DRUGS, COSMETICS, FORENSIC SCIENCES

Multiresidue Method for Simultaneous Determination of Ten Quinolone Antibacterial Residues in Multimatrix/Multispecies Animal Tissues by Liquid Chromatography with Fluorescence Detection: Single Laboratory Validation Study

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Quinolone antibacterials are veterinary drugs authorized for use in food animal production. The analysis of residual amounts of drugs in food from animal origin is important for quality control of products for consumers. For this purpose, Maximum Residue Limits (MRLs) have been set up by a European Union Council Regulation on Veterinary Drug Residues (No. 90/2377/EEC and subsequent), and 8 quinolones received MRLs at concentration levels depending on both the matrix and the animal species of interest. A method was developed for screening and confirming 10 quinolone residues (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, sarafloxacin) in a wide variety of matrixes of different animal species. It involves extraction of the residues from the biological tissues/fluids by acidic aqueous solution, centrifugation and filtration prior to injection on a C18 narrow-bore column, and detection through a 3-step-mode fluorescence detector. The method was validated during a 2-week study for a set of 8 species-matrixes (i.e., bovine raw milk, bovine muscle, porcine muscle, porcine kidney, porcine liver, fish flesh and skin, poultry muscle, whole egg). Residues were quantified down to 15 μg/kg with limits of detection and quantitation ranging from 4 to 11 and 13 to 36 μg/kg, respectively, which are sufficient compared to the wide range of MRLs set for these substances (from 30 μg/kg for danofloxacin in milk to 1900 μg/kg for difloxacin in poultry liver). The limit of performance of the method in terms of CCα and CCβ, the critical concentrations stated in the Decision No. 2002/657/EC and the ISO Standard No. 11843, has been calculated for the authorized (MRL) substances but only estimated in the case of the nonauthorized (non-MRL) substances.

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Quinolone antibacterials are authorized for use in livestock, poultry, and fish farm industries in cases of pulmonary, urinary, and digestive infections. They behave through inhibiting bacterial DNA-gyrase. Analysis of the residual amounts of veterinary drugs is important for quality control of food products for consumers and for evaluation of the correct application of withdrawal times. Under the European Union (EU) Food Law Legislative Framework (Directive Nos. 2001/82/EC and 96/23/EC; 1, 2), several quinolones have been regulated by the so-called Maximum Residue Limits (MRLs) regulation No. 2377/90/EC (3) and subsequents, with MRLs set for different food matrixes of animal origin (muscle, liver, kidney, fish flesh, egg, milk) and from various species (bovine, ovine, porcine, caprine, poultry, rabbit, farmed fish).

Several liquid chromatography/fluorescence detection (LC/FLD) methods (4–8) have been published and are practical for reliably quantifying at least 4 quinolone residues either in meat, fish, egg, or milk matrix at MRL levels. But only 2 LC/FLD methods have been described for the analysis of a complete set of at least 10 quinolones extracted from different matrixes (9, 10). Because of lack of selectivity on the separative columns, both methods proposed a set of 2 or 3 different runs for monitoring the entire set of residues. Consequently, mass spectrometry (MS) detection methods have recently been preferred for this scope of analysis (11–14).

The work presented here proposes a multimatrix method suitable for covering all the quinolone compounds of interest, and is based on a single run of injection into the LC/FLD chromatograph after a short extraction step. In line with the Decision 657/2002/EC (15), validation of the method was particularly designed to evaluate the residual contents upon

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Table 1. Review of the EU MRL (μg/kg) for quinolones in force in 2004

Regulation No.	Date	Annex of Reg. 2377/90 (ref. 3)	Compounds	Species	Milk	Muscle	Fat–skin	Liver	Kidney	Eggs
1181/02	01/07/02	I	Danofloxacin	All species other than hereafter	—	100	50	200	200	—
				Bovine, ovine, caprine	30	200	100	400	400	—
				Poultry	—	200	100	400	400	—
1181/02	01/07/02	I	Enrofloxacin + ciprofloxacin	All species other than hereafter	—	100	100	200	200	—
				Bovine, ovine, caprine	100	100	100	300	200	—
				Porcine, rabbit	—	100	100	200	300	—
				Poultry	—	100	100	200	300	—
1181/02	01/07/02	I	Flumequine	All species other than hereafter	—	200	250	500	1000	—
				Bovine, ovine, caprine, porcine	50	200	300	500	1500	—
				Poultry	—	400	250	800	1000	
				Fish	—	600 muscle + skin	—	—	—	—
1441/95	26/06/95	I	Sarafloxacin	Chicken	—	—	10	100	—	—
1850/97	25/09/97	III		Fish	—	30 muscle + skin	—	—	—	—
546/04	24/03/04	III	Oxolinic acid	Bovine	—	100	50	150	150	—
				Porcine	—	100	50	150	150	—
739/03	28/04/03	I		Chicken	—	100	50	150	150	Do not use for laying hens
				Fish	—	100 muscle + skin	—	—	—	—
1181/02	01/07/02	I	Difloxacin	All species other than hereafter	—	300	100	800	600	—
				Bovine, ovine, caprine	—	400	100	1400	800	—
				Porcine	—	400	100	800	800	—
				Poultry	—	300	400	1900	600	—
282/96	14/02/96	III	Decoquinate	Bovine, ovine	—	500	500	500	500	
2338/00	20/10/00	I	Marbofloxacin	Porcine		150	50	150	150	

the same frame of calibration, even though quinolone MRLs have been set on a particularly large range of concentrations considering all the compounds and all the matrixes, i.e., from 30 μg/kg for danofloxacin in milk to 1900 μg/kg for difloxacin in poultry liver (16–19). Table 1 reviews the 2004 status of EU MRLs for quinolones. An evaluation of the limits of detection (LOD) and quantitation (LOQ) for the different compounds is proposed. The new set of statistical performance limits recommended in the European Decision No. 657/2002/EC, i.e., the limit of decision, CCα, and the capacity of detection, CCβ, were also evaluated along with the principles proposed in the ISO Standard No. 11843 (20), specifically in the case of authorized substances.

Experimental

Reagents and Standards

(**a**) *Ciprofloxacin and enrofloxacin*.—Bayer AG Pharma (Zurich, Switzerland).

(**b**) *Danofloxacin*.—Pfizer, Inc. (Groton, CT).

(**c**) *Flumequine, norfloxacin, oxolinic, and nalidixic acids*.—Sigma Chemical Co. (St. Louis, MO).

(**d**) *Methanol and acetonitrile*.—Analytical reagent grade (Fisher Scientific, Loughborough, Leicestershire, UK).

