Journal of Food and Drug Analysis, Vol. 18, No. 2, 2010, Pages 87-97 藥物食品分析 第十八卷 第二期

Simultaneous Determination of 18 Quinolone Residues in Marine and Livestock Products by Liquid Chromatography/ Tandem Mass Spectrometry

CHUI-SHIANG CHANG[1,2], WEI-HSIEN WANG[1,3] AND CHIN-EN TSAI[4*]

[1.] Asia-Pacific Ocean Research Center, Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan, R.O.C.

[2.] Kaohsiung Branch, Bureau of Standards, Metrology and Inspection, MOEA, Kaohsiung, Taiwan, R.O.C.

[3.] National Museum of Marine Biology and Aquarium, Pingtung, Taiwan, R.O.C.

[4.] Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan, R.O.C.

(Received: January 5, 2009; Accepted: January 19, 2010)

ABSTRACT

A simple and efficient LC/ESI/MS/MS method was developed to determine 18 (fluoro)quinolone (QNs) residues in milk, chicken, pork, fish and shrimp. This method is capable of screening and confirming the presence of 12 amphoteric QNs (marbofloxacin, norfloxacin, enrofloxacin, ciprofloxacin, desethylene ciprofloxacin, lomefloxacin, danofloxacin, sarfloxacin, difloxacin, ofloxacin, orbifloxacin and enoxacin) and 6 acidic QNs (oxolinic acid, nalidixic acid, flumequine, cinoxacin, piromidic acid and pipemidic acid). The drugs were extracted from matrix with acetonitrile containing 1% formic acid, diluted in 10% acetonitrile and defatted by extraction with hexane. The LC separation was conducted on an XDB C-8 (150 mm × 4.6 mm, 5 μm) column with gradient elution of 20 mM ammonium formate in 0.1% formic acid-acetonitrile as the mobile phase. Mass spectrum acquisition was completed in the positive ion mode by applying multiple reaction mode (MRM). The decision limit (CCα) and detection capability (CCβ), stated in the Decision No. 2002/657/EC and the ISO standard No.11843, have been calculated in the case of nonauthorized substance. The values of CCα ranged from 0.18 to 0.68 ng/g and CCβ ranged from 0.24 to 0.96 ng/g under specified conditions.

Key words: quinolones, residue, LC/MS/MS

INTRODUCTION

Quinolones (QNs) are broad-spectrum synthetic antimicrobial agents used in the treatment of bacterial infection of livestock and in aquaculture. They behave through inhibiting bacterial DNA-gyrase and topoisomerase Ⅳ enzyme activities[(1)]. QNs can be grouped into acidic QNs and amphoteric QNs. Acidic QNs are represented by quinolones carboxylic acid, such as flumequine (FLU), oxolinic acid (OXO), nalidixic acid (NAL), cinoxacin (CIN), piromidic acid (PIR) and pipemidic acid (PIP). Amphoteric QNs (fluoroquinolones) 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), sarfloxacin (SAR), difloxacin (DIF), ofloxacin (OFL), enoxacin (ENO) and orbifloxacin (ORB). The major metabolites of ENR were reported to be CIP, and desethylene ciprofloxacin (des-CIP)[(2,3)]. SAR (principal metabolite of DIF) is different only by methyl group at 7-(4-piperazinyl) position[(2)]. The widespread administration of fluoroquinolones to calves[(4)], poultry and swine[(5)] leads to the occurrence of residues in food products. These residues are of great concern for public health in the emergence of antibiotics-resistance to foodborne pathogens[(6-8)]. Delsol[(6)] indicated that a single course of enrofloxacin treatment contributes directly to the emergence and persistence of fluoroquinolone resistance in *Campylobacter coli*. The resistance of bacteria to fluoroquinolones has increased significantly. Mutations in the genes that encode the topoisomerases (gyrA, gyrB, parC and parE) or in the genes that affect

* Author for correspondence. Tel: +886-8-7740-316; Fax: +886-8-7740-312; Email: chinen@mail.npust.edu.tw

cell permeability or drug export are the main causes of quinolone resistance[7]. To ensure that food consumers are not exposed to residues at potentially harmful concentrations, maximum residue limits (MRLs) for residues of veterinary drugs in food animal tissues and products have been established. The MRLs for QNs among Taiwan[9], Japan[10] and the European Union[3,11] are set, as shown in Table 1. Monitoring of QNs residues in animal products is very important to ensure that human food is entirely free of potentially harmful residues.

Many methods have been established for determining QNs in food-producing animals. Typically, these methods employed high-performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence (FLD)[12-25] detection or mass spectrometric detection[26-37]. Although most of these methods can achieve the quantification of QNs at very low concentration levels, they are usually restricted to the numbers of QNs [maximum eleven[17,26,33]] determined simultaneously. This is often due to the co-elution properties for compounds belonging to the same chemical family and also due to pKa differences between the acidic and the amphoteric QNs[33]. Strict guidelines in European Union (EU)[38] for confirmatory techniques state that liquid chromatography/tandem mass spectrometry (LC/MS/MS) monitoring two or three transition-product ions gives sufficient data to confirm the identity of a residue. Other methods, such as LC with FLD or UV detection, must combined with another technique to meet the criteria. LC/MS/MS was the technique of choice, due to its high specificity and sensitivity, monitoring two transition-product ions gives sufficient data to confirm the identify of a substance found in the complex matrix. Consequently, a confirmatory method using LC/MS/MS would supplement the existing screening methods.

The study was aimed to develop a multi-residue method for screening and confirming a group of QNs in various food matrices by triple quadrupole mass spectrometry after a simple liquid-liquid extraction. Method validation was performed for each matrix and validation parameters including selectivity, accuracy, matrix effect, decision limit (CCα) and detection capability (CCβ) were discussed.

