

Development and Validation of a Method for the Determination of Quinolones in Muscle and Eggs by Liquid Chromatography-Tandem Mass Spectrometry

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Received: 6 September 2015 / Accepted: 11 January 2016 / Published online: 28 January 2016
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Abstract In this study, the development and validation of a multiresidue method for the detection of 11 quinolones (marbofloxacin, norfloxacin, ciprofloxacin, danofloxacin, lomefloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid, flumequine) in muscle and eggs were reported. The method involved an extraction with a methanol/metaphosphoric acid mixture and a clean up by Oasis hydrophilic-lipophilic balance (HLB) cartridge. The validation was performed according to the Commission Decision 2002/657/EC. Linearity, specificity, decision limit ($CC\alpha$), detection capability ($CC\beta$), recovery, precision (repeatability and within-laboratory reproducibility), and ruggedness were determined. Depending on the analytes, $CC\alpha$ and $CC\beta$ ranged from 113 to 234 $\mu\text{g}/\text{kg}$ and from 126 to 282 $\mu\text{g}/\text{kg}$ in muscle samples, whereas in eggs, these parameters were between 5.6 and 7.4 $\mu\text{g}/\text{kg}$ and between 6.1 and 9.8 $\mu\text{g}/\text{kg}$, respectively. In both the examined matrices, the recovery values were always higher than 90 % and precision, calculated as relative standard deviation, was equal to or lower than 16 % for repeatability and 23 % for within-laboratory reproducibility. The described method can be considered adequate for the

simultaneous determination and quantification of quinolones in the tested food matrices.

Keywords Quinolones · Muscle · Eggs · Liquid chromatography-tandem mass spectrometry · Method validation

Introduction

Quinolones are synthetic antibiotics with high effectiveness against both Gram-negative and Gram-positive bacteria that are resistant to other antibacterial agents (Hernández et al. 2011). They have also important effects in the treatment of intestinal, respiratory, and urinary tract infections in humans and food-producing animals such as cattle, turkey, pig, and poultry. In the last years, the significant progressive increase of their use caused inevitable residues in food that represent a potential health hazard for consumers (Herme et al. 2008). There are many adverse effects commonly associated with quinolones including gastrointestinal, hepatic, and central nervous system toxicity, disrupted glucose metabolism, phototoxicity, hypersensitivity, and skin disorders (Liu 2010). Moreover, some excitatory events such as confusion, weakness, loss of appetite, tremor, or depression are of particular concern in the elderly population (Stahlmann and Lode 2010), while arthropathy and tendinopathy have been observed in children treated with these pharmacologically active substances (Rosanova et al. 2010). Due to these possible effects, maximum residue limits (MRLs) have been established for some quinolones in foodstuffs of animal origin in the European Union (Commission Regulation 2010). According to Commission Regulation (EU) No. 37/2010, these antibiotics are forbidden in laying hens which produce eggs for human

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consumption (Table 1). Therefore, a zero tolerance policy is applied for these antibacterial compounds in eggs, which means that the presence of any quinolone is illegal at any level. Nevertheless, quinolones are still used in animal production systems, particularly in the poultry sector, for the prophylaxis of *Salmonella* spp. infections in laying hens (San Martín et al. 2005) but, at the same time, they are the antimicrobials of choice for treatment of severe or systemic human salmonellosis. Therefore, the use of antimicrobial agents in animals intended to human consumption is probably the main cause for the occurrence and spread of resistant *Salmonella* strains causing failure of clinical treatment (Souza et al. 2010).

The Council Directive 96/23/EC (Council Directive 1996) requires member states of the European Union to adopt and implement a national residues monitoring plan for specific groups of substances. The member states target the groups of animal/gender/age combinations where the probability of finding residues is the highest, and quinolones belong to the antibiotics investigated in muscle tissue of different animal species as well as in eggs. It appears then evident that the simultaneous determination of quinolones in muscle and eggs would be particularly advantageous in terms of cost and analysis time considering the monitoring plans.

Several methods have been developed for the determination of quinolone residues in a single matrix (Yorke and Froc 2000; Bailac et al. 2006; Hassouan et al. 2007). They generally involve a liquid–liquid extraction and a solid-phase extraction (SPE) to pre-concentrate and clean up the extracts. Then, these methods make use of high performance liquid chromatography (HPLC) separation with ultraviolet (UV) (Bailac et al. 2006) or fluorescence (FLUO) detection (Yorke and Froc 2000; Hassouan et al. 2007). Simultaneous detection of quinolones both in muscle and eggs by HPLC-UV has been previously proposed by Christodoulou et al. (2007), but nowadays, the liquid chromatography-mass spectrometry (LC-MS/MS) is the preferred technique due to

its high selectivity and sensitivity (Durden and MacPherson 2007; Rubies et al. 2007; Gajda et al. 2012). The aim of this study was to develop a LC-MS/MS confirmatory method for the simultaneous determination of the following quinolones: marbofloxacin (MAR), norfloxacin (NOR), ciprofloxacin (CIP), danofloxacin (DAN), lomefloxacin (LOM), enrofloxacin (ENR), sarafloxacin (SAR), difloxacin (DIF), oxolinic acid (OXO), nalidixic acid (NAL), and flumequine (FLU) in two different matrices (muscle and eggs) by using the same extraction and clean up procedure. The analytical method was validated according to the performance criteria of the Commission Decision 2002/657/EC (Commission Decision 2002) in terms of specificity, linearity, precision, decision limit, detection capability, and ruggedness. The method was also applied to the national residues monitoring plan for quinolones detection and quantitation.

Materials and Methods

Reagents and Standard Solutions

All reagents and solvents were of analytical or HPLC grade quality and supplied by Sigma (St. Louis, MO, USA). Ultrapure water was obtained by Elga Labwater (Wicomb, UK). Oasis hydrophilic-lipophilic balance (HLB) SPE cartridges (60 mg/3 mL) were from Waters (Milford, MA, USA).

Metaphosphoric acid 1 % solution was prepared by dissolving metaphosphoric acid in ultrapure water.

The extraction solution was obtained by mixing methanol and 1 % metaphosphoric acid solution (40:60, v/v).

Orthophosphoric acid 0.025 M solution pH 3 was prepared by diluting orthophosphoric acid 85 % in ultrapure water and adjusting pH with sodium hydroxide 1 N (washing solution for SPE columns).