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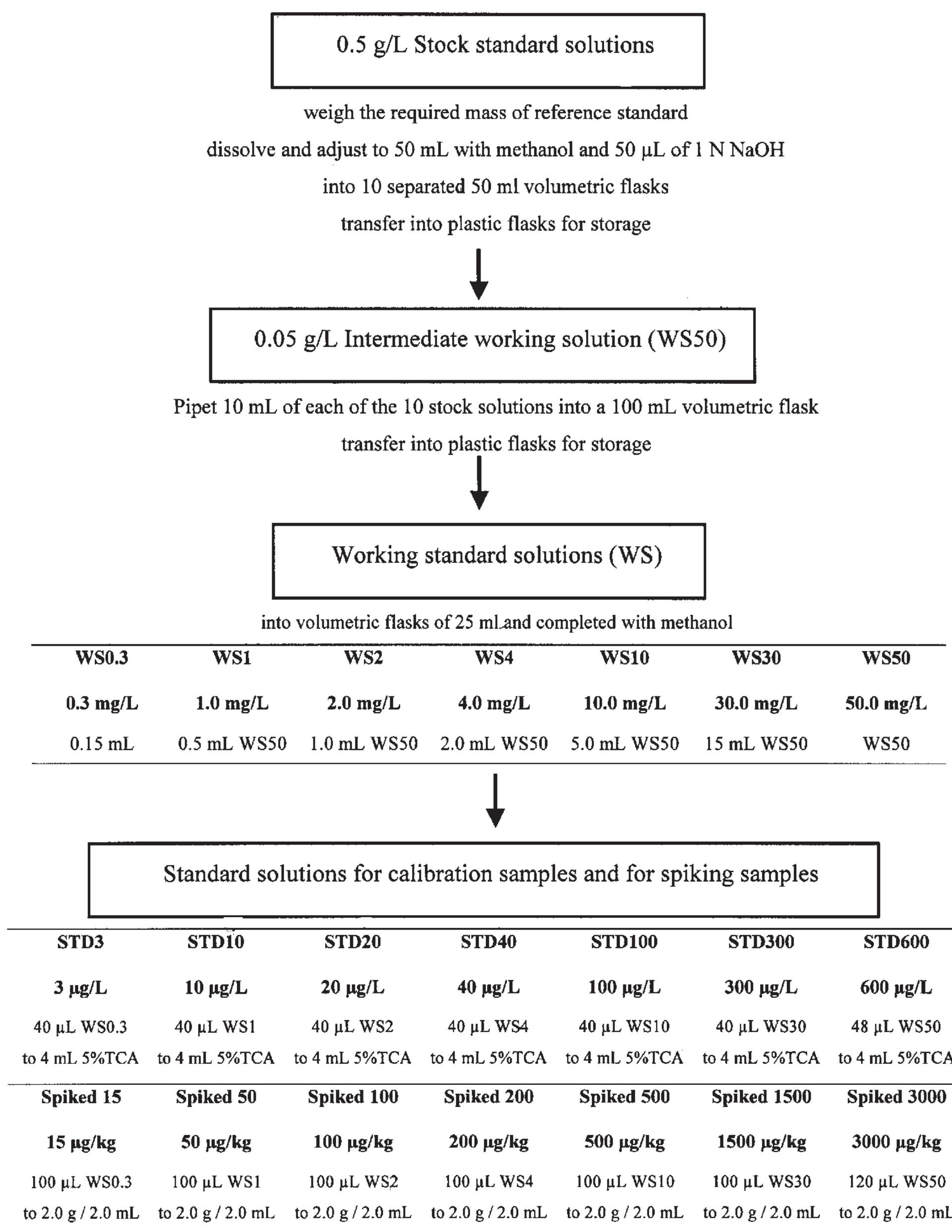

Figure 1. Preparation of working standard solutions and calibrating samples.

(**e**) *Demineralized ultrapure water*.—Obtained from an Alpha-Q ultrafiltration unit from Millipore (Molsheim, France).

(**f**) *Formic acid, trichloroacetic acid, and sodium hydroxide*.—Merck (Darmstadt, Germany), Fisher Scientific Labosi (Elancourt, France), and Prolabo (Fontenay-sous-bois, France), respectively.

Apparatus

(**a**) *Centrifuge*.—Ultraspeed centrifuge (model MR22i; Jouan, Nantes, France).

(**b**) *Liquid chromatograph*.—Composed of a P1000XR pump (Thermo Separation Products, San Jose, CA) with a membrane degassing device (Thermo Separation Products);

Table 2. Liquid chromatographic gradient mode

Time, min	0.1% Formic acid, %	Acetonitrile, %
0	93	7
13	93	7
17	60	40
20	60	40
30	93	7
45	93	7

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Table 3. FLD multistep detection

Time, min	Exc λ, nm[a]	Em λ, nm[b]	Gain
0.0–11.8	294	514	100
11.9–20.5	328	425	100
20.6–30.0	312	366	100

[a] Excλ = Excitation wavelength.

[b] Emλ = Emission wavelength.

an AS100XR autosampler fitted with 100 µL loop and 0.25 mL syringe (Thermo Separation Products); a C18 narrow-bore analytical column (150 × 2.0 mm id; 3.5 µm), model Luna (Phenomenex, Inc., Torrance, CA); a C18 guard column (10 × 2.1 mm; 3.5 µm), model Sentry Guard (Waters Corp., Milford, MA); an FP1520 spectrofluorimetric detector device (Jasco Co., Tokyo, Japan).

The data acquisition and LC system were controlled on a Pentium III station equipped with Chromquest software version 2.51 (Thermo Separation Products).

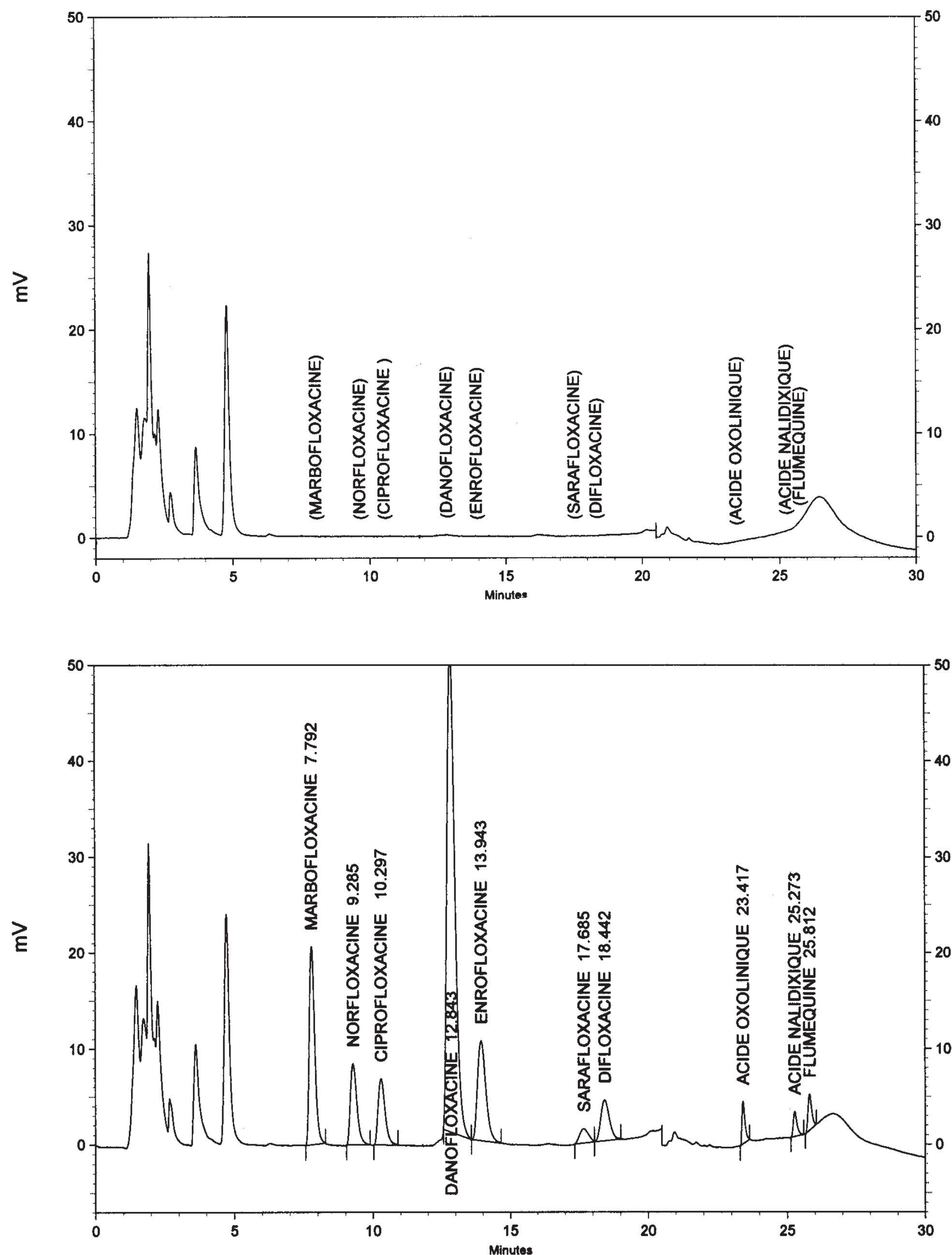

Figure 2. Typical chromatograms obtained from blank tissue samples and tissue samples: chicken muscle fortified to 100 µg/kg with the 10 quinolones.

