ANALYSIS OF QUINOLONES IN POULTRY MUSCLES USING LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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ABSTRACT. Residues of quinolone antibiotics were determined in poultry muscle using an LC-MS/MS method coupled with electrospray ionization. The procedure was optimised and validated for simultaneous identification, confirmation and quantitation of 12 quinolones in poultry muscle. The antibiotics included nalidixic acid, flumequine, cinoxacin, pipemidic acid, norfloxacin, enoxacin, ciprofloxacin, danofloxacin enrofloxacin, ofloxacin, sarafloxacin and difloxacin. The samples were extracted with glycine/HCl and purified using Oasis HLB cartridges. LC separation was achieved using Atlantis C-18 column. Multiple reactions monitoring (MRM) was used for selective detection of each quinolone while D5-norfloxacin was used as internal standard. The calibration curves were linear in the 10–300, 20-600, 30-900 and 40-1200 µg/kg range, with typical (R^2) values higher than 0.98 and 0.99. The CCα ranged between 13.94 and 433.61 µg/kg, while CCβ were ranged between 23.69 and 461.49 µg/kg. The LOD and LOQ were much lower than the respective Maximum Residue Limits. The accuracy of the method ranged from 88 % to 119%, the coefficient of variation (CV, %) for intra-day was lower than 15%. This validated method was successfully applied to real samples for the analysis of quinolones in poultry muscles.

Keywords: Quinolone; Poultry; Liquid chromatography–tandem mass spectrometry

INTRODUCTION

Veterinary drugs are generally used in farm animals for therapeutic and prophylactic purposes and they include a large number of different types of compounds which can be administered in the feed or in the drinking water (McEvoy, 2002). Veterinary drugs most commonly used for treatment and prevention of disease can be classified into major classes such as antibacterials, anthelmintics, anticoccidials, and other ptotozoals (Botsouglou *et al.,* 2001). The most commonly used antimicrobials in food-producing animals include β-lactams, tetracyclines, aminoglycosides, quinolones, macrolides and sulfonamides

(Kirbiš, A., 2007). Subtherapeutic levels of antibiotics could increase feed efficiency and growth in farm animals and have been shown to reduce the incidence or severity of a number of animal diseases. It has been suggested that they also prevent irritation of the intestinal lining and improve digestive processes and metabolic processes of the animal (Doyle, M.E., 2006).

Quinolones are synthetic antibacterial agents with broad spectrum activities and have been widely used in food-producing animals, aquaculture, and humans to treat bacterial infections (Zhao *et al.,* 2007). Residues of quinolone in tissues of food producing animals are of concern due to reports of antibacterial resistance. (Pederson *et al.,* 2003; San Martin *et al.,* 2005). The FDA has banned the use of enrofloxacin in poultry because of the emergence of *Campylobacter* resistance to quinolones, which may result in ineffective treatment of human diseases by these antibiotics (FDA, 2005).

To protect the health of consumers, many countries have established maximum residue limits (MRLs) for selected quinolones in food-producing animals (Food act, 1985). Thus, the establishment of sensitive methods for the analysis of residual amounts of these drugs is required for the quality control of animal based food products.

There are various publications on residue analysis of quinolones in food-producing animals and several techniques have been employed in the monitoring of these compounds in animals based food products. These includes microbiological assay (Okerman *et al*., 2007; Ashwin *et al.,* 2008), immunoassay (Huet *et al*., 2006; Scortichini *et al.,* 2009), thin-layer chromatography (TLC) (Juhel-Gaugain *et al*., 1998) , biosensor (Marchesini *et al.,* 2007), high performance capillary electrophoresis (HPCE) (Lara *et al*., 2008), high performance liquid chromatography (HPLC) with fluorescence (FLD) (York *et al*., 2000; Zhao *et al*., 2007), ultraviolet (UV) (Bailac *et al*., 2004, 2006) and mass spectrometric (MS) detection (Vyncht *et al*., 2002; Toussaint *et al*., 2002, 2005; Van Hoof *et al*., 2005; Clemente *et al*., 2006; Hermo *et al*., 2008). Some of these methods, however, focused on a limited number of quinolones. This paper reports on the development and validation of a method that allows the determination of 12 quinolones simultaneously.