Table 1 Maximum residue limits for quinolones in muscle tissues of food producing animals according to Commission Regulation (EU) no. 37/2010

Quinolone	Marker residue	Animal species ^a	MRL (µg/kg)
Danofloxacin	Danofloxacin	Bovine, ovine, caprine, poultry	200
		All other food producing species	100
Difloxacin	Difloxacin	Bovine, ovine, caprine, porcine	400
		Poultry and all other species	300
Enrofloxacin	Sum of enrofloxacin and ciprofloxacin	All food producing species	100
Flumequine	Flumequine	Bovine, ovine, caprine, porcine, and all other species	200
		Poultry	400
		Fish	600
Marbofloxacin	Marbofloxacin	Bovine, porcine	150
Oxolinic acid	Oxolinic acid	All food-producing species	100
Sarafloxacin	Sarafloxacin	<i>Salmonidae</i>	30

^a Not for use in animals from which eggs are produced for human consumption

The solution used for the analyte elution from SPE columns was prepared by mixing ammonium hydroxide 30 % and methanol (5:95, v/v).

The quinolone reference substances were of the highest available purity. Flumequine, danofloxacin, nalidixic acid, oxolinic acid, and marbofloxacin were supplied by Fluka (Steinheim, Germany), norfloxacin and lomefloxacin by Sigma (St. Louis, MO, USA), and ciprofloxacin, enrofloxacin, difloxacin, and sarafloxacin were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The internal standard norfloxacin d-5 (NOR d-5) was obtained by Witega (Berlin, Germany).

Individual stock standard solutions (1000 mg/L) were prepared for each analyte and for the internal standard in methanol containing 0.1 % sodium hydroxide 1 N, then they were stored in screw-capped glass tubes at +4 °C and were stable for 7 months.

A working standard solution for muscle analysis, containing ciprofloxacin, oxolinic acid, danofloxacin, enrofloxacin at 5 mg/L, marbofloxacin at 7.5 mg/L, flumequine at 10 mg/L, difloxacin at 15 mg/L, norfloxacin, lomefloxacin, sarafloxacin, and nalidixic acid at 1 mg/L, was prepared in methanol.

A working standard solution for eggs analysis was prepared in methanol at 0.5 mg/L.

Working internal standard solutions were prepared at 5 and 0.5 mg/L in methanol for muscle and egg analysis, respectively.

Sample Treatment

An aliquot of 5-g muscle sample was spiked with 100 µL of internal standard solution at 5 mg/L (100 µg/kg), while 5 g of eggs was spiked with 100 µL of internal standard solution at 0.5 mg/L (10 µg/kg).

The analytes were extracted by adding 20 mL of methanol/metaphosphoric acid 1 % (40:60, v/v), then shaken at 200 rpm for 20 min on a horizontal shaker and sonicated for 10 min in ultrasonic bath. After centrifugation at 3200×g for 10 min, the supernatant was transferred into a 50-mL round bottom flask. The extraction was repeated once again, and the combined supernatants were collected and centrifuged. Twenty milliliters of supernatants was evaporated under a stream of nitrogen at 50 °C until volume reduction to about 10 mL. The extract was diluted with 10 mL orthophosphoric acid 0.025 M solution.

The supernatant was loaded into an Oasis HLB cartridge previously conditioned with 2 mL methanol and 2 mL ultrapure water. The column was washed with 5 mL metaphosphoric acid 1 % solution and 5 mL ultrapure water. The elution of analytes was obtained with 5 mL of ammonium hydroxide 30 %/methanol (5:95, v/v). The eluate was evaporated to dryness under a stream of nitrogen at 50 °C. The dry residue was dissolved in 500 µL (muscle) or 250 µL (eggs)

0.1 % formic acid in water, then transferred into a vial for LC-MS/MS analysis.

Liquid Chromatography-Mass Spectrometry

LC analysis was carried out by a Perkin Elmer HPLC system (Perkin Elmer, Waltham, MA, USA) constituted of a model 200 microbinary pump, a model 200 autosampler, equipped with a degasser, and a column oven. Chromatographic separation was obtained using a reversed-phase HPLC column (100 mm × 2.1 mm internal diameter (i.d.) 3.5 µm) Xterra MS C18 Waters (Milford, MA, USA) with a guard column Xterra MS C18 Waters (10 mm × 2.1 mm i.d. 3.5 µm). Column was kept at 25 °C, and the flow rate was set to 0.2 mL/min. The LC mobile phase solvents were acetonitrile (eluent A) and 0.1 % formic acid in water (eluent B). After an isocratic step at 2 % A for 5 min, eluent A was increased by a linear gradient from 2 to 70 % in 4 min, then brought to 100 % in 0.5 min and held for 4.5 min to rinse the column. Finally, the eluent A was lowered to 2 % in 1 min and the column re-equilibrated for 10 min. The injection volume was 10 µL. The mass spectrometer was an API 3000 triple quadrupole (Applied Biosystems, Toronto, ON, Canada) equipped with an electrospray interface set in the positive ionisation mode (ESI+), operating in multiple reaction monitoring (MRM), selecting one precursor ion and two productions for each analyte. The optimization of MS parameters was carried out by infusing individual solutions of analytes at concentration 1 mg/L in 0.1 % formic acid in water/methanol (50:50, v/v) at a flow rate of 10 µL/min. The individual MRMs with their transition parameters were reported in Table 2. The capillary voltage was set at 5.5 kV and the ion source temperature at 350 °C. Analyst 1.4.2. software was used for instrument control and data processing.

Validation Study for Muscle

The developed method was fully validated as quantitative confirmatory method according to the Commission Decision 2002/657/EC (Commission Decision 2002). The parameters taken into account were instrumental linearity, specificity, precision, trueness, decision limit (CC α) and detection capability (CC β), and ruggedness.

The instrumental linearity was evaluated by five-point calibration curves, containing the internal standard at 500 µg/L (Table 3). For each analyte, the curve concentration range was chosen to cover the three spiking levels tested in the validation study.

The specificity was tested by analysing 20 blank muscle samples of different species (poultry, bovine, swine, fish) in order to verify the absence of potential interfering compounds at the retention time of the substances under investigation. Method trueness and precision were evaluated by spiking six aliquots of a blank poultry muscle at three concentration levels depending on individual compounds. This experiment was

Table 2 MRM transitions for MS/MS analysis

Analyte	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	DP (eV)	CE (eV)	CXP (eV)
MAR	363.1	320.1	42	24	9
		345.1	42	27	17
NOR	320.1	276.1	36	27	17
		302.1	36	29	14
CIP	332.2	288.0	43	28	26
		314.0	43	30	15
DAN	358.2	340.1	60	33	15
		283.0	60	32	13
LOM	352.2	265.1	30	35	25
		308.2	30	26	8
ENR	360.2	316.1	35	29	19
		342.1	35	28	23
SAR	386.1	342.1	28	26	5
		368.0	28	37	11
DIF	400.2	356.1	35	28	15
		382.1	35	29	13
OXO	262.1	244.1	32	40	21
		216.1	32	25	19
NAL	233.1	215.2	29	25	9
		187.1	29	37	18
FLU	262.1	244.1	37	29	15
		202.0	37	45	13
NOR-d5	325.2	281.1	47	26	17
		307.1	47	29	9