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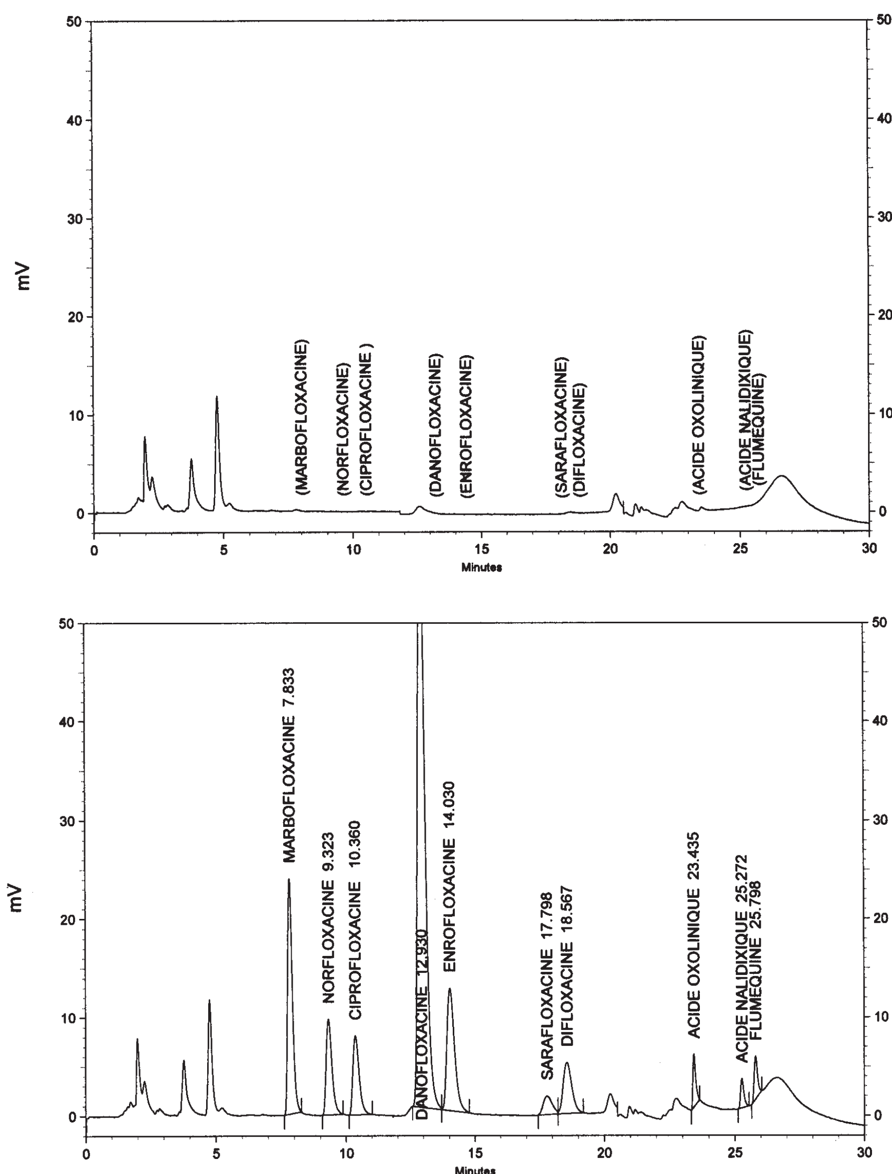

Figure 3. Typical chromatograms obtained from blank tissue samples and tissue samples: whole egg fortified to 100 μg/kg with the 10 quinolones.

Solutions

(**a**) *Mobile phase*.—A 1 L 0.1% formic acid solution was prepared by mixing 1 mL formic acid reagent with ultrapure water. The mobile phase, consisting of 0.1% formic acid and acetonitrile, was mixed by the pump in a gradient mode.

(**b**) *Extraction solution*.—A 5% trichloroacetic acid solution was prepared by dissolving 50 g trichloroacetic acid in 1 L ultrapure water.

(**c**) *Working standard solutions and external calibration curve*.—A 0.5 g/L stock solution was prepared for each of the 10 quinolones by dissolving the appropriate amount of standard in alkaline methanol (50 μL 1N sodium hydroxide in 50 mL methanol). A 0.05 g/L multiquinolone intermediate working solution was prepared by diluting 10 mL of each of the 10 stock solutions into a 100 mL glass-ambered volumetric flask. Stock solutions and intermediate solutions were stored in plastic ambered flasks in a cool, dark place for at least 3 months without any degradation observed. A range of 7 working standard solutions, each containing the 10 quinolones from 50 to 0.3 mg/L, was prepared by dilutions of the intermediate working solution and used for both the fortification of tissues from 3 mg/kg to 15 μg/kg and the external standard calibration from 600 to 3 ng/mL. Figure 1 describes the scheme of preparation for all working standard solutions.

Tissue/Liquid Sample Fortification

Thawed blank tissue sample (either bovine muscle, poultry muscle, fish flesh and skin, porcine muscle, porcine kidney, or

