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Simultaneous Determination of Eleven Quinolones Antibacterial Residues in Marine Products and Animal Tissues by Liquid Chromatography with Fluorescence Detection

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ABSTRACT

A simple and efficient multiresidue method was developed for determining 11 quinolones (QNs; marbofloxacin, norfloxacin, ciprofloxacin, lomefloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid and flumequine) in chicken, pork, fish and shrimp. The analytes were extracted with 0.3% metaphosphoric acid and acetonitrile (1:1, v/v), followed by a HLB cartridge clean-up procedure. The HPLC separation was carried out on a symmetry column C-18 (250 mm × 4.5 mm i.d., 5 μm) with linear gradient elution of 0.1% formic acid and acetonitrile as mobile phase and programmable fluorescence detection. The method was validated by spiking blank animals tissues at three different levels (25, 50 and 250 ng/g; except 6.25, 12.5 and 62.5 ng/g for DAN) while the linearity, detection limit, quantification limit, precision and accuracy were checked. Mean recoveries of 11 QNs from edible animal tissues were 71.7-105.3%. The limits of quantification in different muscle tissues ranged from 5.0 to 28.0 ng/g. The results showed this method was simple, rapid, sensitive and suitable for routine tests.

Key words: Quinolones, residue, HPLC

INTRODUCTION

Quinolones (QNs) were represented by quinolone carboxylic acids (flumequine, oxolinic acid and nalidixic acid) and fluoroquinolones (FQs). Structurally, all FQs contain fluorine at C-6 position and piperazinyl at C-7 position, such as marbofloxacin (MAR), norfloxacin (NOR), ciprofloxacin (CIP), lomefloxacin (LOM), danofloxacin (DAN), enrofloxacin (ENR), sarafloxacin (SAR), difloxacin (DIF). Flumequine (FLU) and oxolinic acid (OXO) are used as aquaculture therapeutic agents in Taiwan[(1)]. FQs are important synthetic antimicrobials with broad spectrum of antibacterial activity, good absorption after oral administration, and extensive tissue distribution. The major metabolite of ENR is reported to be CIP, its de-ethylated product. SAR (principal metabolite of DIF) is different only by methyl group in the 7-(4-piperazinyl) position. Several FQs, e.g. ENR, DAN, SAR, DIF and MAR, have been approved for veterinarian application in several nations[(2)]. Other FQs, including CIP, NOR, ofloxacin (OFL), pefloxacin (PEF) and LOM, are restricted to human treatment but have potential interest for veterinary medicine. These drugs damage bacterial DNA and lead to defects in negative supercoiling by inhibiting bacterial DNA-gyrase and topoisomerase IV enzyme activity[(3)]. A great concern for public health is the emergence of antibiotics-resistance to foodborne pathogens. Because of the emergence of resistance of *Campylobacter* to QNs, the use of enrofloxacin for poultry was withdrawn by the U.S. Food and Drug Administration in 2005[(4)].

To protect human health, the European Union have established maximum residues limits (MRL) for residues of veterinary drugs in animal tissues. The 8 QNs (DAN, FLU, MAR, OXO, DIF, SAR and sum of ENR and CIP) have been included to set up MRL in the EU Council Regulation 2377/1990[(5)]. According to Taiwan's MRL standard[(6)], limits for residues of 4 QNs (OXO, FLU, SAR and DAN) were included in the regulation. There are 11 QNs (DAN, FLU, MAR, OXO, DIF, ENR, NOR, SAR, OFL), orbifloxacin (ORB) and nalidixic acid (NAL)) have been included in the Japan's MRL list[(7)]. Monitoring of these residues is necessary to ensure that human food is entirely free of potentially harmful residues. Thus, methods for multiresidue determination are required to screen samples and confirm the presence of any detected residues.

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Current methods for QNs analysis in food-producing animals are based on high performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence detection (FLD)[8-22] or mass spectrometric (MS) detection[23-27]. QNs are naturally fluorescent compounds due to the quinolone resonance structure. Good sensitivity was observed using FLD detection for most of the QNs. Also, low detection limits were obtained particularly when FLD detection was used[18, 21]. Many different HPLC conditions for the determination of QNs are described in the literatures, especially concerning the mobile phases of various ionic strength or acidity (citric acid and formic acid[10,11,14]), or ion pair reagents (tertiary amines[9], SDS[16,21]) addition to reduce peak tailing effect. In general, these methods usually employ liquid-liquid extraction (0.15 M hydrochloric acid[8], 5% trichloracetic acid[14], acidified ethanol[11,24], phosphate buffer (pH 7.4)[9,11], trichloracetic acid: acetonitrile[13,18,23], metaphosphoric acid: acetonitrile[20,21,26,27]), followed by clean up with solid-phase extraction (SPE). Various types of SPE involving reversed-phase (C-18[8,9,13,21], HLB[11,20,25], SDB[10,18], ENV+isolute[26,27]), cation-exchange (PRS[12]) and mixed cartridge with cation-exchange solvent (MPC[10], SDB-PRS[10]) have been used.

This study was aimed to develop a simple, rapid, and sensitive method for the simultaneous determination of 11 QNs, including MAR, CIP, DAN, NOR, ENR, LOM, SAR, DIF, OXO, FLU and NAL. QNs were extracted with solvent, followed by HLB cartridge for clean up, and then injected into the LC/FLD in a single run. Results from the analysis of 25 fish, 15 shrimp, and 5 Black Bone chicken (*Silkie bantams*) samples using the newly developed method are also presented.

