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Determination of Fluoroquinolones in Milk Samples by Postcolumn Derivatization Liquid Chromatography with Luminescence Detection

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A liquid chromatography (LC) method with luminescence detection for the determination of eight quinolone antibiotics is reported. The system encompasses three consecutive steps: (a) chromatographic separation using reverse-phase mode (RP-LC), (b) postcolumn derivatization reaction, and (c) luminescence detection by monitoring fluorescence (FL) and time-resolved (TR) signals. The derivatization step is based on the reaction between quinolones and terbium(III) to form luminescent chelates, which were determined at λ_{ex} 340 and λ_{em} 545 nm (FL mode) or at λ_{ex} 281 and λ_{em} 545 nm (TR mode). Dynamic ranges of the calibration graphs, obtained with standard solutions of analytes and FL and TR modes, respectively, were 190−3500 and 316−2000 ng mL^{-1} for marbofloxacin, 8−3500 and 8.1−1500 ng mL^{-1} for ciprofloxacin, 6.2−3500 and 13−1500 ng mL^{-1} for danofloxacin, 7.4−3500 and 8.4−1500 ng mL^{-1} for enrofloxacin, 14−3500 and 20−2000 ng mL^{-1} for sarafloxacin, 12.5−3500 and 13.9−1200 ng mL^{-1} for difloxacin, 7.6−3500 and 13−3000 ng mL^{-1} for oxolinic acid, and 9−2000 and 130−3000 ng mL^{-1} for flumequine. Limit of detection values obtained using FL and TR modes, respectively, were 60 and 95 ng mL^{-1} for marbofloxacin, 2 and 2.4 ng mL^{-1} for ciprofloxacin, 1.9 and 3.9 ng mL^{-1} for danofloxacin, 2.2 and 2.5 ng mL^{-1} for enrofloxacin, 3.8 and 7 ng mL^{-1} for sarafloxacin, 4 and 4.2 ng mL^{-1} for difloxacin, 2.3 and 4 ng mL^{-1} for oxolinic acid, and 2.7 and 40 ng mL^{-1} for flumequine. The precision was established at two concentration levels of each analyte and expressed as the percentage of relative standard deviation with values ranging between 1.9 and 7.8%. The validation procedure for the analysis of samples was carried out using European Community recommendations, and the decision limit and detection capability were calculated for bovine whole milk. The method was applied to whole, semiskimmed, and skimmed milk samples spiked with the target analytes, and the recoveries ranged between 93.3 and 106.0%.

KEYWORDS: Postcolumn derivatization; fluoroquinolones; terbium; luminescence detection; milk samples

INTRODUCTION

Quinolones are a group of antibiotics used for the prevention of diseases caused by several bacterial agents. Some of them can be used for the treatment of different animal species, including those destined for food production. Maximum residue limits (MRL) for quinolones in different food samples are set by authorities (*1*) to prevent the growth of antibiotic-resistant bacteria in humans. This is such an important issue that the characterization of analytical methods and the interpretation of results have been recently revised in the European legislation (*2*).

The antibacterial activity of these antibiotics has given rise to different microbiological methods for their determination in milk samples (*3*, *4*). Liquid chromatography (LC) methods with mass spectrometry (MS) (*5*), tandem mass spectrometry (MS/MS) (*6*, *7*), ultraviolet (UV) (*8*, *9*), and fluorometry (*8*, *10*−*16*) as detection systems have been reported for the determination of mixtures of quinolones in different samples. The methods focusing on the analysis of milk samples usually involve the deproteinization and subsequent cleanup and/or preconcentration steps by solid-phase extraction (SPE). Thus, potential interferences from sample matrix can be removed and the required sensitivity levels can be achieved, taking into account the low MRL defined for these antibiotics in milk samples (*1*). Although an automated approach for SPE involving column-switching chromatography has been reported (*10*), this step is usually carried out off-line before the chromatographic separation (*5*−*9*, *13*, *15*), which can decrease the effective sample throughput of these methods.