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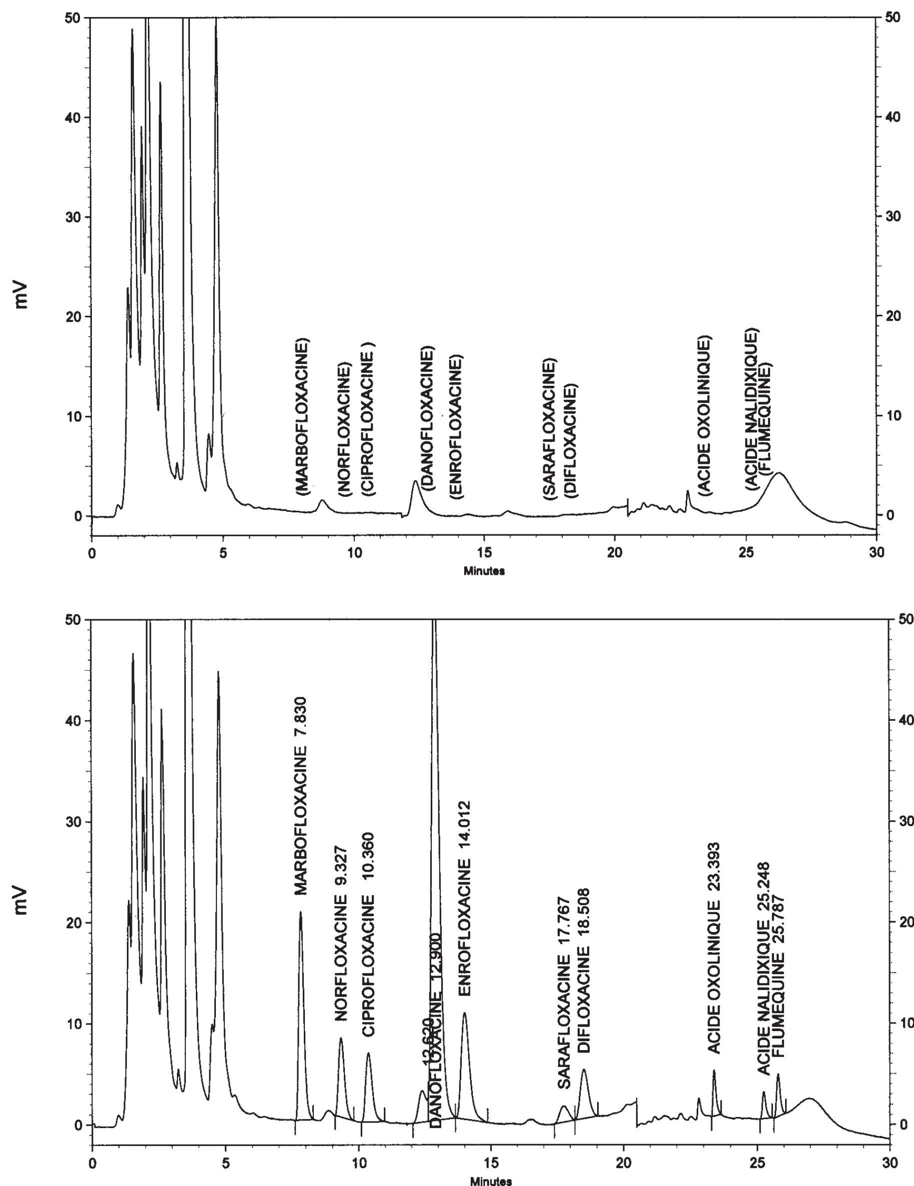

Figure 4. Typical chromatograms obtained from blank tissue samples and tissue samples: porcine kidney fortified to 100 μg/kg with the 10 quinolones.

porcine liver) was ground, or liquid sample (raw milk or whole egg) was mixed, and a 2.0 g/2.0 mL portion was transferred for extraction into a 16 mL clean plastic tube for extraction. A 100 μL volume of the appropriate spiking solution (WS) was added to the blank sample, mixed thoroughly for 1 min, and allowed to stand at least 15 min to obtain a set of quinolone-spiked samples at different levels of desired concentration ranging from 3 mg/kg to 15 μg/kg.

Extraction, Cleanup, and Analytical Procedure

Acidic extraction.—8 mL 5% trichloroacetic acid solution was added to the fortified sample or to the sample to be controlled. The sample was successively mixed for 1 min with a Vortex mixer and for 10 min with a rotary homogenizer (Model RheaxII-Heidolph, Kelheim, Germany), and then centrifuged for 5 min at 14 000 × *g* and thermostatted at +4°C. The aqueous supernatant was transferred to a clean tube and subsequently filtrated on a PVDF 0.45 μm filter in preparation for chromatographic autosampling in ambered vials. The extract should be stored in a cool, dark place or on the autosampler tray, protected from light and from excessive temperature by means of a cooling device (+10°C).

LC determination.—A 100 μL volume of the filtered extract was injected into the LC system operating in a gradient mode, as described in Table 2. The flow rate was set at 0.3 mL/min. The peak area of each of the 10 quinolones was detected by means of their native fluorescence. The fluorimeter was set in a multistep detection mode (Table 3).

Calculations

Recovery calculation.—To determine method recoveries, the fluorimetric responses for quinolones in fortified

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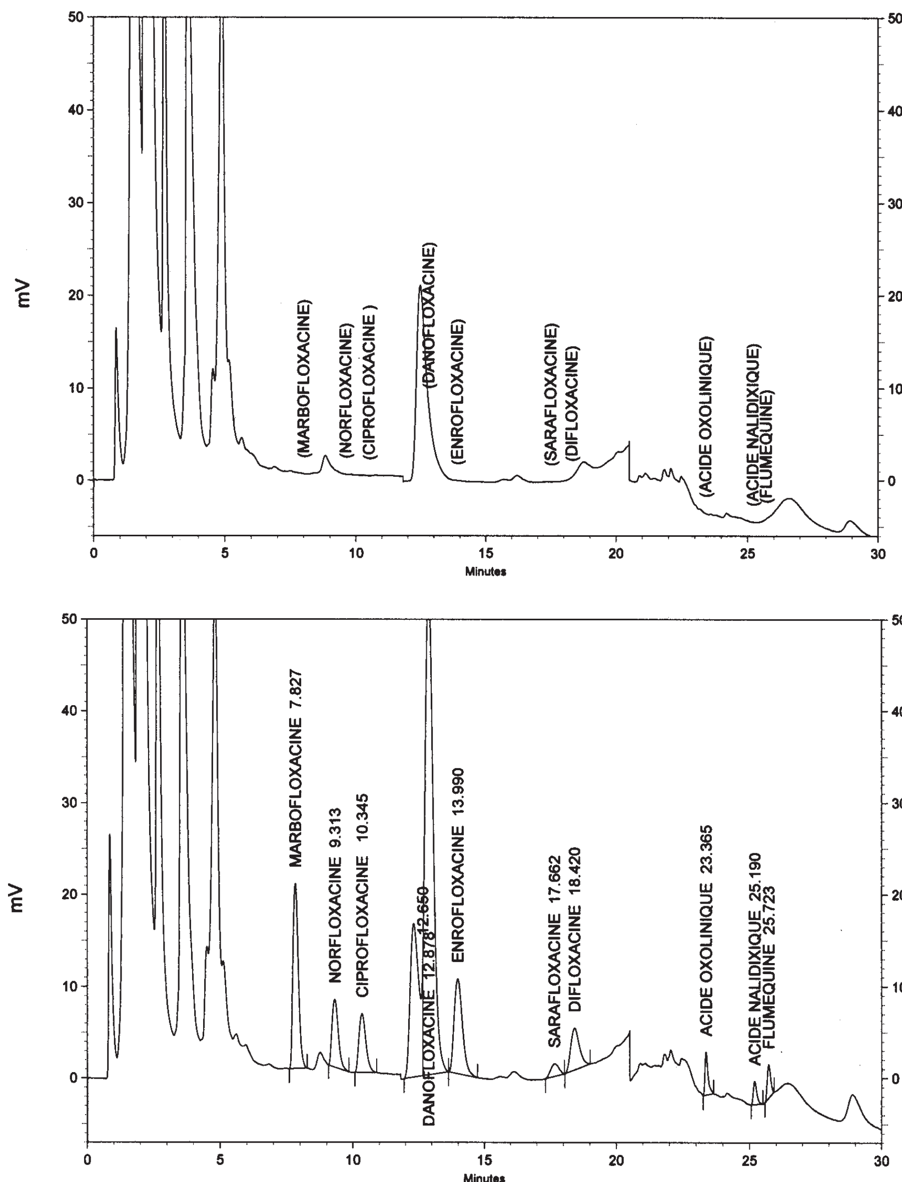

Figure 5. Typical chromatograms obtained from blank tissue samples and tissue samples: porcine liver fortified to 100 μg/kg with the 10 quinolones.

tissue/liquid samples subjected to extraction, cleanup, and LC analysis were compared with those of equivalent external quinolone standards, taking into account the dilution (×5) for the tissue/liquid samples. Recovery was assessed as:

$$R, \% = (S \times 100)/Se$$

where R is the recovery of the fortified sample, S is the peak area of the fortified tissue or liquid sample, and Se is the peak area of the corresponding external standard solution. Furthermore, the mean recovery of the method for each of the 10 quinolones was calculated, considering all the values corresponding to the 7 levels of concentration obtained during the whole validation study ($n = 112$).