MATERIALS AND METHODS

I. *Chemicals*

Norfloxacin (NOR, 100.0%), oxolinic acid (OXO, 99.9%), nalidixic acid (NAL, 99.5%), flumequine (FLU, 99.9%) and lomefloxacin (LOM, 99.1%) were purchased from Sigma (Steinheim, Germany). Marbofloxacin (MAR, 99.8%), ciprofloxacin (CIP, 100.0%), danofloxacin (DAN, 98.4%), sarafloxacin HCl (SAR, 99.7%) and difloxacin HCl (DIF, 98.4%) were from Riedel-de Haen (Seelze, Germany) Enrofloxacin (ENR, 98%) was from Fluka. Desethylene ciprofloxacin HCl (des-CIP, 98%) was from Toronto Research (North York, Canada). The chemical structures of all 18 QNs are shown in Figure 1. Sodium hydroxide and ultra pure sodium sulfate anhydrate were from Nacalai Tesque (Kyoto, Japan). Formic acid and acetonitrile were of HPLC grade and purchased from J.T.Baker (Deventer, Holland). All other reagents were of analytical grade.

Table 1. Comparison of MRLs (ppm) for quinolones in Taiwan[9], Japan[10] and the EU[11]

Quinolone	Species	Taiwan	Japan	EU
NAL	Bovine	–	0.03	–
FLU	Fish	0.05	0.5	0.15
	Bovine	0.05	0.5	0.1
	Porcine	0.05	0.5	0.1
	Sheep	0.05	–	0.1
	Chicken	0.05	0.5	0.1
OXO	Bovine	–	1.0	0.1
	Porcine	–	1.0	0.1
	Chicken	–	1.0	0.1
	Fish	0.05	–	0.3
NOR	Bovine	–	0.02	–
	Chicken	–	0.02	–
DAN	Bovine	0.2	0.2	0.2
	Chicken	0.2	0.2	0.2
	Porcine	0.1	0.1	–
	Milk	–	0.05	–
The sum of ENR and CIP	Bovine	–	0.05	0.1
	Chicken	–	0.05	0.1
	Porcine	–	0.05	0.1
	Milk	–	0.05	0.4
OFL	Chicken	–	0.05	–
MAR	Bovine	–	0.2	0.15
	Porcine	–	0.2	0.15
	Milk	–	0.08	0.075
SAR	Chicken	0.01	0.01	0.01
	Turkey	0.01	0.01	0.01
	Salmon	–	0.03	0.03
ORB	Bovine	–	0.02	–
	Chicken	–	0.02	–
	Porcine	–	0.02	–
	Milk	–	0.02	–
DIF	Bovine	–	0.4	0.4
	Chicken	–	0.3	0.3
	Porcine	–	0.02	0.4
	Fish	–	0.3	–

— : No MRL has been fixed.

Journal of Food and Drug Analysis, Vol. 18, No. 2, 2010

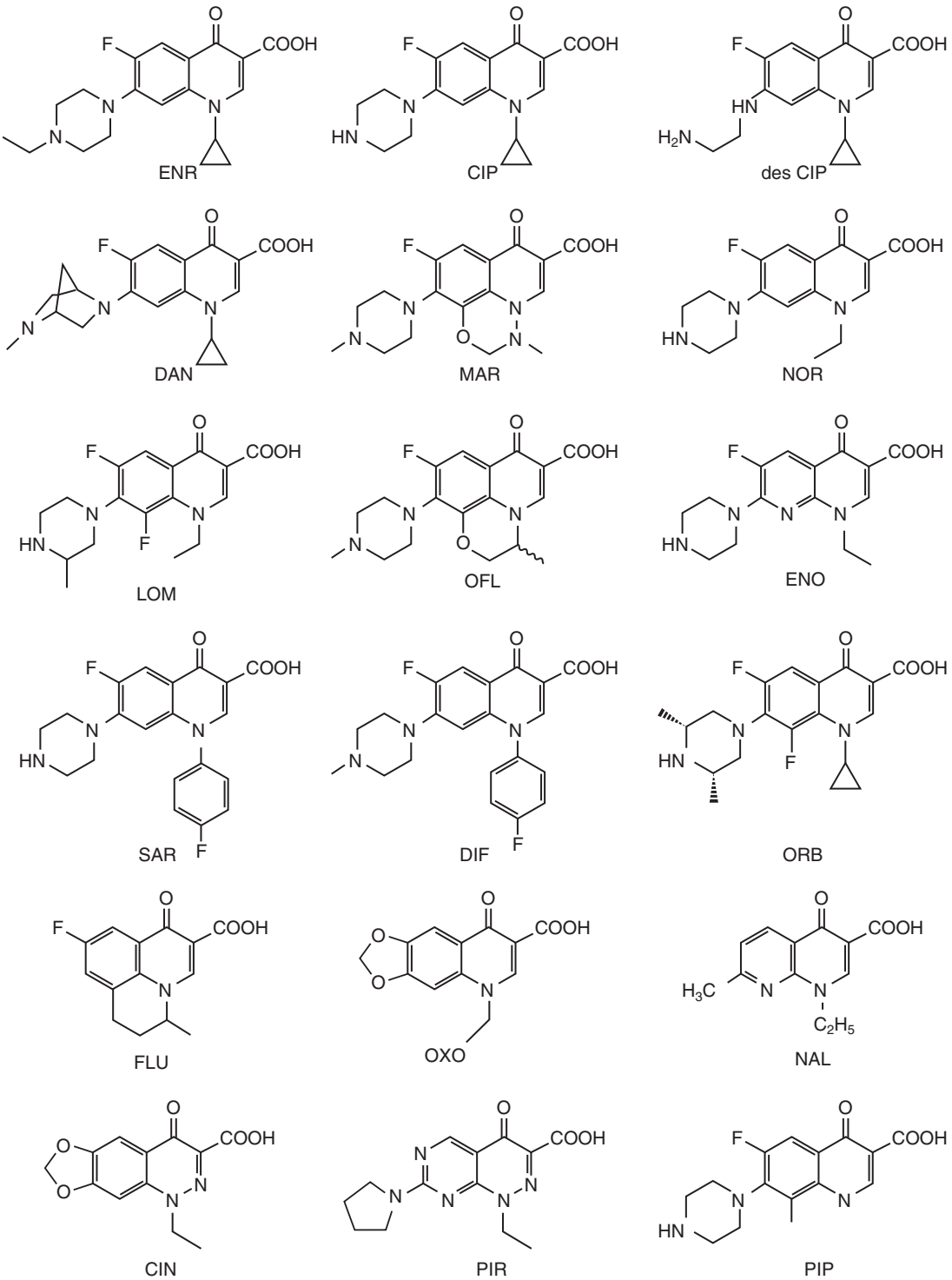

Figure 1. Chemical structures of 18 (fluoro)quinolones.