MATERIALS AND METHODS

Chemicals and reagents

The quinolone standards, namely norfloxacin (NOR) (98%), enoxacin (ENO), ofloxacin (OFX), cinoxacin (CIN) and nalidixic acid (NAL) (100%), pipemidic acid (PIP) (99%) and flumequine (FLU) (98.1%) were obtained from Sigma–Aldrich (St Louis, MO, USA) while enrofloxacin (ENR) and danofloxacin (DAN) (99.9%), sarafloxacin (SAR) HCl (99.7%), difloxacin (DIF) HCl (98.4%), ciprofloxacin (CIP) (98%) were from Riedel-de Haen (Seelze, Germany). Norfloxacin-D5 (99%) was

used as internal standard for MS–MS quantification and was purchased from Sigma–Aldrich (St Louis, MO, USA).

All chemicals and chromatographic reagents used were of HPLC or analytical grade. Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany) Hydrochloric acid 1M, glycine and formic acid (98%, analytical reagent grade) more obtained from Fisher Scientific. Ultrapure water was filtered through a Millipore Rios system followed by a Milli-Q Biocell system (18.2 MΩ cm^{-1} resistivity) (Millipore, Bedford, MA, USA). Nitrogen (99.999%) (for desolvation and nebuliser gas) and argon (99.999%) (MS/MS collision gas) were obtained from Malaysian Oxygen Berhad (MOX).

Preparation of standard solutions

Individual stock solutions (1000 µg/mL) were prepared by dissolving the appropriate amount of quinolone in 100 µL of 1M NaOH and adjusting to 10 mL with methanol (correction was made for the standard purity). The stock solutions were kept at +4°C for 3 months. An intermediate standard solution containing 100 µg/mL of each quinolone was prepared by diluting the stock solution with methanol. These solutions were kept at +4°C for 1 month. A mixture of working standards used for calibration standards were prepared at concentrations 2.5 µg/mL for NAL, CIN, PIP, NOR, ENO, CIP, ENR, OFX and SAR, 5 µg/mL for DAN, 7.5 µg/mL for DIF and 10 µg/mL for FLU. Mixed working standard was freshly prepared for each analysis.

The stock solution of internal standard D_5-norfloxacin (200 µg/mL) was prepared the same way as the other stock solutions. Then 5 µg/mL of working internal standard was prepared by diluting it with methanol.

Instrumentation

A T25 Ultra-Turrax from IKA Labortechnik (Darmstadt, Germany), a REAX2 end-over-end rotator (Heidolph, Kelheim, Germany) and a Jouan CR3i centrifuge (St. Herblain, France) were used for sample extraction. A Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA), and a Vortex (Heidolph, Kelheim, Germany) were used for sample clean-up. An AX26 and PB1502 analytical balance (Mettler, Greifensee, Switzerland) were used in the preparation of standard solutions and sample weighing. SPE cartridges OASIS HLB C18 cartridges (3 ml, 60 mg) were obtained from Waters (Harbor City, USA). Gilson ASPEC XL4 automatic sample preparation system (Gilson, Middleton, USA) was used for solid-phase extraction (SPE) clean-up.

The LC system consisted of a 2695 Alliance Separations Module equipped with a 2695 micro vacuum degasser, a 2695 thermostated autosampler and a 2695 thermostated column compartment (Waters, Manchester, UK). Separations of the compounds were performed using Atlantis d C_{18} (150 mm L × 2.1 mm D; 3

μm particle diameter) column from Waters. Instrument control and data analysis were performed using MassLynx 4.0 application software from Micromass (Manchester, UK).

The ESI-MS/MS detection of the quinolones was carried out with a Quattro Ultima Pt triple-quadrupole mass spectrometer from Micromass Co. Inc. (Manchester, UK). Positive ionisation mode was used and the ions were monitored in the multiple reaction monitoring (MRM) mode. Direct infusion experiments were performed through a 250-ml Hamilton (Reno, NV, USA) gas-tight syringe and a Harvard Apparatus (South Natick, MA, USA) model 11 syringe pump for peak identification and mass fragments determination.