DP declustering potential, CE collision energy, CXP collision cell exit potential

repeated in two different occasions for a total of 54 analyses. The spiking concentration levels, reported in Table 4, were selected as follows: (i) for analytes with the same MRL for all food-producing species, the spiking levels were 0.5–1–1.5 times the corresponding MRL; (ii) for analytes with different

Table 3 Calibration curve points for muscle

Analyte	Concentration ($\mu\text{g/L}$)				
MAR	187.5	375	750	1125	1500
NOR	25	50	100	150	200
CIP	125	250	500	750	1000
DAN	125	250	500	1000	1500
LOM	25	50	100	150	200
ENR	125	250	500	750	1000
SAR	25	50	100	150	200
DIF	375	750	1500	2000	3000
OXO	125	250	500	750	1000
NAL	25	50	100	150	2000
FLU	250	500	1000	1500	3000

MRLs depending on animal species (i.e., danofloxacin, difloxacin, flumequine), the spiking levels were established so as to include the concentration range of interest; (iii) for analytes with no MRL, the three spiking levels were C_0 – $2C_0$ – $3C_0$, being C_0 the lowest concentration at which both qualitative and quantitative acceptance criteria were fulfilled.

Trueness was expressed in terms of recovery (percentage of measured concentration versus fortified concentration) and precision (as relative standard deviation, %RSD). CC α and

Table 4 Validation data for muscle

Analyte	<i>r</i>	Spiking levels ($\mu\text{g/kg}$)	Recovery (%) (<i>n</i> = 18)	Repeatability (%RSD) (<i>n</i> = 6)	Within-laboratory reproducibility (%RSD) (<i>n</i> = 18)
MAR	0.993	75	97	8	11
		150	103	6	9
		225	99	4	4
NOR	0.983	10	97	10	16
		20	102	7	14
		30	99	7	8
CIP	0.988	50	98	9	12
		100	102	6	10
		150	99	5	5
DAN	0.986	50	91	14	23
		100	107	8	16
		200	99	6	7
LOM	0.987	10	95	9	13
		20	105	8	12
		30	98	6	7
ENR	0.989	50	100	8	9
		100	100	7	8
		150	100	5	5
SAR	0.991	10	98	5	7
		20	101	6	8
		30	99	5	5
DIF	0.988	150	98	8	8
		300	102	7	8
		400	99	10	10
OXO	0.995	50	99	6	7
		100	101	8	9
		150	96	11	11
NAL	0.988	10	99	7	11
		20	101	6	10
		30	100	6	7
FLU	0.987	200	97	9	13
		400	103	6	12
		600	99	6	7

r correlation coefficient of matrix calibration curve

$CC\beta$ were calculated according to the matrix calibration curve procedure reported in the Commission Decision 2002/657/EC (Commission Decision 2002). The method ruggedness was estimated for minor and major changes by means of the Youden approach (Youden and Steiner 1975).

Validation Study for Eggs

The validation approach above described for muscle was also used for eggs. The instrumental linearity was evaluated by five-point calibration curves (12.5–25–50–100–200 $\mu\text{g/L}$), each one containing 100 $\mu\text{g/L}$ of internal standard.

The specificity was tested by analysing 20 blank egg samples collected from different laying hen flocks in order to verify the absence of potential interfering substances with the examined quinolones. Matrix calibration curves obtained by spiking six aliquots of a blank egg sample at three concentration levels (5–10–15 $\mu\text{g/kg}$) on three separate days were used again to evaluate trueness and precision.

$CC\alpha$ and $CC\beta$ were calculated by the matrix-matched approach. Method ruggedness was estimated only for minor changes.

Results and Discussion

Sample Clean Up

Sample preparation represents a critical step for the analysis of quinolones at trace level in food samples, as muscle and eggs, which may contain many different endogenous compounds. In order to develop a suitable method to analyze these antibiotics at low concentration level, it is important to isolate the analytes from food matrices as much as possible and separate them from co-extracted interfering compounds.

In this study, the quinolones were extracted from muscle and eggs with a mixture of methanol and metaphosphoric acid 1 % solution (40:60, v/v). Methanol was selected for its affinity for quinolones though it must be eliminated by evaporation