II. *Instrumentation and Conditions*

(I) *Instrumentation*

The instruments for sample preparation consisted of a shaker (model KM, IWAK, Japan), a centrifuge (model himac CF 16RX, Hitachi kok, Hitachinaka, Japan) and a rotary evaporator (EYELA, Japan). Water was purified in a Milli-Q system (Millpore, Bedford, USA).

The HPLC system was Agilent 1100 series (Agilent Technologies, Palo Alto, USA) consisted of a quaternary pump (G1311A), a vacuum degasser (G1322A) and an automatic sampler (G1313A). The separation of QNs was achieved on a Zorbax Eclipse XDB-C8 (150 mm ×

4.6 mm, 5 μm) analytical column (Agilent Technologies).

The triple-quadrupole API 4000 mass (Applied Biosystems/MDS Sciex, Foster, USA) was coupled to HPLC using an electrospray ionization interface in positive ionization mode (ESI+). Data acquisition was conducted using the Analyst 1.4 software (Applied Biosystems/MDS Sciex).

(Π) *LC and MS/MS Conditions*

The injection volume was 20 μL and the analysis was carried out with gradient elution using A eluent (20 mM ammonium formate in 0.1% formic acid) and B eluent (acetonitrile) as the mobile phase at a flow rate 0.7 mL/min. The program of gradient elution is listed in Table 2.

In order to establish the optimized Multiple reactions monitoring (MRM) conditions for individual compounds, the mass spectrometric conditions were optimized using infusion with a syringe pump and direct injection of each QNs individually at a concentration of 0.5 μg/mL and at flow-rate of 0.01 mL/min to select the most suitable ion transitions.

Due to the presence of the amino group in most QNs that is easily protonated in acidic medium, the turbo ion spray source was set in positive mode. The ESI/MS/MS conditions were set as ionspray voltage (IS) 5500 V and collision gas (CAD) 7 (arbitrary units). The nebulizer gas (synthetic air) and the curtain gas (nitrogen) were set at 45 and 12 (arbitrary units), respectively. The heater gas temperature (TEM) was set at 500°C. MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of

Table 2. Timetable of gradient elution program

Time (min)	A (%)*	B (%)
0	85	15
7	30	70
8	5	95
9	5	95
10	85	15
12	85	15

*A: 20 mM ammonium format in 0.1% formic acid, B: acetonitrile.

Table 3. Optimized MS/MS for MRM transitions selected for quantification and identification of the quinolones

Quinolone	Retention time (min)	Precursor ion (*m/z*)	Quantification (Collision energy) *m/z* (V)	Identification (Collision energy) *m/z* (V)
NAL	6.83	233	233→215 (22)	233→187 (36)
FLU	7.27	262	262→244 (25)	262→202 (46)
OXO	6.13	262	262→244 (24)	262→216 (42)
CIN	5.60	263	263→217 (31)	263→245 (22)
PIR	7.38	289	289→271 (26)	289→243 (41)
PIP	3.57	304	304→286 (26)	304→217 (31)
des-CIP	4.25	306	306→288 (24)	306→268 (36)
NOR	4.35	320	320→302 (32)	320→276 (24)
ENO	4.11	321	321→303 (28)	321→234 (31)
CIP	4.50	332	332→314 (27)	332→288 (24)
LOM	4.58	352	352→308 (24)	352→265 (32)
DAN	4.52	358	358→340 (31)	358→283 (25)
ENR	4.76	360	360→342 (29)	360→316 (29)
OFL	4.35	362	362→318 (28)	362→261 (36)
MAR	4.23	363	363→345 (22)	363→320 (28)
SAR	5.24	386	386→368 (31)	368→342 (27)
ORB	4.93	396	396→352 (25)	396→295 (32)
DIF	5.42	400	400→382 (30)	400→356 (28)

triple-quadrupole mass spectrometer. Mass was determined in the second analyzer of the instrument. MRM experiments were performed using a dwell time of 80 ms and the collision energy transition (depending on the compound) as shown in Table 3. Two transitions were used for the identification each QNs but only one was used for quantification.

III. *Preparation of Standard Solutions*

For the QNs with good solubility in alkaline solution[15], a 200 μg/mL stock standard solution was prepared for each of 18 QNs by dissolving the appropriate amount of standard in 50% acetonitrile containing 2% of 0.1 N NaOH. Working standard solutions were prepared by serial dilution of standard solutions with 10% acetonitrile. Stock and working standard solutions were stored at 4°C in brown volumetric flasks for at least 3 months without any degradation observed. Five working standard solutions ranging from 0.5 to 10 ng/mL were prepared for external standard calibration.

IV. *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 spiked into matrix samples to the desired concentrations (0.25, 0.5, 1.0, 2.5 and 5.0 ng/g). Then, the fortified sample was allowed to stand for 15 min at room temperature. Twenty milliliters acetonitrile containing 1% formic acid was added to the sample, which then was reversely shaking for 5 min at high setting. Two grams of sodium sulfate anhydrate was added to each tube, which then was reversely shaking again for another 5 min, followed by centrifugation for 10 min (6000 rpm, 4°C). The acetonitrile extract was evaporated in a rotary evaporator at 40°C. The residue was resuspended in 2 mL of 10% acetonitrile containing 0.1% formic acid, poured into a 16 × 12 mm glass tube and defatted by extraction with 4 mL hexane while mixing on a Vortex mixer in 15 sec twice. The mixture was centrifuged for 5 min (4000 rpm, 4°C) and the aqueous supernatant was transferred and filtered through 0.22 μm Nylon membrane before injection into LC/ESI/MS/MS system.

V. *Matrix Effect and Recovery*

The effect of matrix and recoveries of QNs were determined by comparing the responses obtained from standards injected in mobile phase (standard calibration curve, SCC), standards spiked into the fish homogenate before extraction (method matched calibration curve, MMCC), and standards spiked into the fish extracts followed by the extraction process (matrix calibration curves, MCC) as described by Durden *et al*[22]. Matrix effect can be estimated by dividing the MMCC slope by the SCC slope. The recovery can be estimated by dividing the MMCC slope by the MCC slope.