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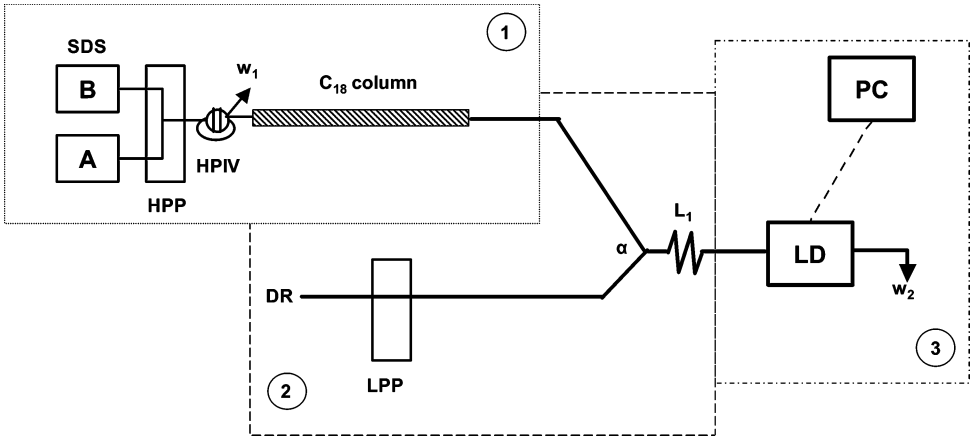

Figure 1. Integrated separation–derivatization and detection approach. 1, 2, and 3 depict the chromatography, derivatizing, and detection subsystems, respectively. A and B denote 30 mM acetic acid and acetonitrile solutions. SDS, solvent delivery system; HPP, high-pressure quaternary gradient pump; HPIV, high-pressure injection valve; LPP, low-pressure pump; L_1, mixing reactor; LD, luminescence detector; PC, personal computer; DR, derivatizing reagent; w_1 and w_2, waste.

Table 1. Excitation and Emission Wavelengths for Quinolones and Their Terbium Chelates

	wavelength, nm					
	quinolone FL[a]		quinolone–Tb(III) chelate			
			FL[a]		TR[a]	
	λ_{ex}	λ_{em}	λ_{ex}	λ_{em}	λ_{ex}	λ_{em}
marbofloxacin	355	498	355	545	295	545
ciprofloxacin	332	446	332		281	
danofloxacin	335	440	335			
enrofloxacin	332	445	332			
sarafloxacin	333	450	333			
difloxacin	333	453	333			
oxolinic acid	333	366	333		250	
flumequine	337	380	337			

[a] Detection mode: FL, fluorescence; TR, time-resolved.

Table 2. Study of Variables

type	variable	range studied	optimum value
liquid chromatography	column (stationary phase)		C_{18}, 4 μm, 250 × 4.6
	flow rate, mL min^{-1}	0.5–2.0	1.0
	initial mobile phase (HAc/acetonitrile)		82:18
	gradient/isocratic program		(see **Figure 2**)
	pH	2.3–3.5	3.0
	HAc concn, mM	10–200	30
postcolumn derivatization	flow rate, mL min^{-1}	0.3–1.0	0.6
	length of reactor L_1, cm	20–100	50
	pH	2.0–8.0	6.0
	Tb(III) concn, mM	10–200	10
	HAc/NH_4Ac buffer concn, M	0.2–2.0	0.5

Terbium-sensitized luminescence (TSL) has been widely used for the development of relatively sensitive luminescent methods for the direct determination of organic species (*17*). In general, the sensitivity of these methods depends on the efficiency of the intramolecular energy transfer between the ligand and the lanthanide and on the relatively long emission wavelength of these chelates, at which the contribution of background signals is lower than that at shorter wavelengths. In some instances, the sensitivity of these methods can be also improved by using the time-resolved (TR) mode, owing to the relatively long lifetime of these chelates. In addition, some potential interferences from sample matrix, which usually emit at shorter wavelengths, can be avoided. With regard to the determination of quinolone antibiotics, TSL has been proposed for the individual determination (*17*−*22*) or for the resolution of binary mixtures of quinolones (*17*), the latter performed by using kinetic methodology. Most of these methods have been applied to the analysis of biological fluids (*17*, *19*−*22*), their application to food analysis (*18*, *23*, *24*) being more limited to date. The carboxylic and keto groups of quinolones are involved in the chelate formation, determining the intensity of the luminescent signal on the substituent attached to the nitrogen atom of the pyridone ring (*20*). Nevertheless, the luminescent behaviors of these antibiotics are similar enough to prevent the individual determination of a quinolone in the presence of other members of this group. The use of a separation technique can improve the selectivity of the above-mentioned methods, allowing the simultaneous determination of a mixture of several quinolones to be tackled.