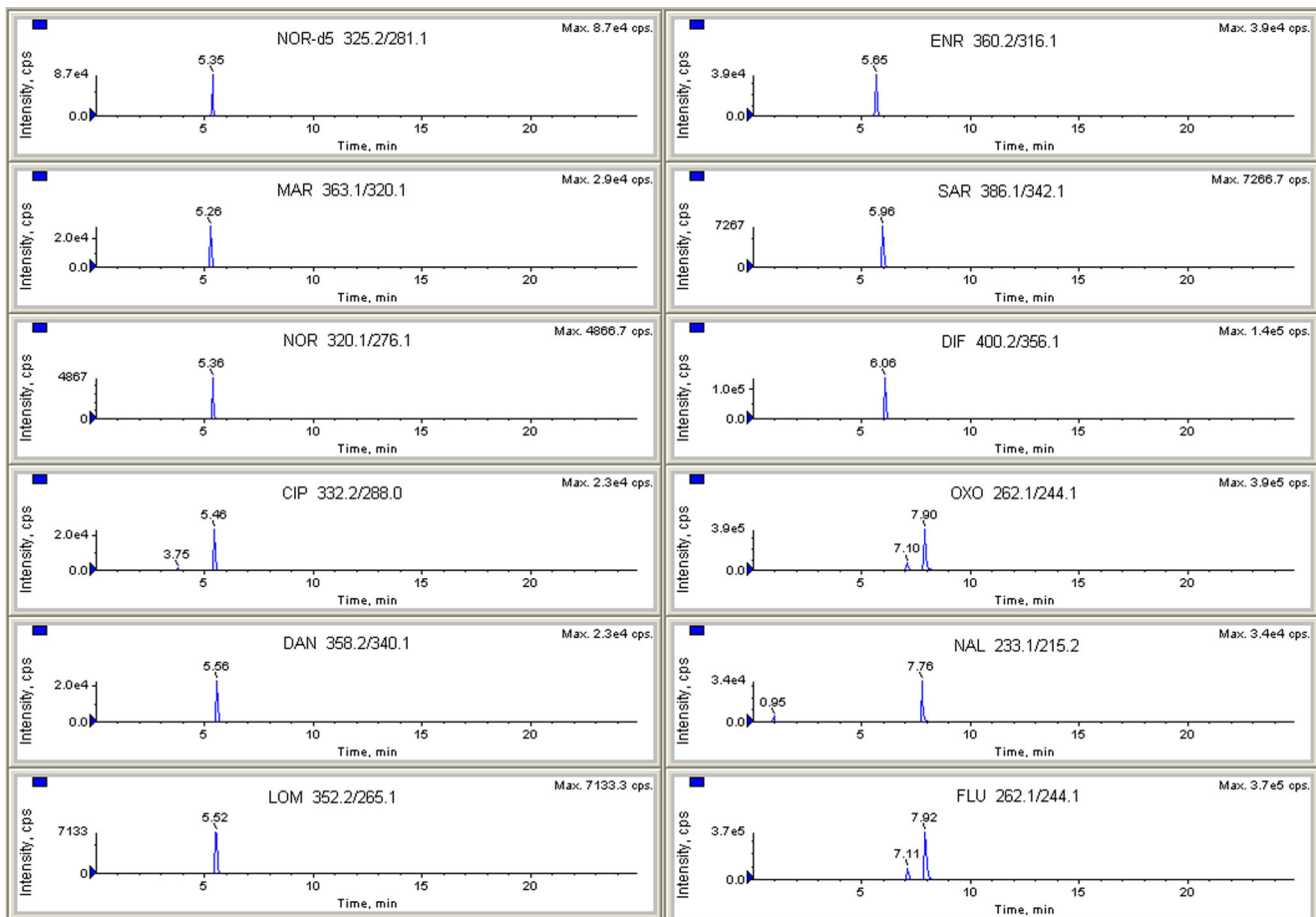


Fig. 1 Chromatogram of standard solution for muscle at the following concentrations: norfloxacin, lomefloxacin, sarafloxacin, and nalidixic acid at 25 $\mu\text{g/L}$, ciprofloxacin, danofloxacin, enrofloxacin, and oxolinic

acid at 125 $\mu\text{g/L}$, marbofloxacin at 187.5 $\mu\text{g/L}$, flumequine at 250 $\mu\text{g/L}$, difloxacin at 375 $\mu\text{g/L}$, and norfloxacin-d5 at 500 $\mu\text{g/L}$. For each analyte, the most intense product ion was selected

before the clean up. This step is necessary to avoid analytes loss in loading on the SPE cartridges. The Oasis HLB stationary phase was chosen for sample purification because its sorbent copolymer represents a hydrophilic-lipophilic balance system able to provide high and reproducible recoveries for quinolones, even if the cartridge runs dry.

Liquid Chromatography-Mass Spectrometry

The column and chromatographic gradient reported above allowed to obtain a good resolution for all the analytes. This is necessary for a quantitative detection of all the analytes. As in low-resolution mass spectrometry oxolinic acid and flumequine have the same precursor ion as well as the most intense product ion (Table 2), they need a good separation during the chromatographic run in order to be unequivocally identified on the basis of the retention time corresponding to 7.1 and 7.9 min, respectively.

The LC-MS/MS method was developed according to the performance criteria for mass spectrometric detection suggested in the Commission Decision 2002/657/EC (Commission Decision 2002). The analysis was performed

in MRM mode due to high sensitivity and selectivity. This approach allowed to achieve the identification points required by the aforementioned document for the identification of these compounds.

Validation Study

Instrumental linearity was evaluated for all the analytes on five concentration points, three replicates for point. For the muscle, the calibration levels were reported in Table 3 while for the eggs the levels were 12.5–25–50–100–200 $\mu\text{g/L}$. Linearity was estimated by using the least square regression line equation. Calibration curves in solvent were constructed using the area ratio of the analyte peak to the internal standard peak versus analyte concentration except for oxolinic acid, flumequine, difloxacin, and nalidixic acid for which the internal standard was not taken into account. The correlation coefficient (r) indicated a good fit for all the analytes with values included in the 0.998–0.999 range for muscle and 0.997–0.999 range for eggs.

The chromatograms of standard solutions were reported in Figs. 1 and 2, whereas Figs. 3 and 4 showed the