Procedure

Sample extraction and clean-up

The sample extractions were performed as reported by McCracken *et al.*, 2002 with some modifications (Sapar M., 2010). The modifications involved reduction of sample weight, and addition of internal standard. 1 g portions of minced tissues were weighed into 50 mL centrifuge tubes and 50 μL of 5 μg/mL D_5-Norfloxacin was added as internal standard. The tubes were shaken vigorously for 5 min at room temperature, after which they were allowed to sit for 15 min at room temperature to allow the drug to penetrate the homogenized tissue. A 10 mL glycine/hydrochloric acid solution was added to the tubes. The tissues were homogenized using an Ultra-Turrax T25 at the speed of 1000 rpm for 20 s and mixed with end-over-end rotator in 5 min for maximum extraction. The homogenized extracts were centrifuged at 4000 rpm for 10 min at 4°C. The liquid phases were separated from the pellets and filtered through a filter paper (No. 4) to facilitate the SPE extractions.

Samples clean-up were performed by automated SPE. The SPE cartridges were conditioned with 2 mL methanol and 2 mL of water. After the application of the extracts, the cartridges were cleaned with 3 mL of water and dried by air aspiration. The analytes were eluted with 2 mL of methanol into a 3 mL glass tube. The eluates were evaporated to dryness under a nitrogen stream at 50°C. The dried residues were reconstituted in 0.5 mL of 0.1% formic acid. The tubes were vortex-mixed for 30 s. If necessary, the residues would be filtered through a 0.45 μm filter. The supernatants were transferred to injection vials and 15 μl of the samples were injected into the LC system.

Chromatographic conditions

The LC separation of the quinolones was achieved using a gradient elution. The initial mobile phase consisted of 99% A and 1% B, where A was 0.1% formic acid and B was acetonitrile mixed with 0.1% formic acid. From 0 to 6.0 min, the percentage of B increased from 1 to 10%. From 6.1 to 12.0 min, the percentage of B increased

to 45%, increasing to 55% from 12.1 to 14 min, returning to 1% by 14.1 min and holding until 21 min. The flow rate of the mobile phase was 0.25 mL/min and injection volume was 15 µL. The column temperature was maintained at 25°C throughout the run.

RESULTS AND DISCUSSION

Optimisation of MS/MS condition

The optimisation of the operational parameters was performed using electrospray ionization of 0.5 µg/mL solution of selected quinolones, which was dissolved in 0.1% acetic acid/acetonitrile solution (99/1, v/v). Each quinolones was directly infused into the MS source, at a flow rate of 10 µL /min and in full scan mode of data acquisition (m/z 50–500). Parameters were optimised in order to obtain the highest sensitivity of the quinolones. Enrofloxacin and sarafloxacin were chosen for the optimisation of the source and analyser parameters of ESI.

The mass spectrometer was operated in positive-ion mode, with nitrogen as the nebuliser and drying gas. Argon was used as the collision gas. The cone voltage and the collision energy parameters for MRM acquisitions were optimised using direct infusion for each particular antibiotic (described in Table 1). The dwell time was 100 ms/transition with an inter-channel delay of 0.1s. Two transitions were used for identification but only one was used for quantitation (Table 1).

Mixed solutions of quinolones were injected into the LC-ESI/MS system for characterisation of the quinolones. ESI-MS-MS chromatogram was obtained using the optimised conditions of the mass spectrometer.

The optimised ESI-MS/MS conditions were: 3.2 kV capillary voltage, source block and desolvation temperatures at 120 and 350°C, respectively while the desolvation and cone gas (N_2) flows were 500 and 50 L/h respectively. Argon pressure in the collision cell was set at 2.5×10^{-3} mbar. The photomultiplier voltage was adjusted to 650 V.