MATERIALS AND METHODS

I. *Sample Collection*

Three Black Bone chicken samples were randomly purchased from supermarkets in Pingtung county. Two Black Bone chicken samples were collected from a farm in Pingtung county. Fish samples (n = 25) and shrimp samples (n = 15) were collected by government inspectors from the imported or exported in 2006. All samples were stored at -20°C until analyzed.

II. *Chemicals*

Norfloxacin (NOR, 100.0%), difloxacin (DIF, 100.0%), oxolinic acid (OXO, 100.0%), nalidixic acid (NAL, 100.0%), flumequine (FLU, 100.0%) and lomefloxacin (LOM, 100.0%) were purchased from Sigma Chemical Co. Marbofloxacin (MAR, 99.8%), ciprofloxacin (CIP, 100.0%), danofloxacin (DAN, 98.4%), sarafloxacin HCl (SAR, 99.7%) and difloxacin HCl (DIF, 98.4%) were purchased from RDH. Enrofloxacin (ENR, 98%) was purchased from Fluka. The chemical structures of all 11 quinolones are shown in Figure 1. Sodium hydroxide (NaOH) and metaphosphoric acid of ultra high grade were purchased from Nacalai Tesque Inc. Formic acid, methanol and acetonitrile of HPLC grade were purchased from J. T. Backer Co., Ltd. The OASIS HLB solid-phase extraction cartridge (3 mL, 60 mg) was from Waters Co. Water was purified with a Milli-Q system (Millipore, Bedford, MA). All other reagents were of analytical grade.

III. *Instrument and Apparatus*

The shaker (model KM) was purchased from IWAKI Co. and the centrifuge (model himac CF 16RX) was purchased from Hitachi Koki Co. The 12-sample nitrogen evaporator (model MG-2000) with a dry thermo bath was purchased from EYELY Co. A Hewlett Packard series 110 chromatographic system was employed, equipped with Q1312A Agilent fluorescence detector. Data acquisition was done using a Chemstation for LC 3D Res. A 08.03 (847) software (Agilent Technologies). The reverse phase analytical column employed in this study was a Symmetry C-18 (250 mm × 4.5 mm, 5μm) from Waters Co.

IV. *Preparation of Standard Solutions*

A 200 μg/mL stock solution was prepared for each of 11 QNs by dissolving the appropriate amount of standard in 50% acetonitrile containing 2% of 0.1 N NaOH. Working solutions were prepared by diluting the stock solutions to a serial of concentrations with 10% acetonitrile. Stock solutions and working solutions were stored at 4°C in brown volumetric flasks for at least 3 months without any degradation observed. Six working solutions ranging from 25 to 1000 ng/mL (6.25 to 250 for DAN) were prepared for external standard calibration.

V. *Sample Preparation*

Thawed tissue sample was ground first and 2.0 g of tissue was weighed and placed in a 50-mL polypropylene centrifuge tube. The working standard solution was then added to the sample. The sample was allowed to stand for 15 min at room temperature and spiked with a set of quinolone at different concentration ranging from 10 to 100 ng/g. Twenty milliliters of 0.3% metaphosphoric acid: acetonitrile (1:1, v/v) solution was added to the sample, which then was reversely shaking for 10 min, followed by centrifugation for 10 min at 6000 rpm. After the extraction step, acetonitrile was removed in a rotary evaporator and the extract was filtered through a filter paper (ADVANTEC 5A) to prevent SPE cartridge blocking. The filtrate was applied to a HLB cartridge which was previously activated with 3 mL of methanol and 3