VI. *Decision Limit (CCα) and Detection Capability (CCβ)*

The new set of statistical performance limits recommended in the European Decision No. 657/2002/EC[38], i.e., the limit of decision CCα, and the capacity of detection CCβ, were calculated from the lower portions of standard curves using the ISO 11843[39] and assuming Case 1, constant standard deviation and linear response. CCα is the lowest concentration at which a method can discriminate, with a statistical certainty of 1-α (α = 1% in the case of banned compound). At CCα, sample contains target analyte with probability of 0.99. The detection capability (CCβ) is the concentration at which truly contaminated samples can be detected by the method with a statistical certainty of 1-β (β = 5% in the case of banned compounds). Negative matrix was fortified with analytes of 5 levels of concentration (0.25, 0.5, 1.0, 2.5 and 5.0 ng/g) and analyzed in 4 replicates at each level.

RESULTS AND DISCUSSION

I. *MS Detection*

Positive ESI-MS spectra gave intense signals characterized using prominent protonated molecule $[MH]^+$ as precursor ion and the more intense transition was used to quantify QNs. Twelve amphoteric QNs (MAR, NOR, ENR, CIP, des-CIP, LOM, DAN, SAR, DIF, OFL, ORB and ENO) and 6 acidic QNs (OXO, NAL, FLU, CIN, PIR and PIP) were incorporated into this project. QNs are detected in the multiple reaction monitoring (MRM) mode which provides a high level of selectivity for targeting the analytes in a complex biological matrix. These transitions were listed in Table 3. The transition $[MH]^+ \rightarrow [MH\text{-}H_2O]^+$ was used for quantification of NAL, FLU , OXO, CIN, PIR, PIP, des-CIP, NOR, ENO, CIP, DAN, ENR, SAR and DIF, while for quantification of LOM, OFL, MAR and ORB, the transition $[MH]^+ \rightarrow [M+H\text{-}CO_2]^+$ was employed. Other less intense ions were used for the confirmation of QNs. The collision energies for each transition were optimized to maximize the product ion intensity. The retention times of the QNs are shown in Table 3. Although the compounds were not fully separated, monitoring two transition-product ions gave sufficient data to confirm the identity of QNs found in the complex matrix. The use of the precursor ion plus two products ions gave four identification points, which are acceptable proof of confirmation in conjunction with the chromatographic retention times[37].

MS/MS modes verified structural information of the compounds present in the unconfirmed samples. As an illustrative example, the interpretation of the product ions of NOR is shown in Figure 2 where an interpretation

of the main fragment ions (*m/z* 276, 302, 256, 233 and 219) of NOR is proposed.

As observed in Table 3, the same precursor ion (*m/z* 262) is obtained from OXO and FLU. These substances only generated two product ions and one of these product ions (*m/z* 244) is common to OXO and FLU. However, the OXO and FLU can be identified by different retention time and the transition $[MH]^+ \rightarrow [MH\text{-}OCH_2O]^+$ (262 → *m/z* 216) for OXO and $[MH]^+ \rightarrow [MH\text{-}OC_3H_8]^+$ (*m/z* 262 → 202) for FLU.

Ion ratios were determined for each analyte from 4 replicate samples spiked with all the analytes at each of five levels (0.23-5.0 ng/g). Ion ratios were all within tolerance in the EU document 2002/657/EC[38]. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak. Ion ratios of QNs are shown in Table 4. We noticed a small change of ion ratio with concentration, parelled in the standard curves and spikes.

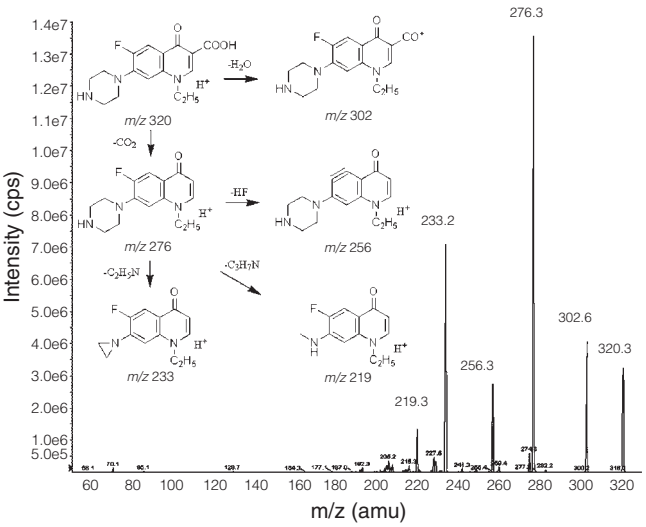

Figure 2. Product ion scan norfloxacin (*m/z* 320) mass spectrum at collision-induced dissociation in q2 at 30 V and proposed fragmentation pathway in norfloxacin.

Table 4. Ion ratios of two transition of 18 quinolones in standard and spiked samples