Validation

Validation is an essential part of any quantitative method development and is required to determine how well a method performs with regards to its accuracy, precision and reproducibility. It is normally carried out when the details of a new method have been finalised, or if any modifications have been made to the method which may affect its performance. The performances of the developed method were validated according to the criteria specified in the Commission Decision 2002/675/EC for a quantitative confirmation method (EC., 2002). Validation parameter includes linearity, specificity, selectivity, limit of detection/quantification, accuracy and precision. For analytes with an established maximum residue limit (MRL), validation parameters were determined at concentration levels

TABLE 1. LC retention times (min), optimised MS–MS conditions and selected multiple reaction monitoring (MRM) transitions for the target quinolones

Quinolones	Ret. Time (min)	Parent ion> daughter ion (m/z)	Cone voltage (V)	Collision energy (eV)
Nalidixic acid	17.51	233.09>**215.17**	35	10
		233.09>187.15	35	22
Flumequine	17.81	262.12>**202.13**	35	27
		262.12>244.19	35	15
Cinoxacin	15.52	263.11>**217.04**	35	20
		263.11>245.04	35	12
Pipemidic acid	12.64	304.26>**217.27**	35	20
		304.26>286.33	35	15
Norfloxacin	13.33	320.36>**276.33**	35	15
		320.36>233.28	35	20
Enoxacin	13.33	321.24>206.02	35	25
		321.24>**257.15**	35	15
Nor-D5 (IS)	13.33	325.28>281.37	35	15
Ciprofloxacin	13.53	332.22>**288.29**	35	15
		332.22>245.24	35	20
Danofloxacin	13.63	358.29>96.26	35	20
		358.29>**314.36**	35	14
Enrofloxacin	13.83	360.31>**316.40**	35	15
		360.31>245.30	35	25
Ofloxacin	13.33	362.23>261.23	35	22
		362.23>**318.31**	35	15
Sarafloxacin	14.23	386.21>299.23	35	20
		386.21>**342.28**	35	15
Difloxacin	14.43	400.29>**356.39**	35	15
		400.29>299.32	35	25

The daughter ion used for quantification is shown in bold

of 0.25 MRL, 0.5 MRL, 1 MRL and 2 MRL. For compound with neither MRL nor minimum required performance limit (MRPL) have been established, such as sarafloxacin, the selected levels were 10, 50, 100 and 200 µg/kg.

Linearity

Linearity was established using matrix-matched calibration curves. The matrix-matched calibration curves were constructed by spiking blank chicken muscle with increasing amounts of quinolones. The calibration curves were determined at six concentration levels depending on the analytes. As shown in Table 2, danofloxacin standard curve was built at 20-600 µg /kg, difloxacin at 30-900 µg/kg, flumequine at 40-1200 µg/kg and other compounds at 10-300 µg/kg. They were prepared following the extraction method described in the procedures. Each level was determined in duplicate. The concentration of the deuterated internal standard was fixed at 250 µg/kg. Calibration standards were injected before and after each series of analysis. The average values were used to construct calibration curves by plotting peak area ratio *(y)* of quinolone to the internal standard, versus concentration of the analyte *(x)*.

The linearity and goodness of fit for each quinolone was determined by the linear regression. The processing of chromatograms, the regression parameters of slope, intercept, and correlation coefficient were calculated automatically by weight (1/*x*) linear regression in Masslynx Software version 4.0. Analysis was carried

TABLE 2. Linear regression data and squares of correlation coefficients for the matrix-matched calibration curves of quinolones