Estimated concentration calculation.—The concentration of the fortified sample was calculated as:

$$C, \mu g/kg = (A \times Ce \times 100 \times 5)/Ae \times R)$$

where A is the peak area of the fortified sample, Ae is the peak area of the corresponding external standard solution, Ce is the concentration of the external standard solution, 5 equals the dilution operated during extraction (10 mL extract for 2 g/2 mL sample), and R is the percentage of recovery of the method for the designed quinolone compound.

Validation Scheme and Statistical Analysis

The method was validated on a single-laboratory validation scheme by fully implementing a set of criteria as requested by the European Decision No. 657/2002 (15) concerning "the performance of analytical methods and the interpretation of results" and, particularly, chapter 2.3 of the

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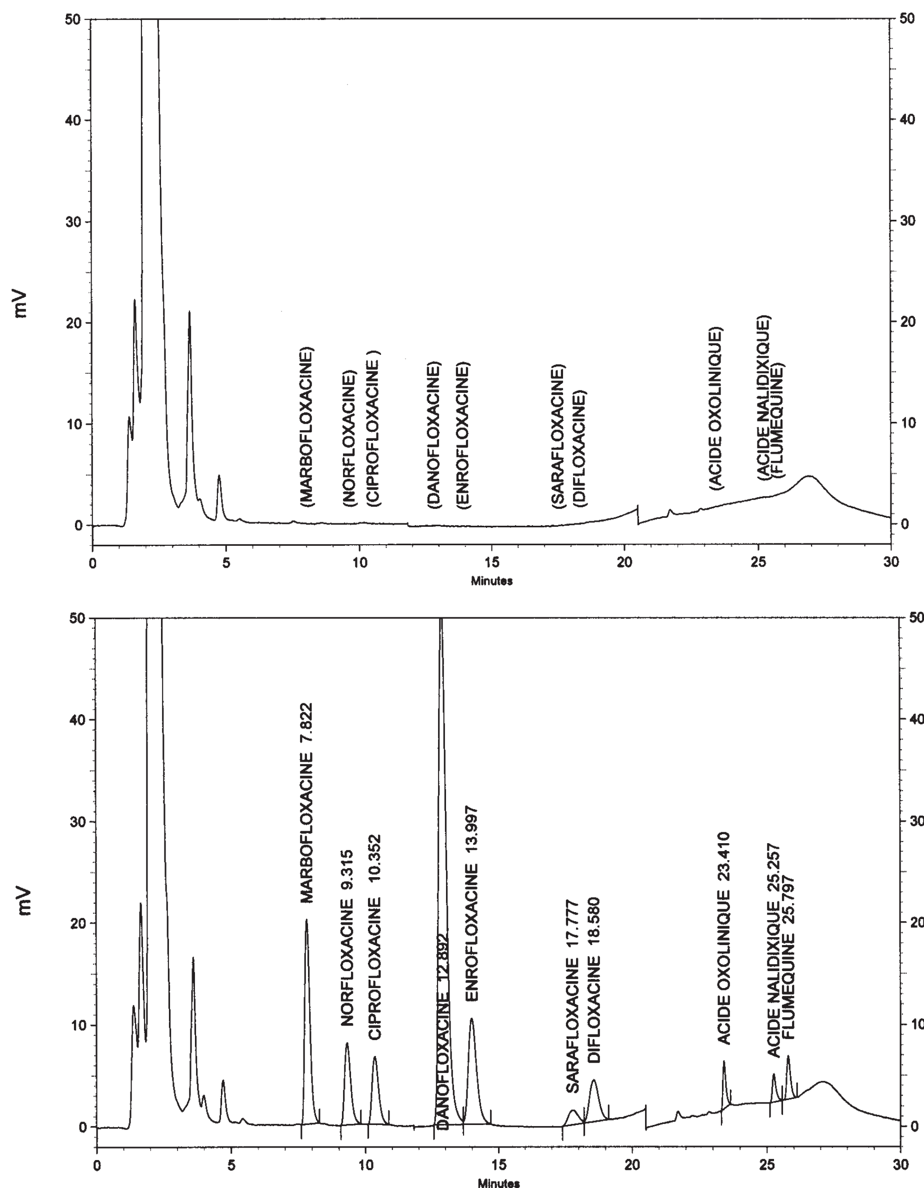

Figure 6. Typical chromatograms obtained from blank tissue samples and tissue samples: fish flesh and skin fortified to 100 μg/kg with the 10 quinolones.

Annex dealing with "confirmatory methods for organic residues and contaminants." For quantitative LC method testing, the main criteria to consider concern the chromatographic separation of the compounds (selectivity and specificity), the quality of calibration (linearity), the accuracy of the results in terms of trueness (taking into account the recovery correction) and precision (both repeatability and intralab reproducibility), and the sensitivity of the method by means of analytical limits (CCα and CCβ calculations).

(**a**) *Linearity*.—The linearity of areas for external standards and fortified samples, and the linearity of estimated concentrations and recoveries were effectively investigated using linear regression and analysis of variance.

(**b**) *Recovery and trueness*.—Dose independence of the recovery was checked (Student's t test). The percentage of trueness of the estimated concentration expressed as the bias to the real spiked concentration was estimated for each quinolone compound and at each level of concentration, taking into account the mean recovery correction of the compound. The mean recovery was measured over the 8-day routine use of the method, by testing the 112 samples prepared by fortification of the 16 different batches of matrixes.

(**c**) *Precision*.—The precision in terms of repeatability and intralaboratory reproducibility was evaluated by calculating the relative standard deviation (RSD) of the results obtained for each quinolone compound and at each level of concentration. The repeatability and reproducibility were