Quinolone	Ion ratios*						
	standard[a]	range[b]	Fish	Shrimp	Pork	Chicken	Milk
NAL	0.41	0.31-0.51	0.41-0.45	0.40-0.41	0.41-0.42	0.41-0.45	0.48-0.50
FLU	0.32	0.24-0.40	0.33-0.38	0.31-0.37	0.34-0.36	0.33-0.36	0.36-0.38
OXO	0.10	0.05-0.15	0.10-0.12	0.09-0.11	0.09-0.11	0.10-0.11	0.09-0.12
CIN	0.32	0.24-0.40	0.32-0.42	0.36-0.41	0.32-0.41	0.32-0.44	0.37-0.44
PIR	0.31	0.23-0.39	0.31-0.35	0.31-0.33	0.32-0.34	0.32-0.34	0.34-0.36
PIP	0.28	0.21-0.35	0.31-0.35	0.20-0.31	0.32-0.34	0.32-0.34	0.24-0.33
des-CIP	0.17	0.12-0.22	0.16-0.19	0.17-0.18	0.17-0.19	0.17-0.19	0.18-0.20
NOR	0.25	0.19-0.31	0.19-0.23	0.21-0.28	0.21-0.24	0.23-0.30	0.27-0.31
ENO	0.04	0.02-0.06	0.03-0.05	0.03-0.05	0.03-0.03	0.04-0.05	0.04-0.06
CIP	0.19	0.13-0.25	0.14-0.15	0.15-0.20	0.15-0.17	0.16-0.22	0.20-0.25
LOM	0.85	0.68-1.02	0.89-0.95	0.92-1.01	0.90-1.00	0.87-0.96	0.85-0.94
DAN	0.02	0.01-0.03	0.01-0.02	0.02	0.01-0.02	0.02	0.01-0.02
ENR	0.34	0.25-0.43	0.24-0.35	0.34-0.44	0.34-0.40	0.39-0.45	0.41-0.53
OFL	0.72	0.58-0.86	0.73-0.82	0.71-0.76	0.67-0.81	0.76-0.78	0.76-0.81
MAR	0.74	0.59-0.89	0.65-0.74	0.77-0.83	0.77-0.88	0.77-0.90	0.83-0.92
SAR	0.24	0.18-0.30	0.21-0.32	0.30-0.36	0.24-0.29	0.30-0.37	0.31-0.39
ORB	0.58	0.46-0.70	0.57-0.64	0.60-0.62	0.60-0.66	0.61-0.64	0.61-0.67
DIF	0.38	0.28-0.48	0.37-0.46	0.43-0.51	0.38-0.42	0.45-0.50	0.49-0.54

*Ion ratio: Identification ion peak area / Quantification ion peak area.

a: Ion ratios of standard solutions.

b: Maximum permitted tolerances according to Decision 2002/657/EC.

II. *Sample Preparation*

Analyses of residual QNs have been reported in many different matrices including fish[16,20,25,32,37], milk[17,24,26], egg[28,29] and animal tissues such as muscle[12,15,18,19,21,23], liver[18,23] and kidney[33-36]. Rather time-consuming combination of liquid-liquid extraction (LLE) and SPE procedures for the extraction and cleanup were commonly used. However, due to the high selectivity of the MRM mode of LC/MS/MS for the determination of QNs in complex biological matrix[23], the sample treatment step was adapted to the simple LLE procedure in this study. The method has been applied to five different matrices (milk, fish, shrimp, pork and chicken) with simple and rapid extraction LLE method. Because acetonitrile has the best capability of extracting and removing protein[26], QNs are initially extracted with acidified (added 1% formic acid) acetonitrile and then dehydrated by anhydrous sodium sulfate. Hatano[26] demonstrated that acetonitrile containing either metaphosphoric acid, acetic acid or formic acid to maintain weak acidic condition may improve the recovery for QNs. He found that the formic acid used in acetonitrile achieved the best recovery.

III. *Matrix Effect and Recovery*

Matrix effect for Nalidixic acid was shown in Figure 3. The SCC was obtained from the analytical values of the beginning and end of the samples set. The individual values were calculated and gave a slope of 1.0514 (solid line with diamond marks, Figure 3). The MCC was compared to this. It can be seen that the matrix, in this case, enhanced the response by about 30 %, as the slope is greater than 1. Finally, the MMCC (solid line with diamond triangle marks, Figure 3) shows slope of 1.0076. By dividing the MMCC slope by the MCC slope it is possible to estimate the recovery, which in this case is about 76.8%. The matrix effect and recoveries for the QNs are shown in Table 5. The matrix effect enhances the responses from a low of 19.9% to a high of 49.3%. The absolute recoveries for CIN and PIR were lower than 60%, whereas the recoveries of the other QNs (NAL, ENO, CIP, ENR, OFL, SAR and DIF) were higher than 80%.

When using ESI ionization, the presence of matrix components (salts, proteins, lipids, carbohydrates, sugars, *etc*.) that affect the ionization of the target analytes may pose a significant problem, by either reducing or enhancing analytes response[37]. This matrix effect can be minimized by an efficient clean-up step or different chromatographic conditions, but usually cannot be completely removed[40]. Khong *et al*.[40] observed that a strong matrix effect of the quantification of tetracyclines (TCs) in honey extracts. Calibration curves of tetracycline from the LC/ESI/MS/MS of standard solution and in spiked honey matrices from different geographical and flower origins, the matrix effect were quite different ranging from 163% (Swiss acacia) to 226% (Swiss multiflower). Samanidou *et al*.[37] accounted matrix effect of the quantification of QNs in seabream (fish). Relative responses ranged from 0.2 to 0.4 for DAN, ENR and FLU indicating signal suppression, 1.8 for CIP indicating signal enhancement, while no significant matrix effect was noticed for SAR, OXO and NAL.

Choice of internal standard (IS) can correct for matrix effects, recovery through the extraction, and any common loss. Toussaint *et al*.[33] used LOM as an internal standard for the quantitation of amphoteric QNs and cincophen for acidic QNs. Durden *et al*.[29] used

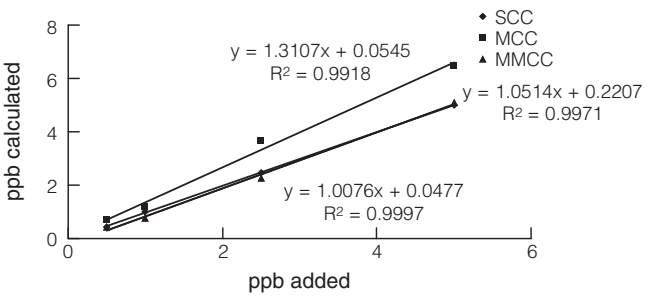

Figure 3. Matrix effect and absolute recovery in Tilapila fish for Nalidixic acid.