Analyte	Injected calibration range (µg /kg)	Regression line [Mean ± SD (n = 3)]		
		Slope	y-intercept	R^2 values
Nalidixic acid	10 - 300	9.1268 ± 4.4412	12.1134 ± 9.5979	0.9902 ± 0.0027
Flumequine	40 - 1200	0.8027 ± 0.6404	2.3956 ± 8.4254	0.9910 ± 0.0013
Cinoxacin	10 - 300	0.7546 ± 0.3094	-0.8841 ± 0.8164	0.9898 ± 0.0032
Pipemidic acid	10 - 300	1.2511 ± 0.5097	0.3515 ± 0.4266	0.9894 ± 0.0078
Norfloxacin	10 - 300	0.7257 ± 0.2984	0.6040 ± 0.7838	0.9871 ± 0.0086
Enoxacin	10 - 300	0.3134 ± 0.0550	0.3308 ± 0.6141	0.9857 ± 0.0127
Ciprofloxacin	10 - 300	0.9271 ± 0.2636	0.2377 ± 0.8589	0.9920 ± 0.0075
Danofloxacin	20 - 600	0.7073 ± 0.2190	0.2493 ± 1.3854	0.9918 ± 0.0037
Enrofloxacin	10 - 300	1.6916 ± 0.6235	-3.1309 ± 0.6719	0.9882 ± 0.0066
Ofloxacin	10 - 300	1.6151 ± 0.1683	0.1212 ± 2.3918	0.9840 ± 0.0156
Sarafloxacin	10 - 300	0.5393 ± 0.1386	-2.1760 ± 1.3790	0.9903 ± 0.0066
Difloxacin	30 - 900	1.2948 ± 0.0794	-10.0867 ± 4.7263	0.9805 ± 0.0086