The present work describes the usefulness of TSL for the simultaneous determination of eight quinolone antibiotics used in veterinary practice. Postcolumn derivatization LC methods using terbium(III) as reagent and FL and TR modes for luminescence detection are described for this purpose, and their features are compared. The LC-FL method provides the required sensitivity level to carry out the determination of four quinolones that can be present in milk samples, namely, ciprofloxacin, enrofloxacin, danofloxacin, and flumequine. The sample treatment consisted of a deproteinization step without any further cleanup. This fact allows the achievement of a higher sample throughput compared to other LC methods with fluorometric detection (*8*, *10*−*16*) described for this purpose with comparable detection limits. The method was validated by performing the recovery study, and some parameters, such as decision limit (CCα) and detection capability (CCβ), were also calculated (*2*).

MATERIALS AND METHODS

Apparatus and Instruments. An SLM Aminco (Urbana, IL) AB2 luminescence spectrometer provided with a 150 W continuous xenon lamp and a 7 W pulsed xenon lamp and equipped with a conventional 110-QS Hellma cell (Hellma Hispania, Barcelona, Spain) was used for monitoring FL and TR batch measurements, respectively. Chromatography was performed on a modular liquid chromatograph that consists

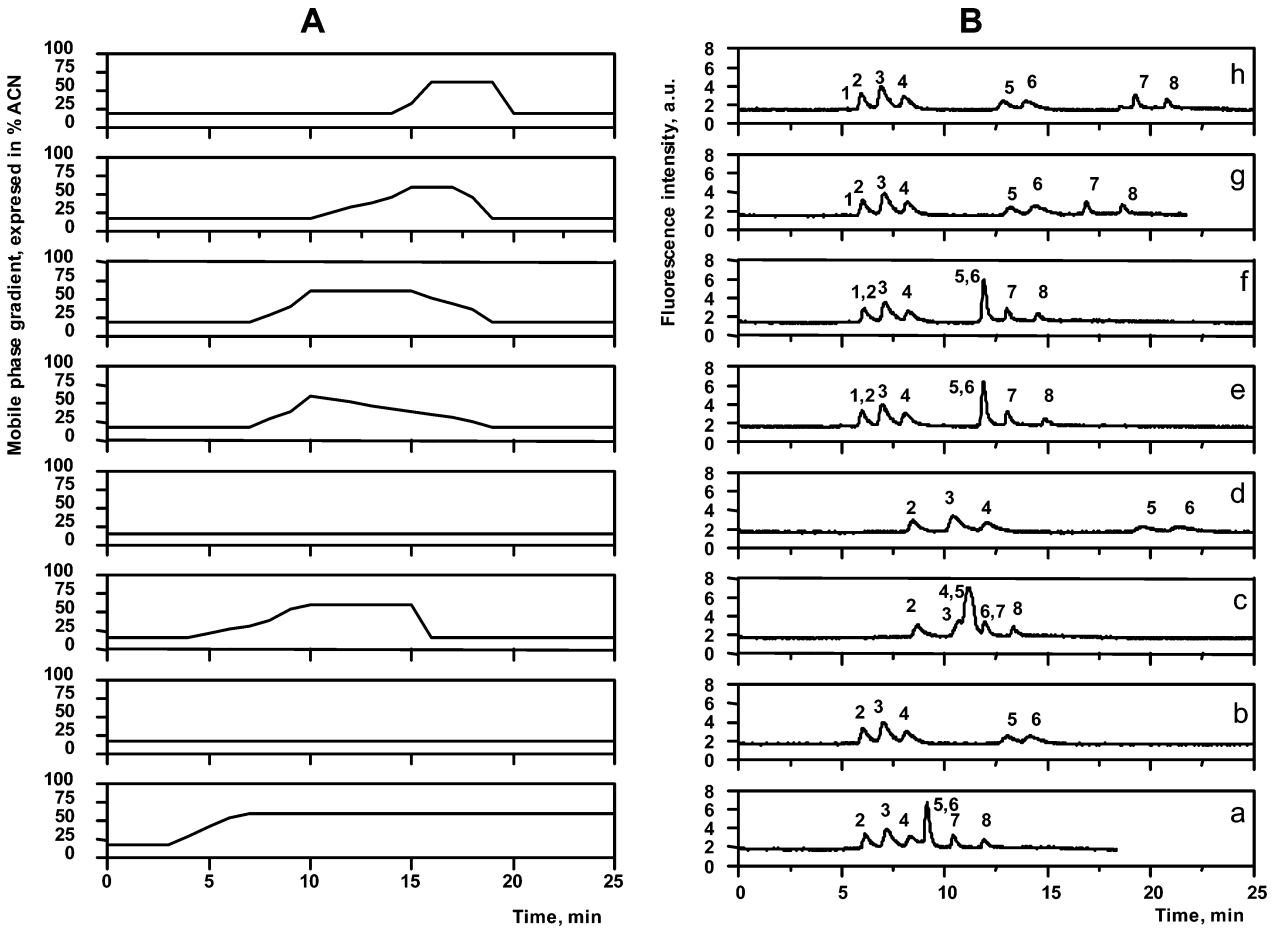

Figure 2. Influence of the mobile phase composition: (**A**) isocratic and gradient programs, expressed as percent of acetonitrile (ACN) in the mobile phase; (**B**) chromatogram profiles achieved in each case (1, marbofloxacin; 2, ciprofloxacin; 3, danofloxacin; 4, enrofloxacin; 5, sarafloxacin; 6, difloxacin; 7, oxolinic acid; 8, flumequine).

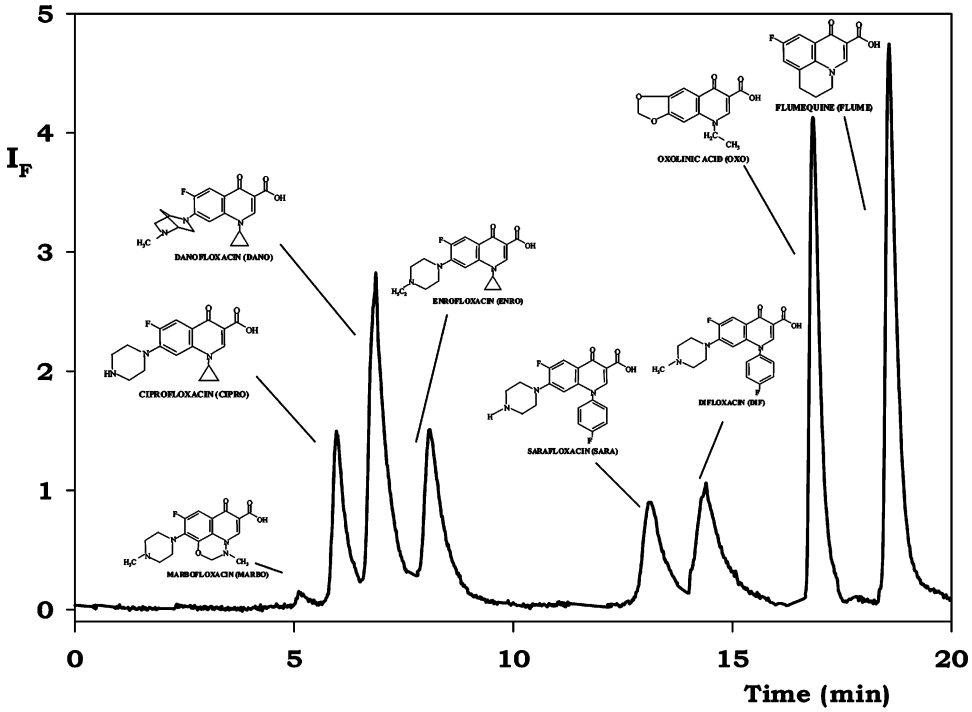

Figure 3. Chemical structures of the analytes and typical chromatogram achieved for the fluorescence detection mode. The chromatogram was achieved by injection of a standard solution containing 1500 ng mL^{-1} of each analyte and processed under optimum experimental conditions.