Table 5. Matrix effects and Absolute recoveries of 18 quinolones in Tilpia muscle (n = 4)

Quinolone	Matrix effect (%)	Absolute recovery (%)
NAL	124.7	95.8
FLU	81.2	67.0
OXO	89.0	72.9
CIN	92.0	57.3
PIR	91.1	51.5
PIP	107.0	61.2
des-CIP	82.3	66.5
NOR	88.5	76.6
ENO	149.3	95.1
CIP	94.9	80.1
LOM	83.6	68.7
DAN	87.8	82.3
ENR	97.8	88.8
OFL	95.1	84.7
MAR	119.1	64.3
SAR	97.2	89.2
ORB	80.1	65.1
DIF	94.8	91.2

NOR as the IS for CIP and the better IS for DAN, ENR, and SAR was LOM. Dufresne *et al.*[28] used LOM and PIP as the IS. Hermo *et al.*[30] chose NOR as the IS. It's difficult for us to select adequate internal standards for all QNs, therefore, an external mode without addition of internal standards was employed in the current study. Quantification by matrix calibration curves (MCC) mode is strongly recommended when sample was detected certain levels of QNs.

Table 6. The different characteristics in CCα and CCβ for 18 quinolones in the fish, shrimp, pork, chicken and milk

Quinolone	Fish		Shrimp		Pork		Chicken		Milk	
	CCα (ng/g)	CCβ (ng/g)	CCα (ng/g)	CCβ (ng/g)	CCα (ng/g)	CCβ (ng/g)	CCα (ng/g)	CCβ (ng/g)	CCα (ng/g)	CCβ (ng/g)
NAL	0.45	0.61	0.49	0.66	0.59	0.80	0.43	0.59	0.18	0.24
FLU	0.44	0.59	0.67	0.91	0.43	0.59	0.48	0.66	0.36	0.49
OXO	0.48	0.66	0.42	0.58	0.50	0.69	0.56	0.77	0.60	0.82
CIN	0.41	0.56	0.51	0.70	0.35	0.47	0.51	0.69	0.51	0.69
PIR	0.39	0.53	0.64	0.87	0.43	0.59	0.53	0.72	0.39	0.54
PIP	0.53	0.73	0.57	0.77	0.68	0.92	0.51	0.70	0.41	0.56
des-CIP	0.60	0.83	0.65	0.88	0.37	0.50	0.55	0.75	0.48	0.66
NOR	0.54	0.75	0.70	0.96	0.59	0.81	0.52	0.71	0.62	0.84
ENO	0.56	0.76	0.59	0.81	0.42	0.57	0.51	0.70	0.61	0.84
CIP	0.50	0.69	0.58	0.79	0.70	0.95	0.40	0.55	0.65	0.89
LOM	0.62	0.84	0.58	0.79	0.60	0.81	0.52	0.71	0.42	0.57
DAN	0.49	0.67	0.64	0.88	0.56	0.76	0.50	0.68	0.56	0.76
ENR	0.54	0.74	0.64	0.87	0.57	0.77	0.39	0.53	0.51	0.70
OFL	0.31	0.43	0.60	0.81	0.57	0.77	0.64	0.88	0.41	0.56
MAR	0.47	0.64	0.53	0.73	0.50	0.61	0.40	0.55	0.41	0.56
SAR	0.50	0.68	0.35	0.48	0.42	0.58	0.38	0.52	0.50	0.68
ORB	0.47	0.56	0.43	0.59	0.28	0.38	0.41	0.56	0.48	0.65
DIF	0.34	0.46	0.47	0.65	0.52	0.70	0.47	0.65	0.50	0.69

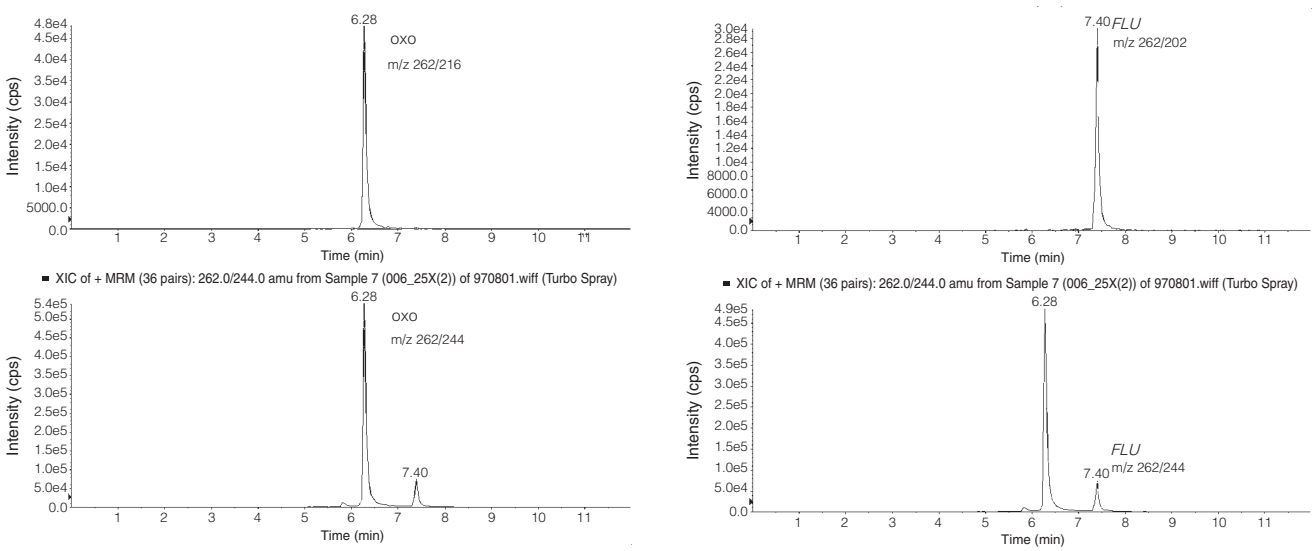

Figure 4. Ion reconstituted chromatographs obtained for a proficiency testing sample (fish muscle).

IV. *Decision Limit (CCα) and Detection Capability (CCβ)*

In Table 6, the CCα and CCβ are summaried for different QNs in each matrix. The values of CC_α ranged from 0.18 to 0.68 ng/g and CCβ ranged from 0.24 to 0.96 ng/g. According to Taiwan's MRLs[9], limits for residues of 4 QNs (OXO, FLU, SAR and DAN) were included in the regulation. The concentration range is wide (1/2 MRL to 2x MRL) owing to the authorized (MRL) substances. As a result, the calibration curve for each of QNs with LC-MS/MS method was hard to be linear in this range. In this study, for the four QNs that have permitted tolerance in Taiwan, those CCα and CCβ were also calculated as unauthorized substance.