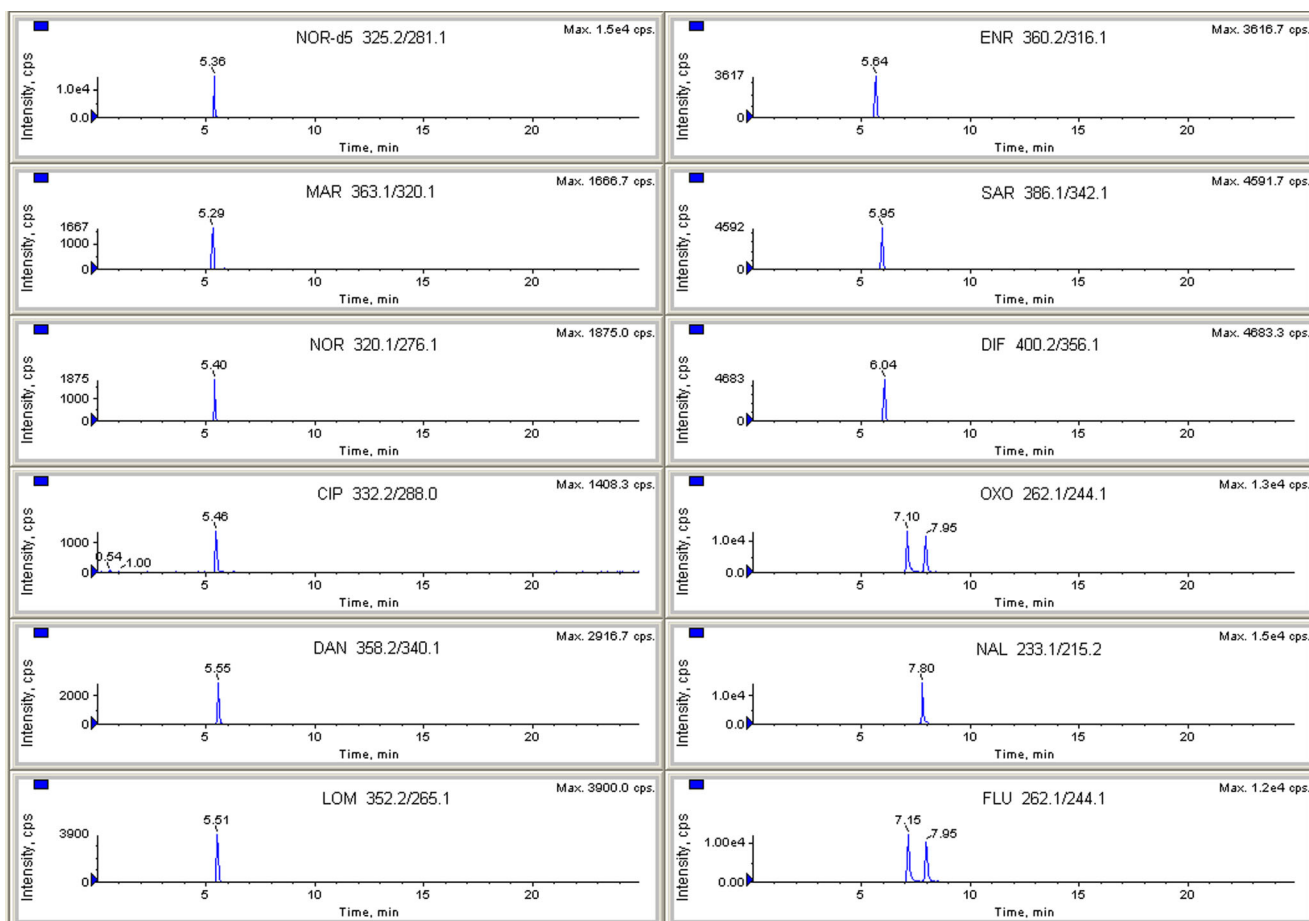


Fig. 2 Chromatogram of standard solution for eggs at concentration of 12.5 $\mu\text{g/L}$ for all the analytes and 100 $\mu\text{g/L}$ for the internal standard. For each analyte, the most intense product ion was selected

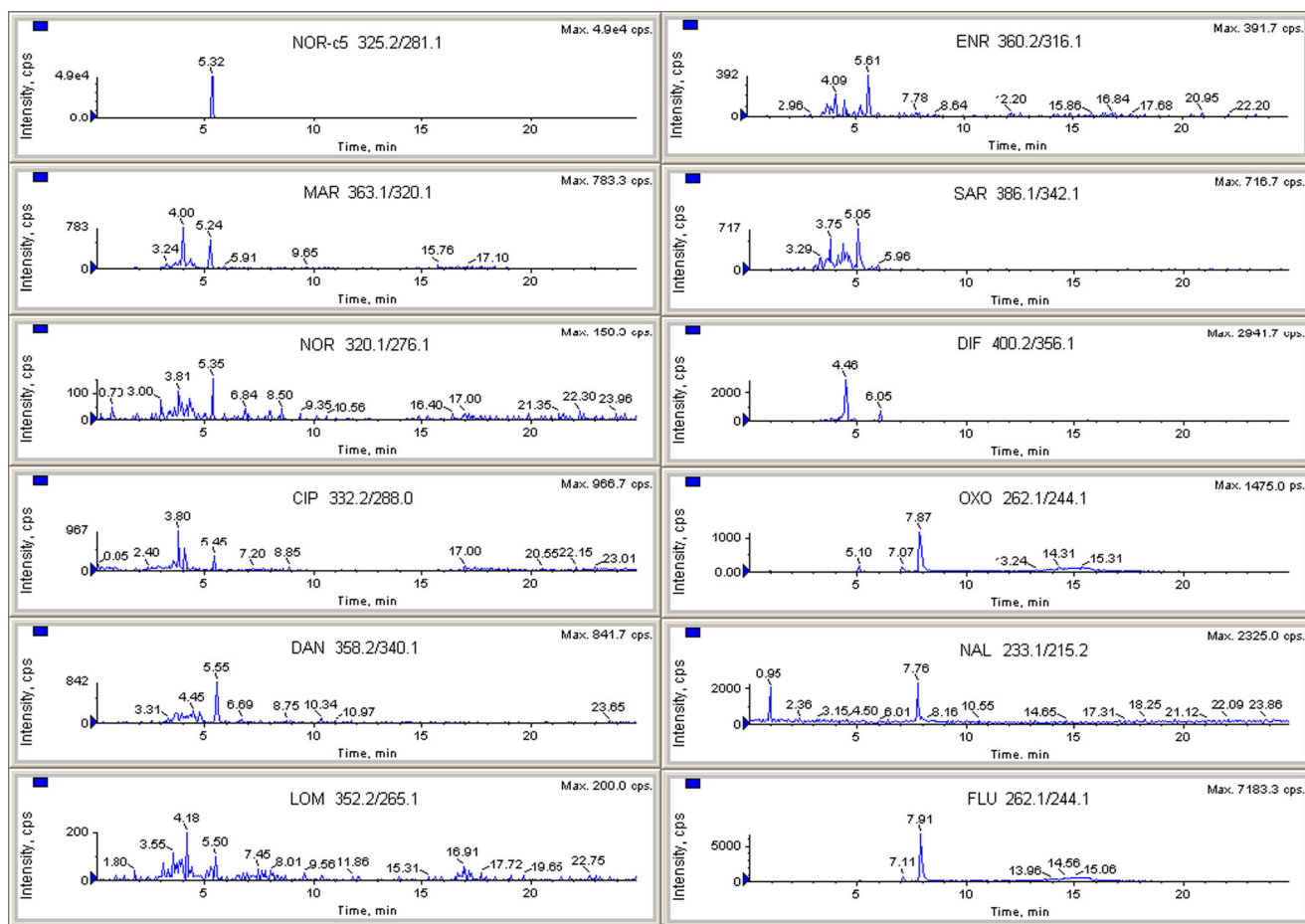


Fig. 3 Chromatogram of a representative blank poultry muscle spiked with 100 $\mu\text{g}/\text{kg}$ for norfloxacin-d5. For each analyte, the most intense product ion was selected

chromatograms of a representative blank sample for muscle and eggs, respectively. Regarding to specificity, the comparison between the chromatogram of the standard solution with the blank samples highlighted the specificity of the described method. The absence of any interfering peaks around the analyte retention times demonstrated the selectivity of the clean up procedure.

Blank muscle and egg samples were spiked with all the selected quinolones at three concentration levels. Figures 5 and 6 showed the chromatograms of spiked muscle and eggs at the lowest validation level, respectively. The signal of the lowest intense product ion appeared fully satisfactory with respect to signal-to-noise ratio.

Precision was evaluated at three spiking levels (six replicates per level, three analytical series) since no certified reference materials were available for the examined quinolones. For all the analytes, the recoveries were calculated by solvent calibration curves plotting area of analyte divided area of internal standard versus the concentration of analyte, except for oxolinic acid, flumequine,

difloxacin, and nalidixic acid for which the internal standard response was not taken into account, because during the sample preparation the internal standard caused a recovery enhancement (above 100 %) and was not suitable for quantitative purposes, but it was used only as process control.