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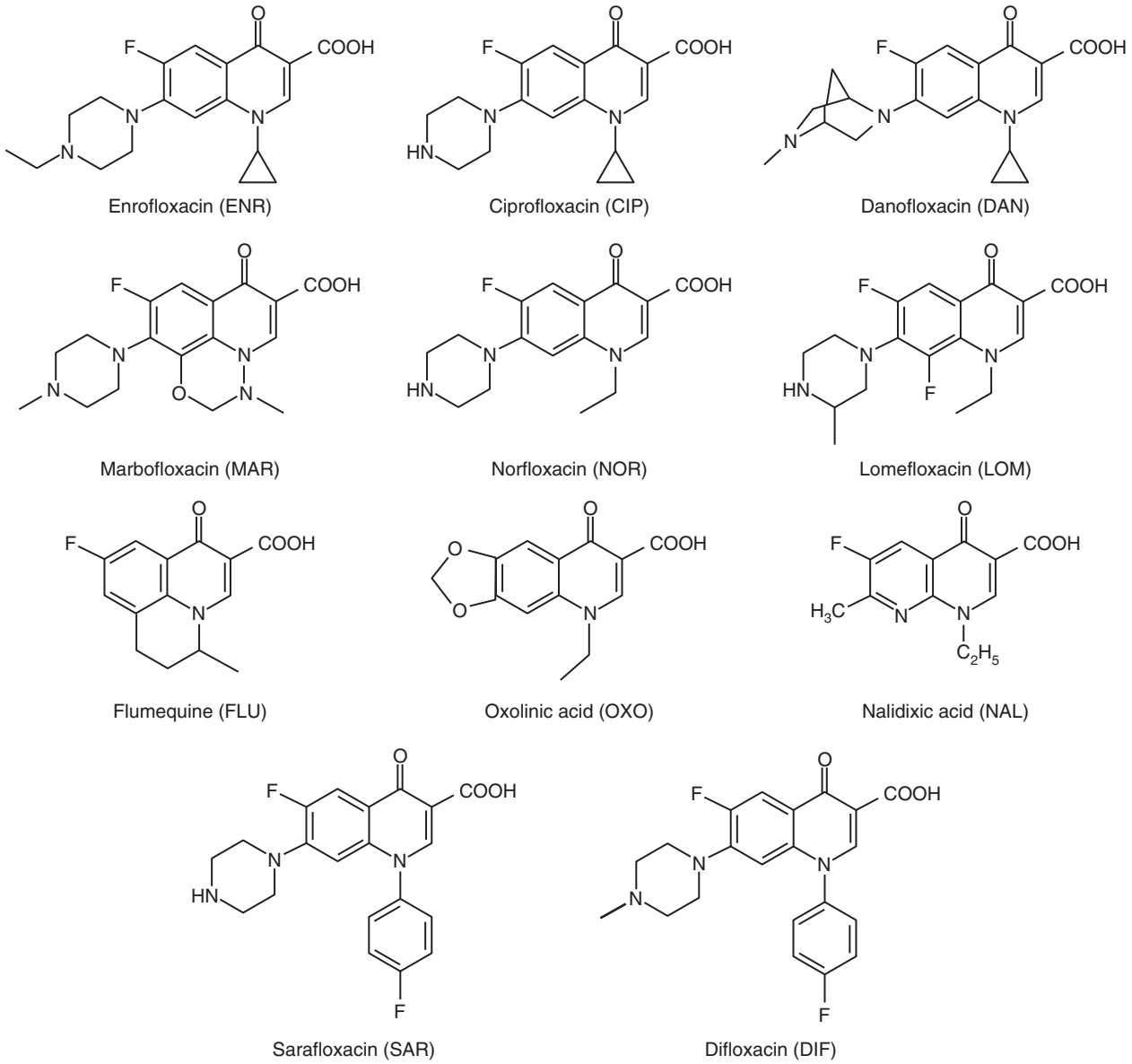

Figure 1. Chemical structures of 11 quinolones.

mL of water. After loading, the cartridge was washed with 3 mL of water and eluted with 4 mL of methanol. The eluent was evaporated to dryness under nitrogen gas at 40°C and resuspended in 1 mL 10% acetonitrile. The sample was filtered through 0.45 μm PVDF membrane before injection to HPLC system.

VI. *HPLC Analysis*

(I) Analytical Condition

The reverse phase analysis column, Symmetry C-18 (250 mm × 4.5 mm i.d., 5 μm; Waters) was maintained at 35°C in a column oven. The mobile phase, consisting of solvent A (0.1% formic acid, pH 2.5) and solvent B (acetonitrile), was run at a flow rate of 1.0 mL/min. The gradient program was as follows: 10% B for 11 min, elevated to 35% B at 17 min, to 45% B at 18 min and held at 45% B until 25 min, elevated to 90% B at 26 min and held at 90% B until 27 min, followed by a return to 10% B at 28 min. All of the analytes were eluted within 28 min, and a 10 min post run allowed for reequilibration of the column. The injection volume was 50 μL. The fluorescence excitation/emission wavelengths were programmed at 297 nm/515 nm for MAR from 0.0 to 5.2 min, at 280 nm/450 nm for NOR, CIP, LOM, DAN, ENR, SAR and DIF from 5.2 to 19.9 min, and at 320 nm/365 nm for OXO, NAL and FLU from 20 to 28 min.

(II) *Calculation of Estimated Concentration*

Amount of each quinolone in sample (ng/g) =

$(C \times V)/ W \times R$

C: concentration of each quinolone calculated from each standard curve (ng/mL)
V: volume of sample (mL)
W: weight of sample (g)
R: percentage of recovery

(III) *Recovery Test*

Each 2 g of homogenized blank samples was spiked to make final amounts equivalent to 25, 50 and 250 ng/g (6.25, 12.5 and 62.5 ng/g for DAN). Recovery tests were conducted for 4 replicates, and the percentage of additive recovery was calculated.

(IV) *Limits of Detection*

Seven replicate measurements were conducted at low concentration (15 ng/g) of mixed QNs. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as 3 times and 10 times of SD, respectively, according to the methods described by Verdon *et al*.[14].

Table 1. Detection of 14 quinolones with UV and FLD methods

Quinolone	UV nm	FLD (Ex/Em) nm
MAR	290	297 / 515
NOR	280	280 / 450
OFL	280	280 / 450
CIP	280	280 / 450
LOM	280	280 / 450
DAN	280	280 / 450
ENR	280	280 / 450
ORB	280	280 / 450
SAR	280	280 / 450
DIF	280	280 / 450
OXO	255	320 / 365
NAL	255	320 / 365
FLU	255	320 / 365
PIR	280	280 / 450