of a Jasco PU-2089 Plus (Jasco, Tokyo, Japan) high-pressure quaternary gradient pump, a 7125 Rheodyne (Cotati, CA) high-pressure manual injection valve with a 20-μL injection loop, and a Synergi Fusion-RP-80A column (Phenomenex, Torrance, CA), 250 $\times$ 4.6 mm i.d., 4 μm,

Table 3. Analytical Features of the Proposed Methods

		LC-TR					LC-FL				
analyte	retention time, min	linear range, ng mL^{-1}	slope ± SD[a]	intercept ± SD	r	LOD,[b] ng mL^{-1}	linear range, ng mL^{-1}	slope ± SD	intercept ±SD	r	LOD, ng mL^{-1}
marbofloxacin	5.12	316–2000	$(1.77 \pm 0.07) \times 10^{-3}$	0.08 ± 0.07	0.9993	95	190–3500	$(1.52 \pm 0.02) \times 10^{-3}$	0.20 ± 0.03	0.9998	60
ciprofloxacin	6.00	8.1–1500	(4.05 ± 0.06) x 10^{-2}	0.2 ± 0.1	0.9997	2.4	8–3500	$(2.57 \pm 0.02) \times 10^{-2}$	0.09 ± 0.03	0.9998	2
danofloxacin	7.05	13–1500	$(5.72 \pm 0.04) \times 10^{-2}$	0.5 ± 0.1	0.9999	3.9	6.2–3500	$(5.01 \pm 0.06) \times 10^{-2}$	0.08 ± 0.05	0.9998	1.9
enrofloxacin	8.18	8.4–1500	$(3.96 \pm 0.02) \times 10^{-2}$	0.4 ± 0.1	0.9999	2.5	7.4–3500	$(2.60 \pm 0.04) \times 10^{-2}$	0.12 ± 0.07	0.9998	2.2
sarafloxacin	13.15	20–2500	$(2.75 \pm 0.08) \times 10^{-2}$	-0.05 ± 0.01	0.9991	7	14–3500	$(1.89 \pm 0.02) \times 10^{-2}$	0.2 ± 0.1	0.9998	3.8
difloxacin	14.43	13.9–1200	$(2.88 \pm 0.05) \times 10^{-2}$	0.5 ± 0.1	0.9995	4.2	12.5–3500	$(2.14 \pm 0.02) \times 10^{-2}$	0.11 ± 0.08	0.9999	4
oxolinic acid	16.85	13–3000	$(1.88 \pm 0.02) \times 10^{-2}$	0.3 ± 0.3	0.9999	4	7.6–3500	$(5.96 \pm 0.09) \times 10^{-2}$	0.2 ± 0.1	0.9997	2.3
flumequine	18.62	130–3000	$(8.7 \pm 0.1) \times 10^{-3}$	0.09 ± 0.01	0.9996	40	9–2000	$(8.96 \pm 0.04) \times 10^{-2}$	-0.1 ± 0.1	0.9999	2.7

[a] SD, standard deviation. [b] LOD, detection limit.

used as analytical column. A Gilson (Villiers-le-Bel, France) Minipuls-3 low-pressure peristaltic pump and Omnifit (Cambridge, U.K.) Teflon tubing of 0.5 mm i.d. were also used for constructing the postcolumn derivatization manifold. A 176-052-QS Hellma (Hellma Hispania) flow cell with an inner volume of 18 μL was used. Luminescence measurements were performed at room temperature.

Reagents. All chemicals used were of analytical reagent grade. All solutions were prepared in doubly deionized water, obtained using a Millipore (Bedford, MA) Milli-Q system. Stock solutions (100 μg mL^{-1}) of ciprofloxacin hydrochloride (Lesvi Laboratories, Barcelona, Spain), danofloxacin mesylate (Pfizer, Amboise, France), difloxacin (Fort Dodge Veterinaria, Girona, Spain), enrofloxacin (Hipra Laboratories, S.A., Girona, Spain), and marbofloxacin (Vetoquinol, S.A., Lure, France) were prepared using bidistilled water. Oxolinic acid (Sigma-Aldrich, Steinheim, Germany), flumequine (Sigma-Aldrich, Steinheim, Germany), and sarafloxacin hydrochloride (Sigma-Aldrich, Seelze, Germany) were prepared at the same concentration in 0.1 M sodium hydroxide. These solutions were stored at 4 °C for a month, and further dilutions were performed in water to obtain working solutions.