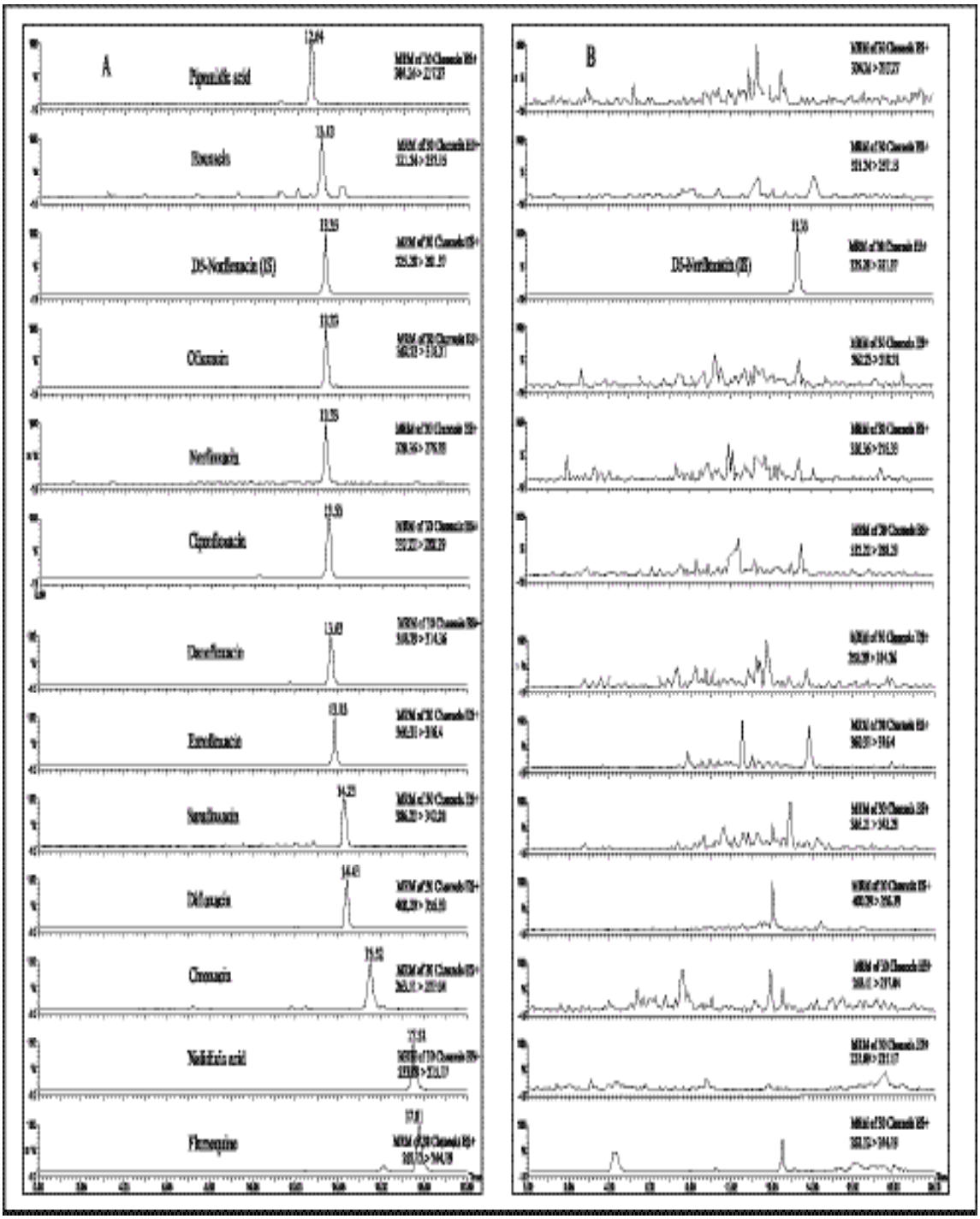

FIGURE 1. MRM chromatograms of chicken muscle sample spiked at 0.25MRL (A) and of a blank chicken muscle (B)

out on three different days to produce independent replicates.

The results of the linearity studies are summarised in Table 2. The correlation coefficients of the calibration curves *(r^2)* in all the quinolone were higher than 0.98 and 0.99. The high correlation coefficient *(r^2)* values indicated a good correlation between quinolone concentrations and peak areas.

Specificity

The specificity is defined as the ability of the method to measure the analyte accurately and specifically in the presence of components present in the sample matrix. The specificity of the method was performed by analysing 20 blank samples collected from different sources to prove if substances can interfere with the retention time of all the quinolones. These samples were called "blank" as they reacted negatively to microbiological test for quinolones by using microbiological six plate method (Myllyniemi, *et al*., 2001). These samples were analysed using the LC–MS/MS method for the detection of the 12 targeted quinolones.

HPLC-chromatograms of a blank sample and a spiked sample of poultry muscle are shown in Figure 1. The result showed that the blank tissue samples were free from endogenous interferences of quinolones and these confirmed the good specificity of the method.

Selectivity

Quinolones are part of group B substances of Annex 1, Council Directive 96/23/EC (EC, 1996). For the confirmation of group B substances in foodstuffs, a minimum of three identification points (IPs) are required and the method fulfilled this requirement with the use of two MRM transitions (one precursor and two product ions) for each compound, which count for four IPs, respectively (EC., 2002). Therefore, in this method, two different transitions were followed for each compound in the MRM mode as shown in Table 1. For the quantification, only one fragmentation path was monitored (indicated in bold in Table 1).

Decision limit (CCα), detection capability (CCβ), limit of detection (LOD) and limit of quantification (LOQ)

Two new parameters had been introduced by Decision 2002/657/EC; CCα (decision limit) and CCβ (detection capability), to replace the old concepts of limit of detection and limit of quantitation. For substances without MRLs, CCα was defined as the limit above which samples were concluded to be non-compliant, with an error probability, α of 1%, while CCβ was defined as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability, β of 5%. In the case of substances with MRLs, decision limit and detection capability must be greater

than MRL and α and β errors must be equal or less than 5%. CCα and CCβ were calculated by the matrix calibration curve procedure according to Decision 2002/657/EC (EC., 2002) and ISO 11843, 2000. For the compound with no set MRL, CCα was established as the concentration at the y−intercept plus 2.33 standard error of the intercept for a set of data with 7 replicates at 3 levels (10, 50, 100 µg/kg). CCα was calculated as the MRL plus 1.64 times the corresponding standard error of analysing blank samples spiked at 1/2 MRL, MRL and 2MRL for the compound with established MRL. The detection capability (CCβ) for both cases was calculated by adding 1.64 times the standard error to the CCα. Table 3 summarises the obtained CCα and CCβ values.