Precision was calculated applying the one-way analysis of variance and expressed in terms of repeatability (intra-day) and within-laboratory reproducibility (inter-day) as %RSD. Validation data obtained for all the analytes in muscle and eggs were summarized in Tables 4 and 5. The mean recoveries for all the quinolones ranged between 91 and 107 % for muscle and 95 and 105 % for eggs. The maximum values of repeatability and within-laboratory reproducibility were, respectively, 14 and 23 % for muscle and 16 and 21 % for eggs, fulfilling the requirements established by the Commission Decision 2002/657/EC (Commission Decision 2002).

Matrix-matched calibration curves deriving from precision experiments were used to calculate $CC\alpha$ and $CC\beta$

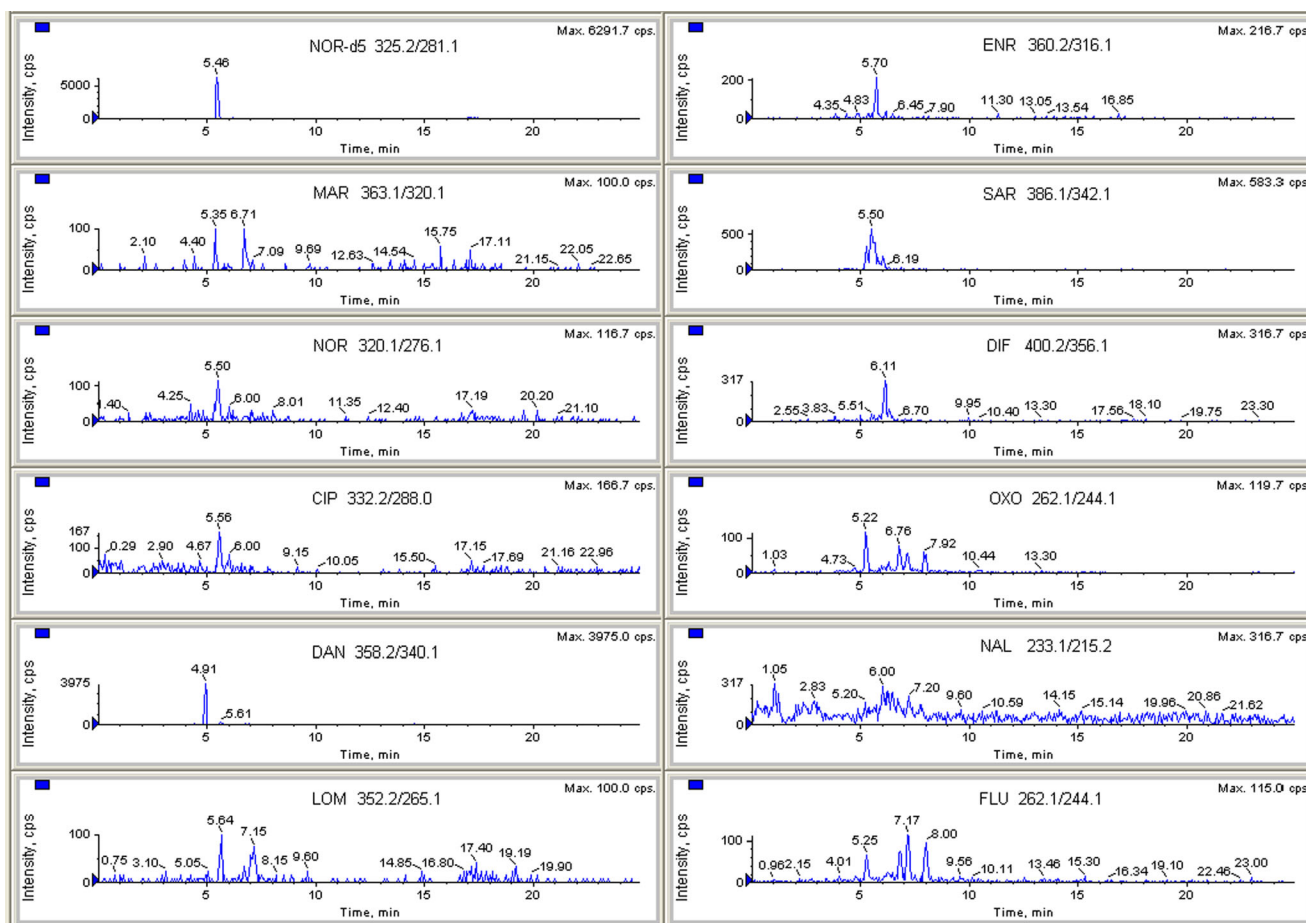


Fig. 4 Chromatogram of a representative blank egg spiked with 10 µg/kg for norfloxacin-d5. For each analyte, the most intense product ion was selected

values (Table 6). These parameters were established for each analyte at the MRL using the following equations:

$$CC\alpha = MRL + 1.64s_{RW,MRL}$$

$$CC\beta = CC\alpha + 1.64s_{RW,MRL}$$

where $s_{RW,MRL}$ was the within-laboratory standard deviation calculated by reproducibility data obtained at MRL.