RESULTS AND DISCUSSION

I. *Optimisation of the HPLC Conditions*

The most common detection methods for analyzing QNs by HPLC are with UV and FLD detector. Recent reports described the simultaneous determination of QNs in biological matrices by FLD detection[9,11,13,14,18,21]. Fourteen QNs (MAR, NOR, OFL, CIP, LOM, DAN, ENR, ORB, SAR, DIF, OXO, NAL, FLU and piromidic acid (PIR)) were analyzed with diarray detector (DAD) and FLD methods in this study. Good sensitivity was obtained using FLD detection for all the QNs, except for PIR that was better monitored by its absorbance at 280 nm. After spectra scanning on 14 QNs with DAD and FLD spectrophotometer, the maximum absorption wavelength of UV as well as the excitation (Ex) and emission (Em) wavelength are shown in Table 1. The FLD responses varied considerably among the QNs. The results indicated that the relative sensitivity fluorescence detection of DAN and PIR to that of OXO were 40 folds and one tenth, respectively. Therefore, it's not feasible to use FLD for PIR measurement.

Nowadays, the trend of the application is to omit the ion-pairing reagent in the mobile phase. The mobile phase consisting of 0.1% formic acid and acetonitrile mixed with a gradient mode[10,11,14] is simple and convenient, so we select 0.1% formic acid-acetonitrile as the mobile phase in this study. Three columns, Cosmosil 5C-18-AR (Nacalai Tesque), Luna 5 μ C-18 (Phenomenex), and Symmetry column C-18 (Waters) were also compared in our study. The best separation performance was achieved by using Symmetry column C-18 column for the separation of polar analytes. This column allows separation with good resolution and peak shapes of all analytes investigated with a single LC run. Ten FQs have very similar structures and similar retention time. We noticed that OFL and ORB can be detected using Ex 280 nm/Em 450 nm, however, it's hard to completely separate OFL and NOR or ORB and ENR due to close retention time under specified conditions.

Eight FQs (MAR, NOR, CIP, LOM, DAN, ENR, SAR and DIF,) and 3 QNs (NAL, FLU and OXO) were incorporated into this method. To detect the 11 QNs in a single run, the experiment was set to time program in 3 steps, Ex 297 nm/Em 515 nm for MAR, Ex 280 nm/Em 450 nm for the other FQs (NOR, CIP, LOM, DAN, ENR, SAR and DIF), and Ex 320 nm/Em 365 nm for OXO, NAL and FLU. The optimized HPLC conditions are described in the method. The chromatogram of the mixed standard solution of 11 QNs shows a satisfactory separation with good resolution and peak shapes for 11 QNs (Figure 2).

II. *Sample Preparation*

Various methods for the determination of QNs in biological matrix have been reported[8-27]. These methods usually employed in organic or aqueous-organic extractions followed by solid phase extraction as a purification step. Recoveries were calculated by comparison with standards prepared in blank fish flesh spiked with 50 ng/g (except DAN at 12.5 ng/g) QNs. We have compared three extract solvents, including 0.3% metaphosphoric acid: acetonitrile (1:1, v/v), 0.3% metaphosphoric acid,

and 0.1 M phosphoric acid buffer (pH 7.0). Following protein precipitation, the QNs were extracted by SPE with HLB cartridge. As shown in Table 3, precipitation with 0.3% metaphosphoric acid: acetonitrile (1:1, v/v) led to significantly higher recovery. This results could be attributed by the addition of metaphosphoric acid with acetonitrile which has the best capability of extracting and removing protein. We found that the recovery of 2 g sample is better than that of 5 g sample, since the capacity of HLB (60 mg) in 5 g sample is overloaded.

III. *Method Validation*

The mixed 11 QNs were prepared as concentrations of 25, 50, 100, 250, 500 and 1000 ng/mL (except 6.25, 12.5, 25, 62.5, 125 and 250 ng/mL for DAN). The peak areas were plotted against concentration of each QNs calibration curves. The linear regression equations of each QNs are given in Table 2. The squares of correlation coefficients (R^2) for the 11 QNs were all above 0.996, indicating a good the linear correlation.

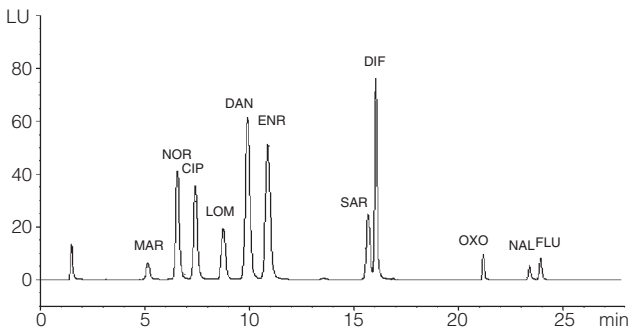

Figure 2. HPLC chromatogram of a mixture of 11 QNs (concentration level 500 ng/mL except DAN at 125 ng/mL, injection volume 50 μL). Chromatographic conditions: see HPLC analysis section.

Swine muscle, chicken muscle, fish flesh and shrimp were spiked with 11 QNs at 25, 50 and 250 ng/g (6.25, 12.5 and 62.5 ng/g for DAN). Good recoveries were obtained for each at all fortification levels as shown in Table 4. The mean recoveries were between 71.7 - 100.3% in swine muscle with relative standard deviations (RSD) of 1.5 - 4.2%. In chicken tissue, recoveries were 74.2 - 105.0% for each analyte with RSD of 1.8 - 4.5%. The recoveries of the 11 QNs in fish flesh ranged from 73.5 to 105.3% with RSD 2.5 - 4.2%. The recoveries of the 11 QNs in shrimp tissue ranged from 76.5 to 105.1% with RSD 1.8 - 4.2%.

