drain the lower layer into a HPLC vial.

The result of the stability experiment of the mixed standard nitrofurans solution at the 100 μg/L concentration level in 46 days showed that furazolidone, furaltadone, nitrofurazone and nitrofurantoin were relatively stable in darkness at 25°C, 4°C and -20°C. The half-life of furazolidone, furaltadone, nitrofurazone and nitrofurantoin in the light at 25°C was 2.85, 3.39, 2.57 and 3.39 days, respectively (Figure 4). Apparently, light was the main cause of nitrofuran decomposition.

II. *LC/ESI/MS/MS Spectrometry*

The highest MS sensitivity for the four nitrofurans and four derived metabolites was obtained using the positive electrospray ionization mode. Parent ions of the molecules were protonated $[M+H]^+$ for furazolidone, furaltadone, nitrofurazone, nitrofurantoin and their corresponding nitrophenyl derivatives NPAOZ, NPAMOZ, NPSC, NPAH at *m/z* values of 226.2, 325.3, 199.2, 239.2, 236.2, 335.2, 209.2 and 249.3, respectively. Two corresponding daughter ions for each nitrofuran and metabolite were determined, one for quantitation and the other for confirmation (Table 2). In all cases, parent ions $[M+H]^+$ were found most abundant. For each parent ion, the most abundant daughter ion was selected for quantitative purposes and the second most abundant for confirmation. Figure 5 shows the typical fragmentation pattern of the mass spectrum of the selected example NPAOZ, where the parent ion is *m/z* 235.9, and the transient *m/z* 134.0 was used for quantification and transient *m/z* 104.0 for confirmation. LC/ESI/MS/MS chromatograms of standard mixtures of the four nitrofurans and four metabolites in the spiked fish muscle are shown in Figure 6.

Table 4. Validation parameters for LC/ESI/MS/MS

Compound	n	CCα (μg/kg)	CCβ (μg/kg)
Furazolidone	24	4.94	6.11
Furaltadone	24	2.93	3.62
Nitrofurazone	24	3.66	4.52
Nitrofurantoin	24	5.01	6.20
AOZ	36	0.19	0.23
AMOZ	36	0.24	0.30
SC	36	0.29	0.36
AH	36	0.43	0.54

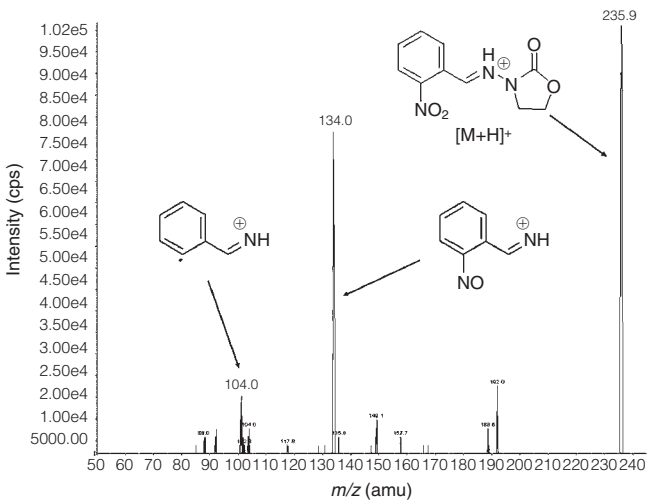

Figure 5. Mass spectrum of NPAOZ and its typical fragmentation pattern.

III. *Method Performance Characteristics*

Selectivity of this method was assessed by use of two transition reactions for each analyte. For confirmation of the substances listed in Group A of Annex I of the Commission Decision 96/23/EC criteria[15], a minimum of 4 identification points were required. The method used in this study conformed to this regulation. The method validation performance data are summarized in Table 3. Recovery of the nitrofurans ranged from 95 to 110% and the metabolites from 82 to 104%; Inter-day precision was satisfactory for both nitrofurans and metabolites (≤ 27%).

CCα and CCβ were calculated following the calibration curve procedure according to ISO 11843[16]. The calibration curve procedure involved the use of 7 concentration levels and was performed by the same operator in triplicate in one day and on twelve separate occasions within a month. Each spiked concentration of the nitrofurans and metabolites was duplicated and determined twice by LC/ESI/MS/MS. The CCα and CCβ values