A 10 mM solution of terbium(III) was prepared by dissolving the appropriate amount of terbium(III) nitrate pentahydrate (Aldrich, Gillingham, Dorset, U.K.) in HAc/NH_4Ac buffer (0.5 M, pH 6.0) (Merck, Darmstadt, Germany). Mobile phase was constituted by solvents A (acetic acid, 30 mM, pH adjusted to 3.0; Panreac Quimica, S.A., Barcelona, Spain) and B (HPLC-grade acetonitrile, Panreac Quimica, S.A.), which were mixed by operating in the gradient mode during the chromatographic separation.

Spiked Milk Samples. Different blank aliquots of milk samples were spiked with minimum volumes of solutions containing the necessary concentrations of the quinolones assayed. The samples were stored at 4 °C for 24 h, thus allowing the incorporation of the antibiotic on the matrix, and were analyzed according to the following procedure: 1.0 g of milk sample was treated with 1.0 mL of a 20% solution of trichloroacetic acid in methanol and shaken for 30 s. The suspension was centrifuged at 3000 rpm for 15 min, and the separated organic extract was adjusted to an apparent pH of 4 with 1 M sodium hydroxide solution. The solution was raised to a 2 mL volume with water and used to determine the concentration of the fluoroquinolones using the LC-FL method below described.

Manifold and Procedure. Figure 1 shows the three-step integrated LC separation−derivatization approach. Standards or sample extracts (20 μL), containing the analytes at concentrations within their respective linear ranges, were injected into the column. The mobile phase was pumped at 1 mL min^{-1} and the system operated under isocratic conditions for 10 min after the injection using an 82:18 acetic acid (30 mM, pH 3.0)/acetonitrile composition. Then, this was changed in 5 min to 40:60 acetic acid (30 mM, pH 3.0)/acetonitrile and held in this proportion for 2 min. Finally, it returned to the initial composition in 2 min, and after 1 min, the system was ready for the next injection.

The eluate was merged at point α with a solution containing 10 mM terbium(III) prepared in HAc/NH_4Ac buffer (0.5 M, pH 6.0) and pumped at a flow rate of 0.6 mL min^{-1}. The mixed solution passed through the reactor L_1, in which the derivatization reaction took place. The analytical signal was monitored at λ_{ex} 340 and λ_{em} 545 nm (FL mode) or at λ_{ex} 281 and λ_{em} 545 nm (TR mode) for 20 min, and the corresponding blank solutions were subtracted. Each solution was assayed in triplicate. Chromatograms were taken using the original software of the luminescence spectrometer, and the raw data of luminescence intensity and time were exported and treated using adequate software packages for the estimation of the main chromatographic parameters.

Table 4. Study of Precision

	RSD%[a]			
	TR mode		FL mode	
analyte	first addition	second addition	first addition	second addition
marbofloxacin	ns[b]	4.4	ns	2.0
ciprofloxacin	7.2	5.1	6.5	5.6
danofloxacin	7.5	5.7	6.8	2.9
enrofloxacin	7.3	5.6	5.5	4.1
sarafloxacin	4.7	3.8	6.3	6.2
difloxacin	5.3	4.8	4.8	4.2
oxolinic acid	7.8	3.7	4.4	1.9
flumequine	6.2	4.3	5.2	2.6

[a] For 50 and 500 ng mL^{-1} for first and second addition, respectively. [b] No signal found.

Table 5. EC Recommendation Parameters for Whole Bovine Milk

EC parameter[a]	ciprofloxacin, μg kg^{-1}	danofloxacin, μg kg^{-1}	enrofloxacin, μg kg^{-1}	flumequine, μg kg^{-1}
MRL	100	30	100	50
CCα	109.8	34.6	111.2	52.1
CCβ	119.7	39.2	122.4	54.3

[a] MRL, maximum residue limits; CCα, decision limit; CCβ, detection capability.