Table 2. Comparison of recoveries (%) using 3 different extraction solution from fortified 50 ng/g quinolones (except DAN at 12.5 ng/g) in fish flesh samples (n = 3)

Quinolone	A Recovery(%)	B Recovery(%)	C Recovery(%)
MAR	98.2	90.3	80.1
NOR	92.5	89.1	78.5
CIP	86.1	74.2	70.2
LOM	87.3	80.1	75.3
DAN	98.0	85.2	81.2
ENR	93.7	81.3	76.5
SAR	81.6	78.2	68.2
DIF	79.5	71.5	65.3
OXO	89.5	72.0	60.1
NAL	88.6	70.1	62.3
FLU	91.5	65.3	61.8

A: 0.3% metaphosphoric acid : acetontrite (1:1, v/v)
B: 0.3% metaphosphoric acid
C: 0.1M phosphoric acid buffer (pH 7.0)

Table 3. Linear regression equations and correlation coefficients of calibration curves for 11 quinolones

Quinolone	retention time (min)	concentration range (ng/mL)	calibration equation	correlation coefficient
MAR	5.18	25-1000	Y=0.1151 X – 0.4564	0.9998
NOR	6.63	25-1000	Y=1.3523 X – 21.334	0.9979
CIP	7.51	25-1000	Y=1.1778 X – 18.102	0.9982
LOM	8.88	25-1000	Y=0.7071 X – 10.042	0.9984
DAN	10.07	6.25-250	Y=9.5647X – 45.425	0.9968
ENR	11.10	25-1000	Y=2.1165X – 38.09	0.9975
SAR	15.83	25-1000	Y=0.7549X – 9.1805	0.9989
DIF	16.14	25-1000	Y=1.5588X – 17.853	0.9984
OXO	21.19	25-1000	Y=0.2009 X – 2.7096	0.9984
NAL	23.39	25-1000	Y=0.1288X – 1.979	0.9984
FLU	23.92	25-1000	Y=0.2598X – 3.9551	0.9982

Table 4. Recoveries of 11 quinolones in fortified tissue samples

Tissue	Quinolone	Recovery (%)		
		25 ng/g	50 ng/g	250 ng/g
Swine muscle	MAR	84.2 (2.8)[a]	84.2 (2.8)	84.2 (2.8)
	NOR	98.3 (4.2)	98.6 (3.5)	100.3 (3.0)
	CIP	71.7 (3.2)	73.2 (3.0)	78.7 (2.8)
	LOM	77.8 (2.5)	78.9 (2.0)	80.2 (1.5)
	DAN[b]	81.0 (3.8)	81.5 (3.2)	83.1 (2.8)
	ENR	79.7 (3.6)	80.1 (3.1)	82.6 (2.6)
	SAR	74.0 (3.0)	75.6 (3.0)	78.3 (3.0)
	DIF	79.8 (3.5)	80.3 (3.4)	82.6 (3.0)
	OXO	77.2 (2.7)	77.6 (2.8)	78.4 (2.3)
	NAL	81.6 (3.5)	82.3 (3.0)	81.6 (2.6)
	FLU	81.2 (3.6)	81.0 (3.3)	82.3 (3.1)
Chicken muscle	MAR	91.6 (3.2)	90.8 (3.2)	92.3 (3.2)
	NOR	105.0 (3.0)	103.5 (2.8)	104.1 (2.0)
	CIP	74.2 (3.7)	76.1 (3.1)	76.8 (3.0)
	LOM	84.4 (3.1)	83.7 (3.1)	86.3 (2.8)
	DAN[b]	86.4 (3.4)	86.2 (2.8)	88.5 (1.8)
	ENR	85.3 (3.1)	88.1 (3.0)	88.5 (2.7)
	SAR	75.2 (3.5)	78.1 (2.9)	79.2 (2.1)
	DIF	82.4 (2.6)	83.6 (2.2)	85.1 (2.3)
	OXO	84.4 (4.5)	82.8 (3.6)	85.1 (3.2)
	NAL	92.3(4.1)	90.5 (3.8)	91.2 (3.5)
	FLU	80.9(3.2)	81.5 (3.3)	82.0 (3.0)
Fish flesh	MAR	92.2 (4.1)	90.6 (3.8)	93.8 (3.2)
	NOR	105.3 (4.2)	103.6 (3.8)	105.1 (3.9)
	CIP	73.5 (3.4)	76.3 (3.0)	78.0 (3.1)
	LOM	84.6 (3.2)	85.4 (3.5)	85.1 (3.0)
	DAN[b]	84.9 (3.5)	86.2 (3.0)	87.9 (2.6)
	ENR	80.5 (3.8)	82.5 (3.5)	83.5 (3.0)
	SAR	76.2 (2.8)	76.8 (2.6)	78.2 (2.5)
	DIF	87.2 (3.5)	86.2 (3.3)	88.3 (3.0)
	OXO	87.3 (3.8)	88.3 (3.0)	89.3 (2.8)
	NAL	89.1 (3.6)	89.5 (3.5)	90.1 (3.0)
	FLU	84.8 (3.1)	85.2 (3.2)	89.3 (2.8)
Shrimp	MAR	85.8 (2.1)	87.3 (2.3)	89.9 (2.0)
	NOR	102.3 (3.2)	104.2 (3.0)	105.1 (2.6)
	CIP	76.5 (1.8)	77.3 (2.0)	79.3 (2.1)
	LOM	83.8 (3.8)	85.0 (3.2)	88.6 (2.8)
	DAN[b]	87.9 (3.2)	88.3 (2.6)	89.6 (2.1)
	ENR	85.5 (4.1)	85.6 (3.8)	86.8 (3.2)
	SAR	79.8 (3.4)	81.2 (3.0)	81.0 (3.1)
	DIF	88.5 (3.0)	89.2 (2.8)	87.6 (3.1)
	OXO	87.5 (3.8)	85.4 (3.2)	86.9 (2.8)
	NAL	83.2 (4.0)	84.8 (3.6)	89.6 (3.1)
	FLU	83.5 (4.2)	85.4 (3.8)	90.1 (3.2)