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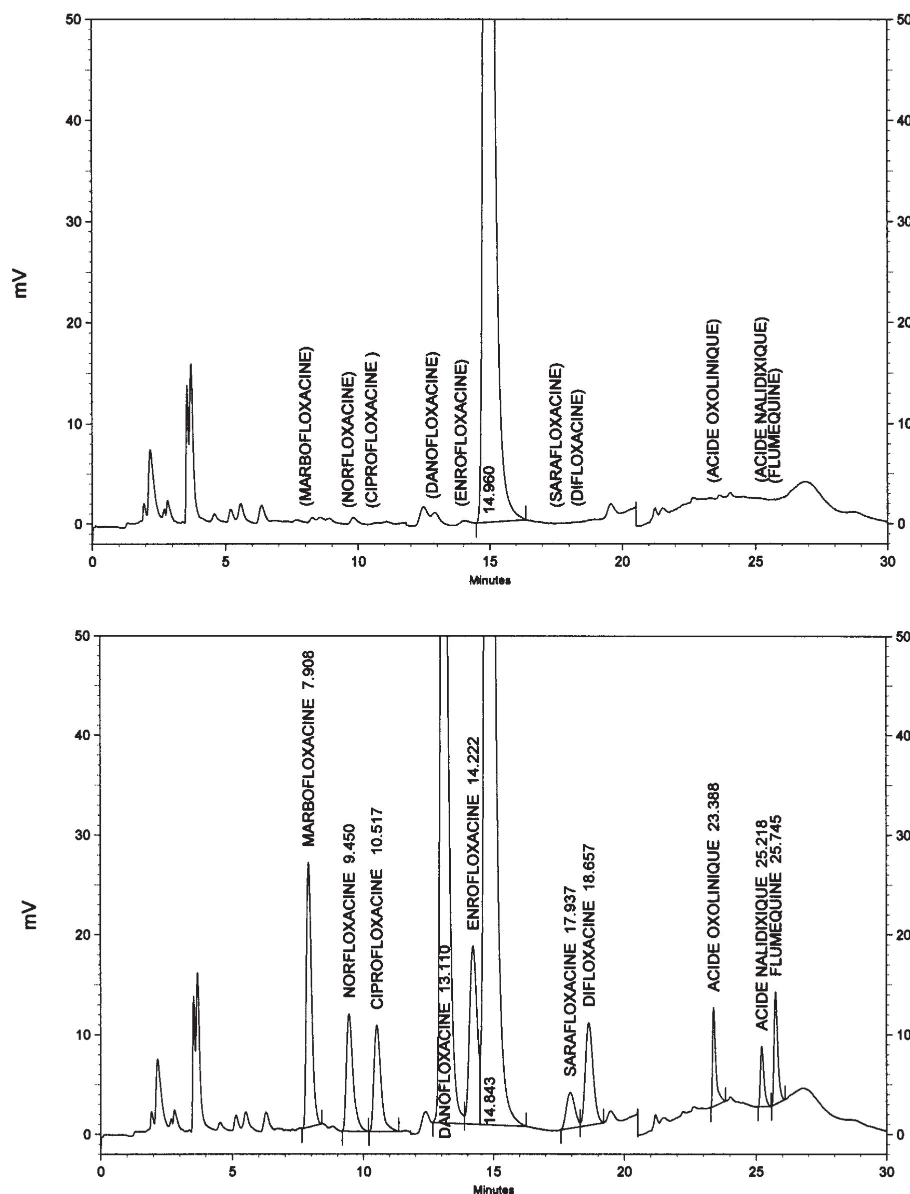

Figure 7. Typical chromatograms obtained from blank tissue samples and tissue samples: bovine milk fortified to 100 μg/kg with the 10 quinolones.

particularly examined to evaluate the within- and between-matrix variations.

Analytical Limits

(**a**) *LODs and LOQs*.—The LOD and LOQ were estimated from the regression equation derived from the calibration curves obtained during the 8-day routine use of the method. They were both calculated considering 3 times and 10 times, respectively, the ratio between the SD of the results (n = 14) obtained at the lowest level of concentration (15 μg/kg) and the slope of the linear regression.

(**b**) CCα and CCβ.—The 2 new analytical limits recommended in the European Decision No. 657/2002/EC (15), CCα, the critical concentration at risk alpha, also called the limit of decision, and CCβ, the critical concentration at risk beta, also called the capacity of detection of the method, were both calculated as stated in the ISO Standard 11843 (20). The decision limit (CCα) means "the limit at and above which it can be concluded with an error probability of a (5% for authorized substances) that a sample is noncompliant" (EC 657/2002 Annex 1, 1.11). The calculation is:

$$CC\alpha = MRL + 1.64 \times SD_{\text{within-lab reproducibility at MRL level}}$$

For authorized substances, CCα is calculated above the MRL level and strongly depends on the precision of the method.

Capacity of detection (CCβ) means "the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (5% for authorized substances)." In the case of substances with an

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Table 4. Validation data for selectivity assessment

	Retention times, min (n = 224[a])		
	Mean, min	SD[b], min	RSD[b], %
Marbofloxacin	7.8	0.1	0.8
Norfloxacin	9.3	0.1	1.2
Ciprofloxacin	10.3	0.1	1.3
Danofloxacin	13.0	0.2	1.5
Enrofloxacin	13.9	0.2	1.5
Sarafloxacin	17.6	0.2	1.4
Difloxacin	18.4	0.2	1.1
Oxolinic acid	23.4	0.1	0.1
Nalidixic acid	25.2	0.1	0.03
Flumequine	25.7	0.1	0.1

[a] 112 Matrix-spiked samples and 112 external standard samples.
[b] SD = Standard deviation; RSD = relative standard deviation.

established permitted limit (MRL substances), this means that detection capability is the concentration at which the method can detect permitted limit concentration with a statistical certainty of 1 - β (95%; EC 657/2002 Annex 1, 1.12). For authorized substances, the detection capability is calculated above the MRL level and above the CCα, and depends on the precision of the method. CCβ can be calculated as follows:

$$CC\beta = CC\alpha + 1.64 \times SD_{\text{within-lab reproducibility at } CC\alpha \text{ level}}$$

Results and Discussion

Applicability

The method was assessed by analyzing the 10 quinolone compounds after their spiking, either in a set of food-producing animal tissues (bovine and porcine muscle, porcine liver, porcine kidney, poultry muscle, and fish flesh/skin) or in bovine raw milk and poultry eggs. It was also successfully controlled by assaying 2 naturally incurred materials for flumequine residue in salmon tissue and for enrofloxacin and ciprofloxacin residues in porcine muscle tissue, respectively.

Stability

The stability of the working standard solutions was investigated. They were considered stable for more than 3 months without significant degradation (<5%). The stability of the extracts before injection was also evaluated. It was demonstrated to be satisfactory for a 24 h period when the samples prepared in capped vials were stored protected from light and placed in the tray of the automatic injection device under cooling conditions (+10°C).

Practicability

The operator was able to prepare up to 20 samples within the same batch in 1 day without automation of the extraction/cleanup procedure. Only the injection step was automated. In fact, the main time-limiting factor in routine

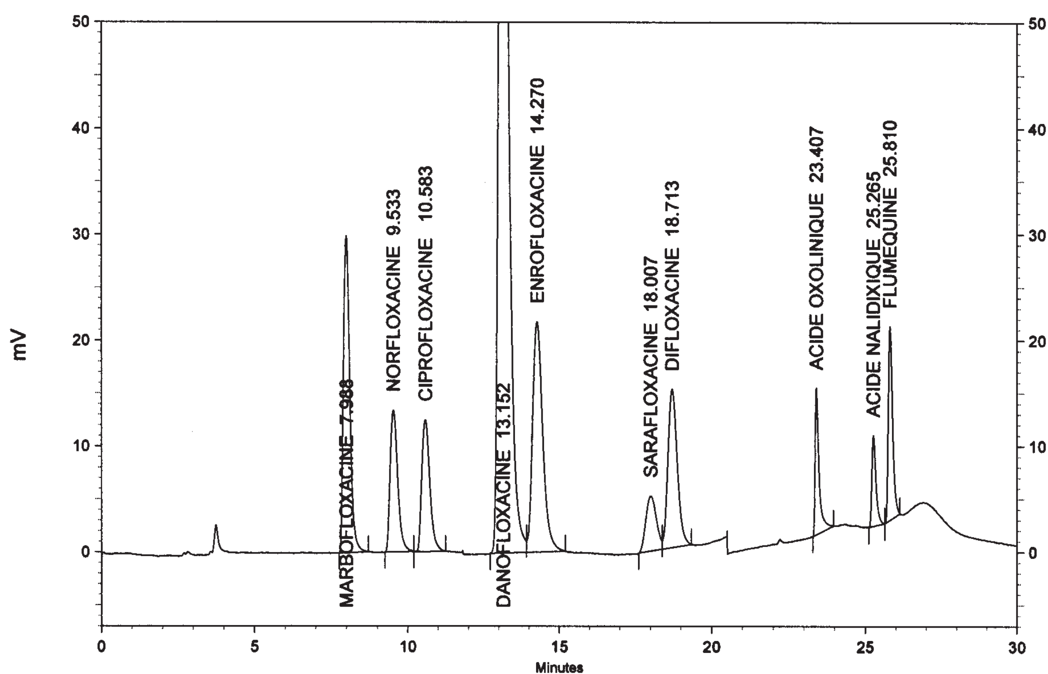

Figure 8. Typical chromatogram obtained from the external standard solution containing 20 μg/L of the 10 quinolones.