The limit of detection (LOD) and limit of quantification (LOQ) were also calculated. The limit of detection of the quantitative analysis indicated the lowest level of the analyte that can be measured with statistical certainty in a sample, which gave a signal-to-noise ratio of 3 (the ratio between the peak intensity and the noise intensity was used), while the LOQ is calculated from the concentration of the analytes that provided a signal-to-noise ratio of 10 on analysis as recommended in Decision 2002/657/EC (EC., 2002) and mentioned by Pozo et al., 2006.

TABLE 3. Decision limits (CCa), detection capabilities (CCb), limits of detection (LOD) and limits of quantification (LOQ) in poultry muscle of the analytes studied

Analyte	MRL (µgkg^{-1})		CCα (µg/kg)	CCβ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
	Malaysia	EU				
Nalidixic acid	ND	ND	26.83	45.63	0.2750	0.9165
Flumequine	400	400	186.54	317.33	0.5444	1.8147
Cinoxacin	ND	ND	22.18	37.73	0.5891	1.9636
Pipemidic acid	ND	ND	32.03	54.49	0.3373	1.1242
Norfloxacin	ND	ND	27.60	46.96	0.3015	1.0050
Enoxacin	ND	ND	39.55	67.29	1.9318	6.4392
Ciprofloxacin	100[a]	100[a]	24.88	42.32	0.2704	0.9014
Danofloxacin	200	200	47.05	80.05	0.4724	1.5748
Enrofloxacin	100[a]	100[a]	44.66	75.97	0.2466	0.8222
Ofloxacin	ND	ND	20.86	35.48	0.2486	0.8288
Sarafloxacin	100	ND	29.29	49.83	0.6335	2.1117
Difloxacin	300	300	107.51	182.83	0.2812	0.9373

ND – not defined by legislation a – MRL for Enrofloxacin + ciprofloxacin = 100 mg/kg

An estimate of LOD and LOQ was performed by extrapolating the S/N ratio of the peak areas obtained from a chicken meat sample fortified at lowest concentration levels. The S/N was calculated by the statistical software of MassLynx v4.0 (Micromass, Manchester, Lancashire, UK). The LOD and LOQ of the method for the quinolone are as shown in Table 3.

TABLE 4. Accuracy and precision (%RSD) for the analytes at 4 spiking levels

Analyte	Nominal Concentration (µg/kg)	Accuracy (%)	Precision (relative standard deviation, %)	
			Intra-day	Inter-day
Nalidixic acid	10	99	9.91	27.01
	50	98	8.83	8.70
	100	107	12.58	14.97
	200	99	7.19	7.00
Flumequine	40	118	9.59	15.15
	200	100	8.40	11.18
	400	88	6.72	22.98
	800	91	11.70	17.74
Cinoxacin	10	110	14.91	21.10
	50	92	11.01	16.04
	100	99	8.49	10.51
	200	98	6.12	7.05
Pipemidic	10	100	12.31	17.69
	50	98	6.43	15.07
	100	104	7.35	11.41
	200	94	9.58	11.25
Norfloxacin	10	113	7.94	16.58
	50	101	5.89	10.38
	100	99	6.05	10.31
	200	94	5.89	11.15
Enoxacin	10	119	9.07	14.13
	50	92	14.67	15.70
	100	90	9.54	14.81
	200	92	12.01	15.35

Analyte	Nominal Concentration (µg/kg)	Accuracy (%)	Precision (relative standard deviation, %)	
			Intra-day	Inter-day
Ciprofloxacin	10	118	8.96	17.31
	50	99	8.18	8.44
	100	97	6.43	7.80
	200	95	8.15	9.74
Danofloxacin	20	117	7.57	11.97
	100	100	11.73	12.79
	200	100	5.32	8.59
	400	99	4.97	6.71
Enrofloxacin	10	117	9.15	12.99
	50	100	8.03	7.77
	100	97	11.29	14.69
	200	93	11.36	18.74
Ofloxacin	10	117	7.86	9.63
	50	101	9.48	9.23
	100	97	5.87	9.18
	200	98	6.07	7.58
Sarafloxacin	10	127	4.10	16.83
	50	101	6.98	8.46
	100	98	12.01	11.35
	200	96	10.07	10.41
Difloxacin	30	117	10.38	21.27
	150	110	6.72	6.51
	300	100	9.19	12.87
	600	92	11.09	12.90