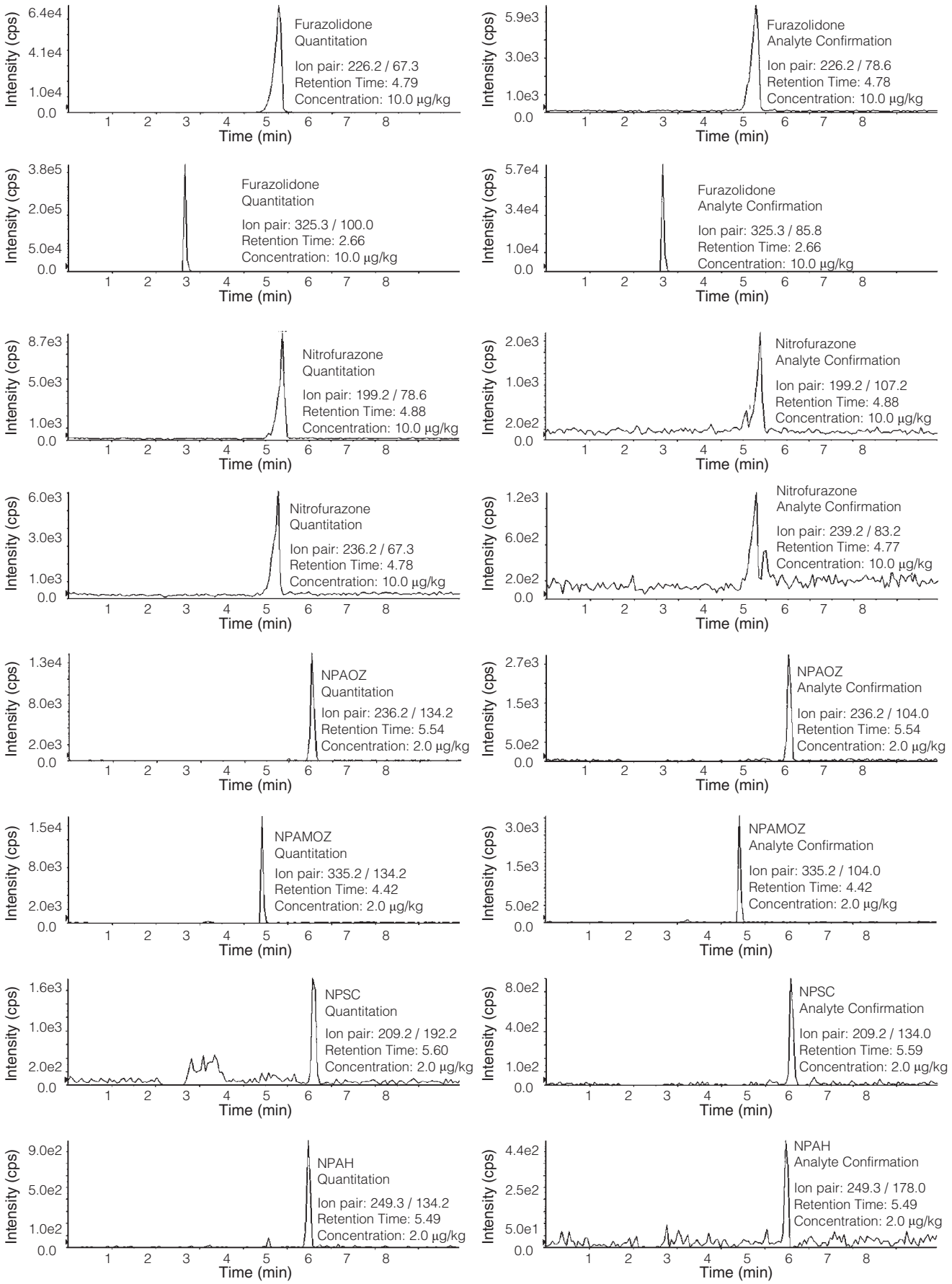

Figure 6. MRM chromatograms obtained by LC-ESI-MS/MS in the positive mode of non-contaminated fish muscle fortified at 10 µg/kg with four nitrofurans and metabolites.

are shown in Table 4. The decision limits (CCα) were 2.93-5.01 μg/kg for nitrofurans and 0.19-0.43 μg/kg for metabolites; the detection capabilities (CCβ) were 3.62-6.20 μg/kg for nitrofurans, and 0.23-0.54 μg/kg for metabolites, which were below the minimum required performance limit (MRPL) set at 1 μg/kg by the EU.

Diaz *et al*. attempted to detect nitrofurantoin, furazolidone and furaltadone in milk by high-performance liquid chromatography with electrochemical detection[4]. The detection limits in that study were between 4 and 6 μg/kg and the working concentration range was 10-60 μg/kg. Draisci *et al*. used liquid chromatography-ionspray mass spectrometry to determine nitrofuran residues in avian eggs. Nitrofuran residues were extracted from 10.0 g sample and lower detection limits of 3.2, 1.6 and 1.0 μg/kg were observed for nitrofurazone, furazolidone, furaltadone[5]. Comparing this developed method promulgated by the Department of Health of Executive Yuan of Taiwan, half quantity of sample (1.0 g) was used in the pretreatment procedure[13]. This is the first study in which four nitrofurans were determined by LC/MS/MS; moreover, the four corresponding metabolites were determined simultaneously.

Solid phase extraction (SPE) is commonly used in the sample pretreatment procedure. Mottier *et al*. described a SPE method for the determination of nitrofuran metabolites in meat[11], in which residue from 5 g of homogenized sample was extracted and reconstituted with 0.25 mL of acetonitrile/distilled water (70:30, v/v). CCα and CCβ were determined to be 0.11 and 0.19, 0.12 and 0.21, 0.20 and 0.34, and 0.21 and 0.36 μg/kg for AOZ, AMOZ, SC and AH, respectively. Thus, the liquid-liquid extraction sample pretreatment method used in this study attained a detection limit similar to that of the SPE method.

CONCLUSIONS

A quantitative method of LC/ESI/MS/MS has been developed for the determination of four nitrofuran and four metabolites in fish muscle. The method was validated following the EU criteria for analysis of metabolites of veterinary drug residues. Based on the CCα and CCβ values obtained in this study, this method is able to measure the presence of the four aforementioned nitrofurans and corresponding metabolites below the MRPL (i.e. 1.0 μg/kg). Applying the methodologies in routine analysis was simple and quick. As only fish muscle was used in method evaluation in this study, we will expand the method for the determination of other nitrofurans and metabolites in different matrices in the future.

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