a: Relative standard deviation (n = 4)
b: DAN spiked levels were 6.25, 12.5 and 62.5 ng/g

The precision of the method was assessed using fish muscle spiked with QNs at 25, 50 and 250 ng/g on the same day (intra-day) and three different days (inter-day). Results (Table 5) showed that the mean RSDs of intra-day were less than 5% and the mean RSDs of inter-day were less than 10%, which suggested the precision of this method is acceptable. Repeatability and reproducibility coefficients lower than 15% in accordance with FDA Guidance for Bioanalytical Validation were obtained[28]. As shown in Table 6, the LODs and LOQs were estimated

Table 5. Validation data for precision of quinolones in fortified fish tissue

Quinolone	Fortified level (ng/g)	Recovery (%)	RSD[a] (%)	
			Intra-day	Inter-day
MAR	25	92.0	4.0	5.2
	50	91.1	3.8	4.8
	250	92.8	3.5	5.6
NOR	25	102.8	4.2	6.1
	50	103.0	3.6	5.8
	250	105.2	3.8	5.6
CIP	25	74.0	3.5	8.9
	50	76.0	3.2	7.5
	250	78.3	3.8	7.2
LOM	25	84.5	3.5	5.8
	50	85.8	3.0	6.0
	250	86.6	3.0	5.5
DAN	6.25	85.0	3.2	5.8
	12.5	86.6	3.0	5.0
	62.5	88.2	2.5	5.1
ENR	25	81.0	3.8	6.1
	50	82.0	3.5	5.2
	250	83.5	3.0	4.8
SAR	25	76.5	3.0	8.2
	50	77.2	2.8	7.6
	250	78.5	2.5	7.5
DIF	25	87.5	3.5	7.0
	50	86.2	3.0	6.5
	250	88.5	3.2	5.8
OXO	25	87.5	3.8	5.6
	50	88.2	3.2	5.4
	250	89.0	3.0	5.4
NAL	25	89.2	3.8	7.5
	50	89.6	3.5	6.2
	250	90.5	3.2	6.0
FLU	25	84.6	3.0	6.5
	50	85.2	3.5	5.8
	250	89.6	3.1	5.6

[a] Relative standard deviation (intra-day, n = 4; inter-day, n = 12)

Table 6. LOD and LOQ of 11 quinolones in fortified tissue samples (n = 7)

Quinolone	Chicken muscle		Fish flesh	
	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)
MAR	3.9	13.1	3.6	12.1
NOR	7.5	25.0	6.9	23.0
CIP	5.4	18.0	5.1	17.0
LOM	3.6	12.0	4.2	14.0
DAN	1.5	5.0	1.8	6.0
ENR	4.2	14.0	3.6	12.1
SAR	6.2	21.0	6.0	20.0
DIF	7.5	25.0	7.2	24.0
OXO	7.5	25.0	6.5	25.0
NAL	8.7	27.0	8.4	28.0
FLU	6.9	23.0	7.2	24.0

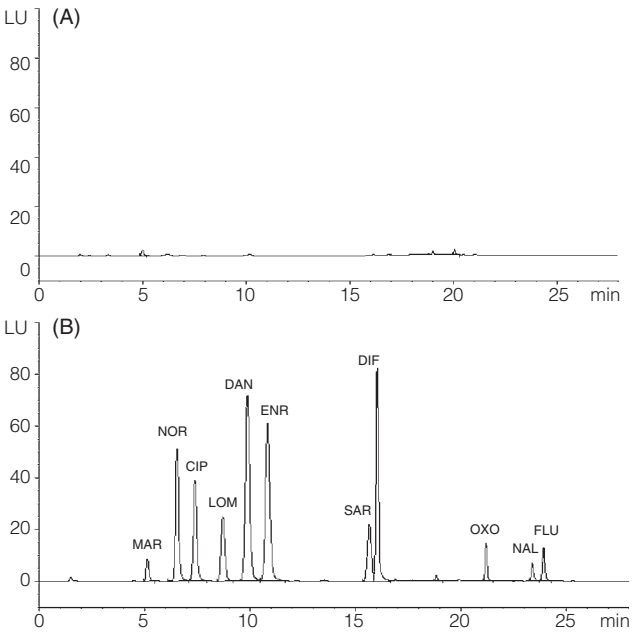

Figure 3. HPLC chromatograms of (A) blank chicken muscle (B) spiked chicken muscle at 250 ng/g (except DAN at 62.5 ng/g).

for each of the 11 QNs from 1.5 to 8.7 ng/g and from 5.0 to 28.0 ng/g, respectively. Similar LODs (4 to 11 ng/g) and LOQs (13 to 36 ng/g) for 10 QNs in different matrix were reported by Verdon *et al.*[14].