V. *Application to a Proficiency Testing Sample*

A fish muscle sample of FAPAS proficiency test 02117[41] was dispatched in July 2008, 56 laboratories took part in the exercise with test 02117, and the proposed method has been applied to determinate a proficiency fish muscle testing sample. In order to deal with the high concentration of FLU and OXO, the final extraction of the sample was diluted 25-fold. The assigned values for FLU and OXO are 90.8 and 286 ng/g, respectively, in the test 02117. Our sample has been tested with the values of FLU 88.6 ng/g and OXO 279 ng/g. Z-score of the two results sample are -0.1. Figure 4 shows the ion reconstituted chromatogram. The results of comparison between and LC/ESI/MS/MS method and HPLC-FLD[25] method are presented in Table 7.

CONCLUSIONS

The LC/ESI/MS/MS multi-residue method allows simultaneous determination 18 QNs within the ng/g level. To the best of our knowledge it is the first method for the determination of 18 QNs simulateously. A simple and rapid extraction method allows the analysis conducted in 5 different food matrixes (chicken, pork, shrimp, fish and milk). The LC-ESI/MS/MS analysis procedure established gives CCα and CCβ down to 1 ng/g. The proposed method has been successfully applied to determine FLU and OXO in fish muscle from FAPAS proficiency testing sample. Thus, the short extraction step with LC/MS/MS detection should easily support a successful switch from HPLC/FLD[25] method and could be applied for determining multiquinolones residues.

Table 7. The results of FAPAS proficiency testing (n = 3)

Quinolone	method			
	LC/MS/MS		LC/FLD[25]	
	(ng/g)	R.S.D. (%)	(ng/g)	R.S.D. (%)
FLU	88.6	5.2	86.9	4.3
OXO	279.0	9.7	281.3	9.5

Z-score for LC/MS/MS method was -0.1.

REFERENCES

1. Zechiedrich, E. L. and Cozzarelli, N. R. 1995. Roles of topoisermase IV and DNA gyrase in DNA unlinking during replication in *Escherichia coli*. Genes Dev. 9: 2859-2869.
2. Riviere, J. E. and Papich, M. G. 2009. Veterinary pharmacology and therapeutics. 9th ed. pp. 993-994. Wiley-Blackwell. U.S.A.
3. Krebber, R., Hoffend, F. and Ruttmann, F. 2009. Simple and rapid determination of enrofloxacin and ciprofloxacin in edible tissues by turbulent flow chromatography/tandem mass spectrometry (TFC–MS/MS). Anal. Chim. Acta 637: 208-213.
4. Catry, B., Croubels, S., Schwarz, S., Deprez, P., Cox, B., Kehrenberg, C., Opsomer, G., Decostere, A. and Haesebrouck, F. 2008. Influence of systemic fluoroquinolone administration on the presence of *Pasteurella multocida* in the upper respiratory tract of clinically healthy calves. Acta. Vet. Scand. 50: 36-39.
5. Yue, L., Jiang, H., Liao, X., Liu, J., Li, S., Chen, X., Chen, C., Lü, D. and Liu, Y. 2008. Prevalence of plasmid-mediated quinolone resistance *qnr* genes in poultry and swine clinical isolates of *Escherichia coli*. Vet. Microbiol. 132: 414-420.
6. Delsol, A. A., Sunderland, J., Woodward, M. J., Pumbwe, L., Piddock, L. J. V. and Roe, J. M. 2004. Emergence of fluoroquinolone resistance in the native *Campylobacter coli* population of pigs exposed to enrofloxacin J. Antimicrob. Chemother. 53: 872-874.
7. Katsuda, K., Kohmoto, M., Mikami, O. and Uchida, I. 2009. Antimicrobial resistance and genetic characterization of fluoroquinolone-resistant Mannheimia haemolytica isolates from cattle with bovine pneumonia. Vet. Microbiol. 139: 74-79.
8. Béraud, R., Huneault, L., Bernier, D., Beaudry, F., Letellier, A. and del Castillo, J. R. E. 2008. Comparison of the selection of antimicrobial resistance in fecal *Escherichia coli* during enrofloxacin administration with a local drug delivery system or with intramuscular injections in a swine model. Can. J. Vet. Res. 72: 311-319.
9. Department of Health, Executive Yuan. 2008. Tolerances for Residues of Veterinary Drugs. DOH Food No. 97040692. Sep. 5, Taipei. (in Chinese).
10. The Japan Food Chemical Research Foundation. Maximum Residue Limits (MRLs) List of Agriculturals in Foods. http://www.m5.ws001.squarestart.ne.jp /foundation/search.html
11. European Commission. Regulation No. 2377/1990.