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Table 5. Validation data for accuracy asessment (recovery, trueness, and precision)

	Mean recovery, %		Intralaboratory precision, %[a]						Trueness, %			
	Milk matrix, n = 14	All other 7 matrixes[b], n = 98	Milk matrix, n = 14			All other 7 matrixes[b], n = 98			Milk matrix, n = 14		All other 7 matrixes[b], n = 98	
	Mean, SD	Mean, SD	Mean	Min	Max	Mean	Min	Max	Min	Max	Min	Max
Marbofloxacin	89 (7)	68 (6)	4.3	1.6	9.8	6.4	5.0	8.5	–8.0	+3.2	–7.8	–0.4
Norfloxacin	87 (8)	60 (6)	5.6	3.3	9.1	8.0	6.2	12.4	–9.0	+7.5	–5.6	+4.1
Ciprofloxacin	84 (5)	54 (5)	5.0	1.2	8.5	8.0	3.8	14.0	–9.0	+2.9	–5.9	+3.2
Danofloxacin	86 (5)	59 (8)	3.7	0.3	10.6	11.5	5.0	19.7	–3.9	+4.6	–11.9	+29.9
Enrofloxacin	76 (6)	48 (4)	3.0	1.4	4.5	8.1	3.9	10.0	–16.1	+7.6	–3.9	+2.4
Sarafloxacin	68 (7)	30 (4)	6.3	0.6	18.0	13.5	7.3	26.7	–6.8	+7.0	–3.4	+4.8
Difloxacin	69 (6)	32 (4)	7.7	5.5	14.4	11.5	8.7	18.6	–8.0	+9.3	–4.9	+2.4
Oxolinic acid	69 (13)	35 (6)	19.8	7.9	30.6	15.4	9.6	24.3	–9.0	+10.9	–2.5	+3.2
Nalidixic acid	70 (7)	32 (5)	11.0	1.3	20.9	13.6	8.9	20.7	–6.4	+5.8	–4.4	+4.1
Flumequine	64 (4)	29 (3)	6.3	1.2	17.1	14.8	9.4	20.4	–7.5	+4.1	–7.5	+7.8

[a] Over the fortification range of concentration from 15 to 3000 μg/kg.
[b] All other 7 matrixes = bovine muscle, porcine muscle, porcine kidney, porcine liver, fish flesh and skin, poultry muscle, whole egg.

analysis might be the sample preparation before extraction specifically designed to avoid cross-contamination.

Specificity

The chromatograms corresponding to the extracts of blank samples for the different food matrixes (muscle, egg, kidney, and fish flesh and skin) reveal no peak interfering with the quinolones (Figures 2–4, 6), except for the liver matrix and for the milk matrix, for which an interfering peak [retention time (RT) = 12.6 min in liver matrix and RT = 14.9 min in milk matrix] are only slightly separated from the peak of the danofloxacin (RT= 12.8 min) and enrofloxacin compound (RT = 14.2 min), respectively (Figures 5 and 7).

In addition, the specificity of the native FLD led to no interference with other major veterinary antimicrobials, as was observed when the method was tested for several aminosides, macrolides, penicillins, sulfonamides, and tetracyclines.

Selectivity

The results provided in Table 4 underline the selectivity of the method in terms of the variability of the RT. A mean RT was calculated for each of the 10 compounds, considering the analysis of all the fortified tissue/fluid samples used during the validation (n = 112). A similar calculation is also provided for the RT variability of the peaks obtained from standard samples used during the validation (n = 112). The resolution between the peaks of the 10 compounds was also evaluated. Most of the compounds were eluted with a satisfactory resolution (Rs > 1.5). Sarafloxacin and difloxacin, however, were not always completely separated (1.0 < Rs < 1.5; Figure 8).

Linearity

The linearity of the response was verified with tissue and fluid samples fortified from 15 to 3000 μg/kg and with standard solutions from 3 to 600 μg/L for all 10 quinolones. The coefficients of determination, R^2, were >0.98.

Recovery and Trueness

During the 8-day routine use of the methodology, the variations in recovery for each compound were statistically estimated. A first important comment concerns the significant difference in the recovery obtained from the milk matrix in comparison with the recoveries obtained from all the other matrixes (muscle, liver, kidney, fish flesh, egg). This information led us to a selective processing of the results obtained from the milk matrix apart from those obtained from the other matrixes, and to make some adjustments on the routine use of the method as discussed in *Conclusions.*

The mean recoveries (n = 14) of all 10 quinolone compounds obtained from the results for the milk matrix ranged from 64 to 89%, depending on the quinolone compound (Table 5). Their RSDs ranged from 4 to 13%. They were controlled independent of the dose of fortification. In contrast, the mean recoveries (n = 98) of the 10 quinolone compounds, when calculated from the results obtained for all the matrixes except milk, ranged from 68 to 21%, depending on the quinolone compound (Table 5). Similarly, their RSDs satisfactorily ranged from 3 to 8%. Unfortunately, they were controlled dose-dependent for marbofloxacin, norfloxacin, ciprofloxacin, and danofloxacin. Because the dose-independence of the recovery is not always verified on the large range of concentrations used for the calibration in

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Table 6. Validation data for analytical limit assessment (n = 14)

	Bovine milk					Chicken muscle					Fish flesh					Whole egg					Porcine liver					Porcine kidney				
	LOD	LOQ	MRL	CCα[a]	CCβ[b]	LOD	LOQ	MRL	CCα	CCβ	LOD	LOQ	MRL	CCα	CCβ	LOD	LOQ	MRL	CCα	CCβ	LOD	LOQ	MRL	CCα	CCβ	LOD	LOQ	MRL	CCα	CCβ
Marbofloxacin	4	13	75	92	111	4	13	NA[c]	29[d]	38	4	13	NA	41[d]	53	4	13	NA	44[d]	57	4	13	150	198	246	4	13	150	165	179
Norfloxacin	6	20	NA	50[d]	66	6	20	NA	24[d]	32	6	20	NA	51[d]	67	6	20	NA	141[d]	184	6	20	NA	21[d]	27	6	20	NA	73[d]	95
Ciprofloxacin	6	22	100	142	184	6	22	100	119	138	6	22	100	127	153	6	22	NA	159[d]	209	6	22	200	215	229	6	22	300	348	396
Danofloxacin	6	22	30	55	80	6	22	200	221	242	6	22	100	130	160	6	22	NA	161[d]	212	6	22	200	243	285	6	22	200	229	259
Enrofloxacin	4	13	100	123	146	4	13	100	120	141	4	13	100	123	147	4	13	NA	155[d]	203	4	13	200	216	231	4	13	300	349	389
Sarafloxacin	11	36	NA	49[d]	64	11	36	100	126	152	11	36	30	57	83	11	36	NA	202[d]	265	11	36	NA	27[d]	35	11	36	NA	71[d]	93
Difloxacin	8	27	NA	57[d]	75	8	27	300	325	349	8	27	NA	58[d]	76	8	27	NA	171[d]	224	8	27	800	813	825	8	27	800	852	904
Oxolinic acid	11	36	NA	111[d]	146	11	36	100	114	129	11	36	100	154	207	11	36	NA	176[d]	231	11	36	150	170	191	11	36	150	222	293
Nalidixic acid	10	34	NA	52[d]	68	10	34	NA	33[d]	43	10	34	NA	119[d]	156	10	34	NA	137[d]	179	10	34	NA	39[d]	51	10	34	NA	129[d]	166
Flumequine	8	28	50	75	99	8	28	400	425	449	8	28	NA	83[d]	108	8	28	NA	235[d]	308	8	28	500	513	525	8	28	1500	1559	1619