Accuracy and Precision

The accuracy of the method was expressed as the mean recoveries of spiked analytes in chicken muscle at four concentration levels. Table 4 showed the recovery data that ranged from 88% to 119%. The recovery values obtained in this study fulfilled the acceptable Decision No. 2002/657/EC for the analysis of residues at ppb-level indicating good accuracy of the method. The accuracy of a confirmatory method should be 80–110% for samples (EC., 2002).

The precision of the method was tested by repeatedly analysing the spiked chicken samples. The intra-day precision of the method was determined in chicken

TABLE 5. Overall Detection Results

Processing Plant ID	Number of samples analysed	Concentration Found (µg/kg)											
		NAL	FLU	CIN	PIP	NOR	ENO	CIP	DAN	ENR	OFX	SAR	DIF
1. A	2	ND	616.94 (1)	ND	ND	ND	ND	ND	ND	22.69 (1) -34.18 (1)	ND	11.55 (1)	ND
2. B	1	ND	261.93 (1)	ND	ND	ND	ND	136.75 (1)	ND	1,487.47 (1)	ND	ND	ND
3. C	3	ND	ND	ND	ND	ND	ND	21.05 (1) 36.81 (1)	ND	4.86 -483.73 (3)	ND	ND	ND
4. D	2	ND	ND	ND	ND	108.96 (1)	ND	ND	ND	ND	ND	ND	ND
5. E	17	ND	ND	ND	ND	ND	ND	3.42 - 170.53 (6)	ND	7.41 - 1,036.07 (17)	ND	ND	ND
6. F	1	ND	ND	ND	ND	ND	ND	ND	ND	31.91 (1)	ND	ND	ND
7. G	2	ND	ND	ND	ND	ND	ND	ND	ND	15.06 – 22.47 (1)	ND	ND	ND
8. H	5	ND	ND	ND	ND	ND	ND	238.11 (1)	ND	3.51 - 1,734.61 (5)	ND	ND	ND
9. I	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10. J	1	ND	ND	ND	ND	ND	ND	ND	ND	127.48 (1)	ND	ND	ND
11. K	2	ND	ND	ND	ND	ND	ND	ND	ND	18.63 (1) 45.35 (1)	ND	ND	ND
Total	**37**												

( ) Number of samples detected positive ND – Not detected

muscle using seven determinations *(n* = 7) at four concentration levels.The intra-day precision analysis was done on the same day. The inter-day precision was determined by repeating the study for two consecutive days. The precision of the entire method was expressed by the RSD of multiple analysis at different concentration levels. Results showed that the RSD ranged from all were lower than 15% for the intra-day precision tests. Overall, good inter-day precision was observed for most analytes at all levels except at lowest spiked level, the RSD for NAL, CIN and DIF was in the range of 21 to 27% (Table 4) and for FLU the RSD was 23% at 400 µg/kg. Only RSD for FLU exceeded the level calculated by Horwitz equation (EC., 2002), the rest of the results were in agreement with the Horwitz acceptable limits for each concentration.

Application to real samples

This method had been successfully applied to detect the quinolones studied in 37 chicken samples detected positive with microbiological six plate method (Myllyniemi *et al*., 2001). Most of the compounds studied were not detected in the samples analysed. Enrofloxacin and ciprofloxacin were the most prevalent residues where 35 of 37 samples were found to contain these two compounds at various concentrations (Table 5). In addition to them, 2 samples contained flumequine, 1 norfloxacin and 1 sarafloxacin.

CONCLUSION

In this work, an improved LC-MS/MS method has been developed and validated for the determination of 12 quinolones from poultry muscle. Satisfactory results were obtained with respect to EU Commission Decision 2002/657/EC. The proposed methods have been successfully applied to confirm poultry muscle samples found positive for quinolones by screening test.

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