2006. Off. J. Eur. Commun. L224: 1-8.
12. Maraschiello, C., Cusido, E., Abellan, M. and Vilageliu, J. 2001. Validation of an analytical procedure for the determination of fluoroquinolone ofloxacin in chicken tissues. J. Chromatogr. B Biomed. Sci. Appl. 754: 311-318.
13. Ramos, M., Aranda, A., Garcia, E., Reuvers T. and Hooghuis, H. 2003. Simple and sensitive determination of five quinolones in food by liquid chromatography with fluorescence detection. J. Chromatogr. B Biomed. Sci. Appl. 789: 373-381.
14. Turiel, E., Bordin, G. and Rodriguez, A. R. 2003. Trace enrichment of (fluoro)quinolone antibiotics in surface waters by solid-phase extraction and their determination by liquid chromatography-ultraviolet detection. J. Chromatogr. A 1008: 145-155.
15. Zhaos, S., Jiang, H., Li, X., Mi, T., Li, C. and Shen, J. 2007. Simultaneous determination of trace levels of 10 quinolones in swine, chicken, and shrimp muscle tissues using HPLC with programmable fluorescence detection. J. Agric Food Chem. 55: 3829-3834.
16. Roybal, J., Walker, C. C., Pfenning, A. P., Turnipseed, S. B., Storey J. M., Gonzales, S. A. and Hurlbut, J. A. 2002. Concurrent determination of four fluoroquinolones in catfish, shrimp, and salmon by liquid chromatography with fluorescence detection. J. AOAC Int. 85: 1293-1301.
17. Yang, G., Lin, B., Zeng, Z., Chen, Z. and Huang, X. 2005. Multiresidue determination of eleven quinolones in milk by liquid chromatography with fluorescence detection. J. AOAC Int. 88: 1688-1694.
18. Verdon, E., Couedor, P., Roudaut, B. and Sanders, P. 2005. Multiresidue method for simultaneous determination of ten quinolones antibacterials residues in multimatrix/multispecies animal tissues by liquid chromatography with fluorescence detection: single laboratory validation study. J. AOAC Int. 88: 1179-1192.
19. Horie, M., Saito, K., Nose, N. and Nakazawa, H. 1995. Simultaneous determination of eight quinolone antibacterials in meat and fish by high performance liquid chromatography. J. Food Hyg. Soc. Jpn. 36: 62-67. (in Japanese).
20. Horie, M., Saito, K., Hoshino, Y., Nose, N., Mochizuki, E. and Nakazawa, H. 1987. Simultaneous determination of nalidixic acid, oxolinic acid and piromidic acid in fish by high performance liquid chromatography with fluorescence and ultraviolet detection. J. Chromatogr. A 402: 301-308.
21. Posyniak, A., Zmudzki, J., and Semeniuk, S. 2001. Effect of matrix and sample preparation on determination of fluoroquinolone residues in animal tissues. J. Chromatogr. A 914: 89-94.
22. Romos, M., Aranda, A., Garcia, E., Reuvers, T., and Hooghusis, H. 2003. Simple and sensitive determination of five quinolone in food by liquid chromatography with fluorescence detection. J. Chromatogr. B Biomed. Sci. Appl. 789: 373-381.
23. Su, S. C., Chang, M. H., Chang, C. L. and Chang, P. C. 2008. Simultaneous determination of quinolones in livestock and marine products by high performance liquid chromatography. J. Food and Drug Anal. 11: 114-127.
24. Marazuela, M. D. and Moreno-Bondi, M. C. 2004. Multiresidue determination of fluoroquinolones in milk by column liquid chromatography with fluorescence and ultraviolet absorbance detection. J. Chromatogr. A 1034: 25-32.
25. Chang, C., S., Wang, W. H. and Tsai, C. E. 2008. Simultaneous determination of eleven quinolones antibacterial residues in marine products and animal tissues by liquid chromatography with fluorescence detection. J. Food and Drug Anal. 16: 87-96.
26. Hatano, K. 2004. Simultaneous determination of quinolones in foods by LC/MS/MS. J. Food Hyg. Soc. Jpn. 45: 239-244. (in Japanese).
27. Dufresne, G., Fouquet, A., Forsyth, D. and Tittlemier, S. A. 2007. Multiresidue determination of quinolone and fluoroquinolone antibiotics in fish and shrimp by liquid chromatography/tandem mass spectrometry. J. AOAC Int. 90: 604-612.
28. Schneider, M. J. and Donoghue, D. J. 2003. Multiresidue determination of fluoroquinolone antibiotics in eggs using liquid chromatography-fluorescennce -mass spectrometry^{n}. Anal. Chim. Acta. 483: 39-49.
29. Durden, A. D. and Macpherson, T. 2007. Quantitation and validation of fluoroquinolones in egg using liquid chromatography/tandem mass spectrometry. J. AOAC Int. 90: 613-625.
30. Hermo, M. P., Barron, D. and Barbosa, J. 2006. Development of analytical methods for multiresidue determination of quinolones in pig muscle samples by liquid chromatography with ultraviolet detection, liquid chromatography-mass spectrometry. J. Chromatogr. A 1104: 132-139.
31. Hermo, M. P., Barron, D. and Barbosa, J. 2006. Confirmatory and quantitative analysis using experimental design for the extraction and liquid chromatography–UV, liquid chromatography-mass spectrometry and liquid chromatography–mass spectrometry/mass spectrometry determination of quinolones in turkey muscle. J. Chromatogr. A 1135: 170-178.
32. Johnston, L., Mackay, L. and Croft, M. 2002. Determination of quinolones and fluoroquinolones in fish tissue and seafood by high-performance liquid chromatography with electrospray ionization tandem mass spectrometric detection. J. Chromatogr. A 982: 97-109.
33. Toussaint, B., Bordin, G. and Janosi, A. 2002. Validation of a liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of 11 (fluoro)quinolone antibiotics in swine kidney. J. Chromatogr. A 976: 195-206.
34. Toussaint, B., Chedin, M. and Bordin, G. 2005. Determination of (fluoro)quinolones antibiotic residues in pig kidney using liquid chromatography–tandem mass

spectrometry. I. Laboratory-validated method. J. Chromatogr. A 1088: 32-39.

35. Toussaint, B., Chedin, M. and Bordin, G. 2005. Determination of (fluoro)quinolones antibiotic residues in pig kidney using liquid chromatography-tandem mass spectrometry. PartII. Intercomparison exercise. J. Chromatogr. A 1088: 40-48.
36. Hoof, N. V., Wasch, K. D. and Reybroeck, S. P. 2005. Validation of a liquid chromatography-tandem mass spectromertic method for the quantification of eight quinolones in bovine muscle, milk and aquaculture products. Anal. Chim. Acta. 529: 265-272.
37. Samanidou, V., Evaggelopuloa, E. and Trotzmuller, M. 2008. Multi-residue determination of seven quinolones antibiotics in gilthead seabream using liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1203: 115-123.
38. EU Commission Decision No. 657/2002. 2002. Off. J. Eur. Commun. L221: 8-36.
39. ISO Standard No. 11843-2. 2000. Capability of Detection, Part 2: Methodology in the Linear Calibration Case, International Organization for Standardization, Geneva, Switzerland.
40. Khong, S., Hammel, Y. and Guy, P. A. 2005. Analysis of tetracyclines in honey by high-performance liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 19: 493-502.
41. United Kingdom, FAPAS Central Science Laboratory. 2008. FAPAS proficiency 02117 Quinolones in Fish Muscle.