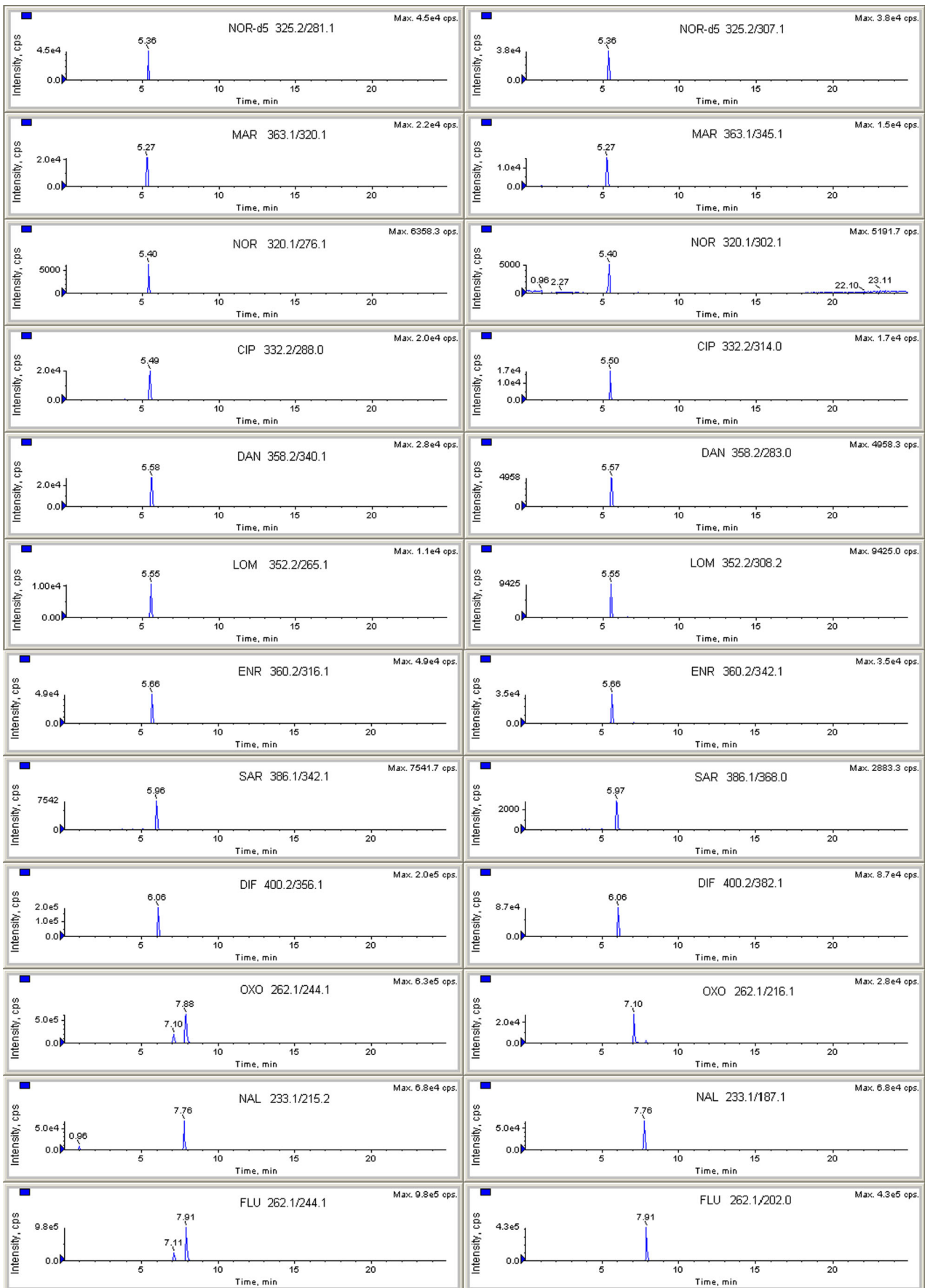
For quinolones with different MRL in muscle based on animal species (danofloxacin, difloxacin, flumequine), $CC\alpha$ and $CC\beta$ values calculated from the lowest MRL were shown in Table 6.

For the analytes without MRL in muscle (norfloxacin, lomefloxacin, sarafloxacin, nalidixic acid), $CC\alpha$ was calculated starting from C_0 (10 µg/kg) plus 2.33-fold the within-laboratory standard deviation calculated by reproducibility data obtained at this level. $CC\beta$ was calculated starting from $CC\alpha$ plus 1.64-fold the within-laboratory standard deviation calculated by reproducibility data obtained at C_0 . On the other hand, additional experiments at $CC\alpha$ did not appear strictly necessary considering that, for the confirmatory methods, $CC\beta$ is a parameter not directly involved in the assessment of sample compliance.

For eggs, $CC\alpha$ and $CC\beta$ were calculated as for the analytes without MRL in muscle, starting from the lowest spiking level that was equal to 5 µg/kg.

The method ruggedness was estimated by means of the Youden robustness test. Seven different critical factors, chosen in the entire analytical process (minor changes), and their levels of variation were reported in Table 7. Eight aliquots of a blank muscle sample spiked at MRL (the lowest one for danofloxacin, difloxacin, flumequine) or at C_0 (for the analytes without MRL) were used for all the experiments. For eggs, the ruggedness test was performed on eight aliquots of a blank eggs sample spiked at C_0 with all the analytes. For both matrices, the standard deviation of the differences between the two levels of each factor was calculated according to the Youden approach. It was demonstrated that all the

Fig. 5 Chromatogram of a representative blank poultry muscle spiked at the following concentrations: norfloxacin, lomefloxacin, sarafloxacin, and nalidixic acid at 10 µg/kg, ciprofloxacin, danofloxacin, enrofloxacin, and oxolinic acid at 50 µg/kg, marbofloxacin at 75 µg/kg, flumequine at 200 µg/kg, difloxacin at 150 µg/kg, and norfloxacin-d5 at 100 µg/kg. For each analyte, all the studied ions were shown