[a] CCα = Critical concentration at 1st order risk α = 5%, according to ISO Standard 11843 and EC Decision 657/2002.
[b] CCβ = Critical concentration at 2nd order risk β = 5%, according to ISO Standard 11843 and EC Decision 657/2002.
[c] NA = No MRL officially set for this compound in that specific matrix, leading to a different calculation for CCα at 1st order risk α = 1% instead of α = 5%.
[d] Critical concentration CCα is calculated at 1st order risk α = 1%, according to EC Decision 657/2002.

this study (i.e., 7 levels ranging from 15 to 3000 μg/kg), it is recommended on the basis of a routine analysis to reduce the calibration to 6 or even 5 levels, and to finally adapt it to the set of considered MRL levels for the different matrixes considered in the batches of analyses (Table 1).

For each compound, the trueness of the method is presented in Table 5. The results are given in 2 separate columns, the first for trueness obtained from the milk samples and the second for those covering the samples of all the other matrixes. The trueness data presented are the minima and maxima of bias (%) from the known amount of fortified compound as calculated for each level of fortification, taking into account all the replicates (i.e., 2 for milk matrix and 14 for the set of other matrixes) and by correcting with the mean recovery of the compound. Only the danofloxacin results show a bias that is outside the limits of –20 and +10% accepted by the EU Decision 657/2002/EC (15).

Precision

The mean RSDs of the within- and between-matrix precision (Table 5) were mainly found within the limits fixed either by EU Decision 657/2002/EC (15) point 2.3.2.2 (based on the Horwitz equation) or by the new proposition introduced by Thompson (21). They both specify a 22–23% acceptable variability for the interlaboratory precision of analytical confirmatory methods used for analysis of chemical residues at a level of concentration of 100 μg/kg, or even at lower concentrations, as stated by Thompson (21). Under the conditions of our validation study, this means that the between-matrix (intralaboratory) reproducibility obtained for all the different matrixes (except milk) should be equal to or lower than 15.3% (2/3 of the recommended interlaboratory precision). Consequently, the routine analysis of samples extracted from different matrixes (muscle, kidney, fish, eggs) within the same batch becomes an acceptable concept. Nonetheless, oxolinic acid in milk matrix (n = 2 per level of concentration) was the single compound actually found exceeding the proposed 15.3% precision with a mean RSD of 19.8% and with max RSDs ranging from 27.3 to 30.6% for samples spiked at the 3 specific levels of 50, 100, and 200 μg/kg.

LODs and LOQs

The LOD and LOQ, were both evaluated by considering the set of results obtained from all the matrixes except milk (n = 98). LOD and LOQ were estimated for each of the 10 quinolone compounds from 4 to 11 μg/kg and from 13 to 36 μg/kg, respectively (Table 6).

CCα and CCβ

Practically, CCα and CCβ were calculated taking into account a calibration range of 6 levels of concentration: 15, 50, 100, 200, 500, and 1500 μg/kg. This range was chosen because it guarantees the dose independence of the recovery, as discussed above. Moreover, the CCα and CCβ were considered for each matrix separately (Table 6) because it is not possible to calculate these 2 analytical limits for the whole

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set of matrixes at the same time. In fact, for authorized substances, CCα and CCβ must be estimated at the MRL level, and quinolone compounds have MRLs set at very different levels of concentration depending on the matrix/species in which they have to be monitored (egg, fish, milk, poultry; Table 1).

Finally, it must be stressed that the CCα and CCβ calculations proposed in our study depart from the principle of the equations of ISO Standard 11843 because a nonequal distance is observed between the 6 calibrating levels. Even if our calculations might have been affected by this fact, it is assumed that the CCα and CCβ presented in this study are undoubtedly excessive in comparison to those that would be derived from a strictly equally distant set of calibrants. Therefore, the results of Table 6 are considered to mimic or, at most, to exaggerate the CCα and CCβ values found with this method on a routine analysis scheme with respect to a more conforming calibration.

Conclusions

The narrow-bore LC/FLD method allows simultaneous determination of 10 quinolone antimicrobial residues within the range of their MRLs (for 8 of them). It allows equal analysis of 8 different food-producing biological matrixes (milk, egg, fish, poultry, bovine and porcine muscle, porcine liver, and porcine kidney). Samples from all these matrixes can be extracted in the same time and chromatographed through the same process, but significant differences in the recoveries were observed with milk samples compared to the other matrixes. This fact damages the accuracy of the results when milk samples are mixed with the other matrix samples. Therefore, when properly applying this method, it is recommended that milk samples be analyzed apart from the others, with their specific calibration taking into account the milk sample recoveries.

Overall, the method is very simple to apply and a trained operator can easily prepare about 20 samples per batch/day without automated systems. Consequently, the method is particularly suitable for screening quinolone residues. Again, one major difficulty in applying such a multiresidue, multimatrix/multispecies method is the extremely wide range of MRLs set for each quinolone compound in different matrix/species, leading to a calibration based on a wide range of concentrations. Obviously, it is recommended that this range be reduced when possible, especially for complying with the status of a quantitative confirmatory method.

The method was validated according to the criteria of the EU Decision 657/2002/EC. Its analytical performances are mainly in line with these criteria, except for the precision of milk samples containing oxolinic acid (a nonauthorized compound in milk). An approach of the new statistical analytical performance limits, CCα and CCβ, for estimation of the compliant/noncompliant status of the routinely controlled samples, is presented along with its specific application to the authorized quinolone substances. Moreover, the method was not specifically validated for testing nonauthorized quinolone substances at the lowest level of sensitivity. Therefore, the data exhibit some excessive CCα and CCβ values compared to the LOD and LOQ values calculated for these compounds. The correct approach is to calibrate nonauthorized compounds as for banned substances, i.e., in the range of lowest concentrations for which the performance of the method in term of accuracy (trueness + precision) is still valid with regard to the officially recognized criteria.

On the basis of a routine analysis, the accuracy of the method could also possibly be improved by implementing an internal calibration using, for example, the nonauthorized quinolones such as norfloxacin (short RT) and nalidixic acid (large RT) as internal standards. Finally, the short extraction step and the acidic chromatographic eluents should easily support a successful switch from the FLD to a tandem mass spectrometric (MS/MS) detection and, therefore, could more widely open the field of application of such a method. This assumption is supported by the recent development of 2 LC/MS/MS methods, one at the Joint Research Center EU-JRC-IRMM (Geel, Belgium) for multiquinolone residues in kidney (12) and one in our laboratory for a multiclass antibiotic residue control in meat and milk, including the 10 quinolones (22).

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