The chromatograms corresponding to the extracts of blank samples from different animal tissues (chicken and fish flesh) revealed no peak interfering with the QNs (Figure 3 and Figure 4). In addition, the specificity of the native FLD led to no interference with other major veterinary antibacterial, when macrolides, penicillins, tetracyclines and sulfonamides were tested by the same method.

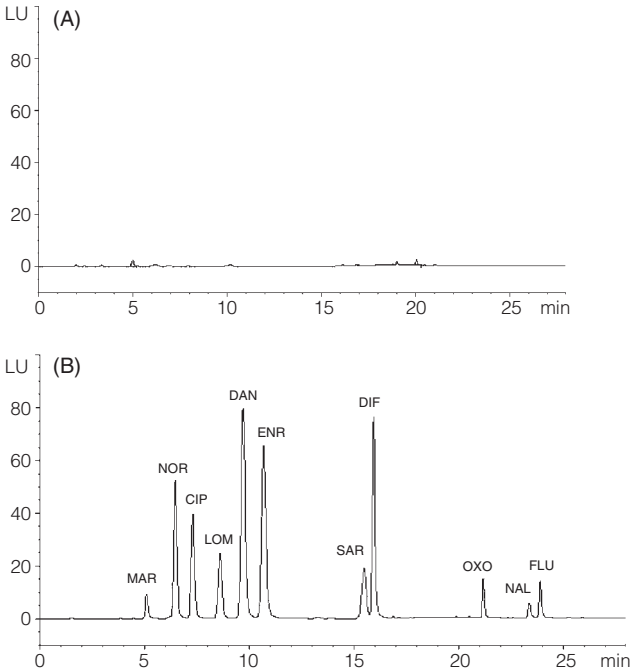

Figure 4. HPLC chromatograms of (A) blank fish muscle (B) spiked fish muscle at 250 ng/g except DAN at 62.5 ng/g.

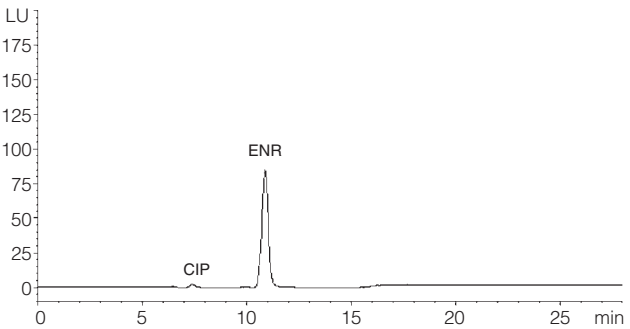

Figure 5. HPLC chromatogram of shrimp (*Paneus vannamei*) sample with CIP (21 ng/g) and NR (368 ng/g) residue.

IV. *Determination of QNs Residues in Market Sample*

QNs residues in 45 samples of Black Bone chicken, fish and shrimp, were examined by this method, and it turned out that ENR and CIP (the major metabolite of ENR) residues were detected in 2 Black Bone chicken tissue and 1 shrimp (*Penaeus vannamei*) sample. HPLC chromatograms of Black Bone chicken tissue and shrimp (*P. vannamei*) with positive residual ENR and CIP are shown in Figure 5 and Figure 6. In the present work, all positive samples were analyzed for duplication and the LC-MS/MS technique was used to the unambiguous identification of ENR and CIP for the 3 positive samples. For the confirmation of ENR and CIP by LC-MS/MS, the precursor-product ions combination for ENR were 360 → 316, 342 m/z and for CIP 332 → 314, 288 m/z. Figure 7 showed the LC-MS/MS chromatograms of Black Bone chicken sample. The amounts of ENR and CIP residues

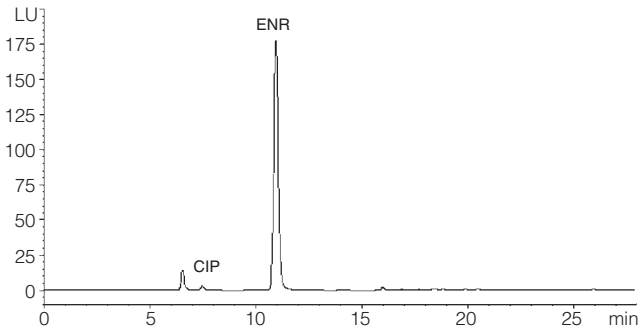

Figure 6. HPLC chromatogram of Black Bone chicken muscle sample with CIP (16 ng/g) and ENR (428 ng/g) residue.

in various Black Bone chicken tissues (serum, muscle, fat, skin, liver, kidney and lung) were listed in Table 7. Black Bone chicken lung contained the highest amount of ENR (1900 ng/g), followed by skin (1210 ng/g), then muscle (428 ng/g). Besides, CIP can not be detected in fat tissue, and up to 11-30 ng/g in the rest of tissues. ENR levels in Black Bone chicken tissues (muscle, skin and liver) were similar to the result by Su *et al.*[21].