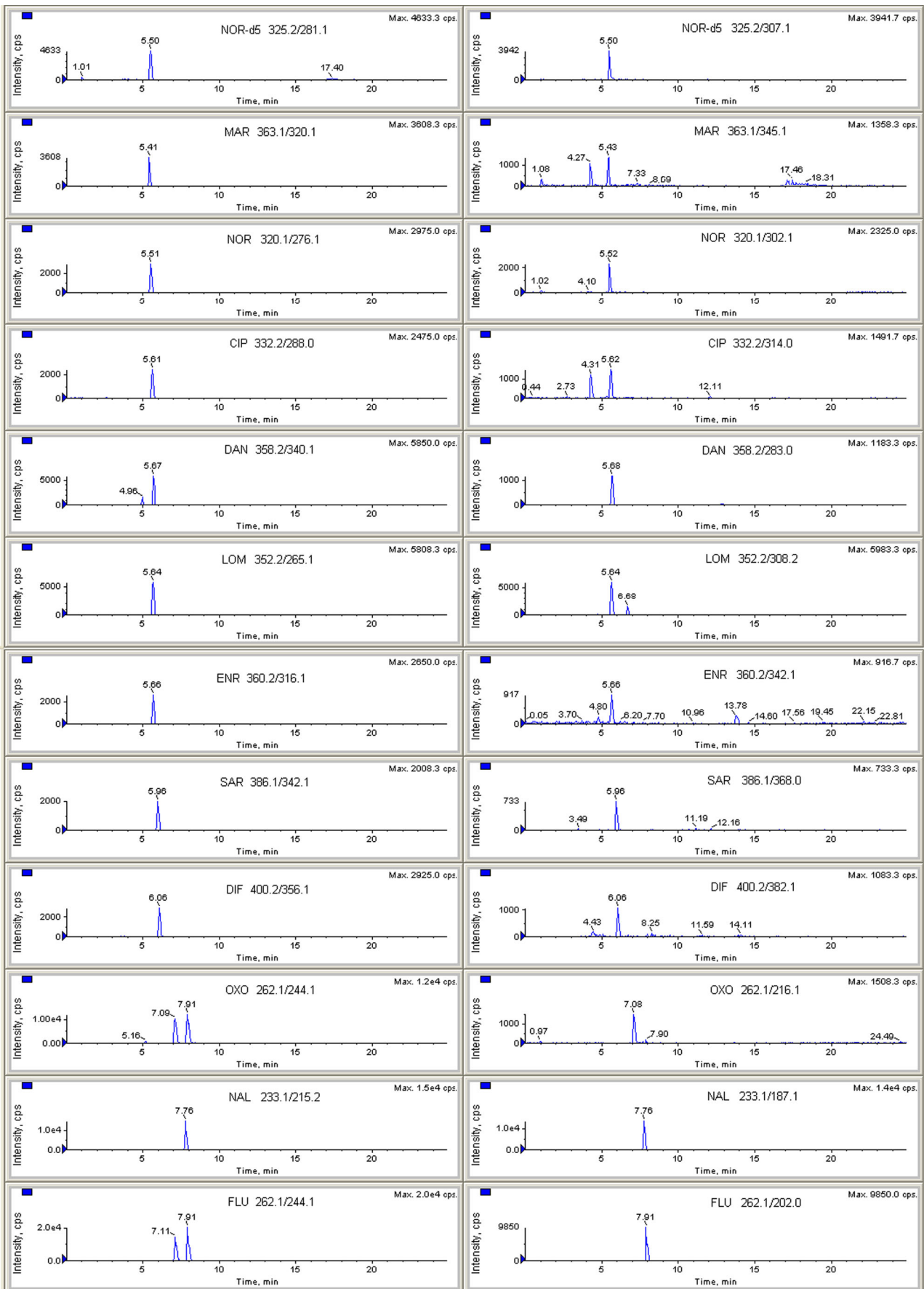


Fig. 6 Chromatogram of a representative blank egg spiked with 5 µg/kg for the analytes and 10 µg/kg for norfloxacin-d5. For each analyte, all the studied ions were shown

selected factors did not significantly affect the analytical performance and therefore they had no significant effect on ruggedness.

For muscle, the Youden approach was also used to test the method applied to different animal species, such as bovine and swine (major changes). Consequently, this

Table 5 Validation data for eggs

Analyte	<i>R</i>	Spiking levels (µg/kg)	Recovery (%) (<i>n</i> = 18)	Repeatability (%RSD) (<i>n</i> = 6)	Within-laboratory reproducibility (%RSD) (<i>n</i> = 18)
MAR	0.994	5	100	4	8
		10	99	5	9
		15	100	6	7
NOR	0.996	5	101	5	5
		10	99	4	4
		15	100	3	3
CIP	0.995	5	98	4	9
		10	102	4	9
		15	99	5	5
DAN	0.975	5	100	9	17
		10	100	11	17
		15	100	13	14
LOM	0.979	5	96	8	11
		10	104	5	9
		15	99	11	11
ENR	0.970	5	99	7	12
		10	101	11	15
		15	100	10	11
SAR	0.971	5	95	11	16
		10	105	6	13
		15	98	9	10
DIF	0.986	5	96	13	18
		10	104	9	15
		15	98	12	12
OXO	0.973	5	100	11	20
		10	100	11	21
		15	100	12	13
NAL	0.983	5	102	11	17
		10	98	9	16
		15	101	15	16
FLU	0.989	5	100	9	13
		10	100	7	12
		15	100	16	17

r correlation coefficient of matrix calibration curve

Table 6 CC α and CC β data (µg/kg) for muscle and eggs

Analyte	Muscle		Eggs	
	CC α	CC β	CC α	CC β
MAR	173	194	6.0	6.8
NOR	14	17	5.6	6.1
CIP	116	132	6.0	6.9
DAN	126	151	6.9	8.8
LOM	13	16	6.3	7.5
ENR	113	126	6.4	7.7
SAR	32	35	6.9	8.7
DIF	339	390	7.1	9.2
OXO	115	135	7.4	9.8
NAL	13	15	7.0	9.0
FLU	234	282	6.6	8.0

method proved to be fairly robust and able to withstand minor fluctuations in the routinely operating factors; moreover, the different animal species did not have significant effect on the analytical results.

By a comparison with other previously published studies, the present method showed some important advantages. First of all, it allowed to apply the same sample preparation to two different matrices (muscle and eggs), whereas other methods (Bailac et al. 2006; Gajda et al. 2012) were carried out only for one matrix. Moreover, it was validated according to the Commission Decision 2002/657/EC (Commission Decision 2002), ruggedness included, whereas other authors as Shen et al. (2008) did not follow the above-cited decision. Finally, this method permitted the identification and confirmation by LC-MS/MS of all the quinolones included in the Commission Regulation 2010/37/EU (Commission Regulation 2010).

Table 7 Robustness test (minor changes): selected factors and levels of variations of experimental conditions

Selected factor	Method level	Low level	High level
Methanol in extraction solution (%)	40	36	44
Evaporation temperature before SPE (°C)	50	45	55
Oasis HLB cartridge lot (no.)	–	080A38157A	084038263A
pH washing solution for SPE	3.0	2.9	3.1
Ammonium hydroxide in elution solution (%)	5.0	4.5	5.5
Elution solution (mL)	5.0	4.5	5.5
Formic acid in mobile phase (%)	0.10	0.09	0.11

Table 8 Proficiency tests

Proficiency test service-year	Material code-matrix	Analyte-contamination level ($\mu\text{g}/\text{kg}$)	<i>z</i> score
Progetto Trieste-2010	M1021A-turkey muscle	Enrofloxacin-144	0.10
FAPAS-2011	2175-fish muscle	Ciprofloxacin-116	-0.10
FAPAS-2013	2200-eggs	Flumequine-953	0.60
		Danofloxacin-241	-0.70
		Norfloxacin-240	0.40
Progetto Trieste-2014	T1433-turkey muscle	Enrofloxacin-172	0.37

Proficiency Testing Results

During the validation study, our laboratory took part in proficiency tests of eggs and muscle of different animal species organized by Food Analysis Performance Assessment Scheme (FAPAS, www.fapas.com) and Progetto Trieste - Laboratory Proficiency Testing (Progetto Trieste, www.testveritas.com) in order to verify the confirmatory technique performance. The data obtained in these proficiency tests were reported in Table 8.

The results showed that the method was also applicable to fish muscle even if the ruggedness test did not include this species. Satisfactory results were obtained for eggs even at concentration levels out of the range considered in the validation study, thus demonstrating the good linearity of the method. Moreover, the method was successfully used for the analysis of samples taken in routine official control.

Conclusions

In the present study, a multiresidue and confirmatory method was developed and validated to be applied to two different matrices such as muscle and eggs. Different methods intended to a single matrix have been described in literature (Bailac et al. 2006; Clemente et al. 2006; Rubies et al. 2007; Shen et al. 2008; Gajda et al. 2012), even if the recoveries were usually equal to or lower than the values reported in our study.

The proposed confirmatory method fulfilled the requirements of the Commission Decision 2002/657/EC (Commission Decision 2002), showing satisfactory results with respect to selectivity and precision. Therefore, the developed method can be applied in laboratories involved in official residue controls for the determination of targeted quinolones.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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