CONCLUSIONS

The analytical method developed in this work for

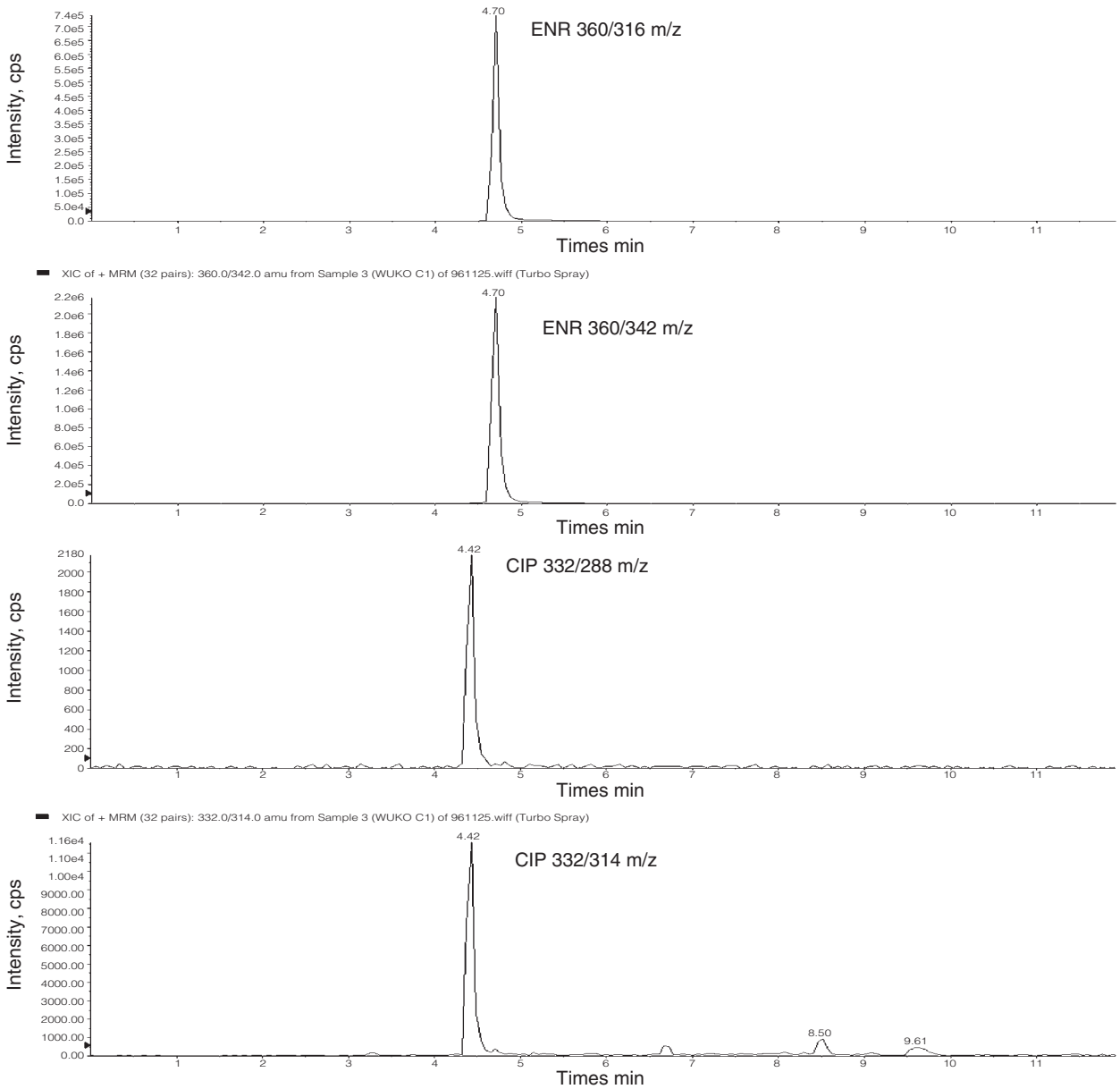

Figure 7. LC-MS/MS chromatograms of Black Bone chicken muscle. Precursor-product ions combination for ENR were 360 → 316, 342 m/z and for CIP were 332 → 314, 288 m/z. The LC-MS/MS was operated in positive electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode. The HPLC separation was carried out on a Zorbax SB C-18 column (5 μm, 150 mm × 4.6 mm) with gradient elution of 20 mM ammonium formate and acetonitrile (95:5, v/v) as mobile phase. The flow-rate of the mobile phase was maintained at 700 μL/min.

Table 7. ENR and CIP residues in Black Bone chicken tissue (n = 2)

Tissue	ENR (ng/g)	CIP (ng/g)
Serum	21	12
Muscle	428	16
Fat	220	ND
Skin	1210	17
Liver	70	11
Kidney	290	13
Lung	1900	30

ND: not detected.

the simultaneous determination of 11 QNs from porcine, chicken, fish and shrimp samples is fast, specific, accurate, precise, reproducible and sensitive. The method consists of extraction with 0.3% metaphosphoric acid: acetonitrile (1:1, v/v), cleaning up on HLB SPE cartridge, separation of 11 QNs by gradient elution of mobile phase, and detection with fluorescence by means of a wavelength program. Overall, the method is simple to apply to a routine analysis and particularly suitable for screening QNs residues in marine and livestock products.

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