RESULTS AND DISCUSSION

Characterization of the Luminescent System. The intrinsic fluorescence of quinolones and the luminescence of their terbium chelates were studied by carrying out their excitation and emission spectra. As has been already described in some methods dealing with individual determinations of quinolones using TSL (*18*, *20*), the excitation spectra showed relatively wide bands, whereas the emission spectra showed a narrow band with maximum emission at 545 nm. **Table 1** shows the values of maximum excitation and emission wavelengths obtained using FL and TR modes in the solvent mixture chosen to provide the mobile phase. Most of the quinolones and their terbium chelates exhibited similar excitation wavelengths, except that for marbofloxacin, which was longer. To make compatible the

Table 6. Recoveries of Fluoroquinolones Added to Milk Samples Using the Fluorescence Detection Mode

	milk samples								
	whole			semiskimmed			skimmed		
analyte	added, μg kg^{-1}	found,[a] μg kg^{-1}	recovery, %	added, μg kg^{-1}	found,[a] μg kg^{-1}	recovery, %	added, μg kg^{-1}	found,[a] μg kg^{-1}	recovery, %
ciprofloxacin	50	51 ± 2	102.0	50	51 ± 3	102.0	50	49 ± 3	98.0
	100	98 ± 6	98.0	100	97 ± 7	97.0	100	100 ± 8	100.0
	150	150 ± 10	100.0	150	150 ± 4	100.0	150	150 ± 4	100.0
danofloxacin	20	19 ± 1	95.0	20	20.8 ± 0.9	104.0	20	21 ± 2	105.0
	30	31 ± 3	103.3	30	29 ± 3	96.7	30	28 ± 2	93.3
	50	51 ± 1	102.0	50	51 ± 2	102.0	50	50 ± 1	100.0
enrofloxacin	50	49 ± 3	98.0	50	48 ± 2	96.0	50	53 ± 3	106.0
	100	98 ± 7	98.0	100	96 ± 6	96.0	100	98 ± 7	98.0
	150	149 ± 8	99.0	150	158 ± 8	105.3	150	143 ± 4	95.3
flumequine	30	29 ± 2	96.7	30	30 ± 1	100.7	30	31 ± 2	104.0
	50	52 ± 1	104.0	50	50 ± 2	99.0	50	51 ± 3	101.2
	80	78 ± 6	97.5	80	81 ± 5	101.3	80	79 ± 1	98.9

[a] Mean ± SD (n = 6).

measurements of these luminescent systems, 340 nm was selected as the optimum wavelength for the simultaneous excitation of the chelates in the FL mode. A similar behavior was observed using TR measurements, and 281 nm was selected as the compromise excitation wavelength.

Optimization of Variables. The hydrodynamic and chemical variables involved in the LC methods were optimized using the univariate methodology. Values chosen were those yielding the maximum luminescence signal with a minimum standard deviation. **Table 2** summarizes the range studied and the optimum value for each variable.

Chromatographic Variables. The luminescence of lanthanide chelates can be quenched by the vibration of hydroxyl groups of water molecules (*17*), which is really notable when RP-LC is used due to the relatively high content of water in the mobile phases used. This effect can be minimized by adding a synergetic agent or by introducing an acid in the mobile phase, which additionally provides the acidic medium necessary to achieve the required chromatographic resolution (*25*). Orthophosphoric and oxalic acids were tried for this purpose, resulting in terbium(III) precipitation. However, an adequate luminescent signal was obtained in the presence of acetate ions, which can be ascribed to the capability of these ions to displace water from the first coordination sphere of terbium(III) (*25*).

A remarkable objective of the optimization of the chromatographic variables was to achieve the best separation of the analytes in the lowest separation time. The study of the effect of the apparent pH of the mobile phase showed that it is a critical variable to attain the required chromatographic selectivity. The shape of peaks was more definite at pH 3.0 and 30 mM acetic acid (**Table 2**). There was no improvement on the results obtained at lower pH, and the long-term stability of the column could be affected by operating at pH values close to 2.0. Binary mixtures of acetic acid (30 mM, pH 3.0) and acetonitrile using isocratic and gradient profiles were tried at the optimum flow rate (1 mL min^{-1}). **Figure 2A** shows the gradient programs used and **Figure 2B** the corresponding separation of quinolones achieved under these hydrodynamic conditions. The best resolutions for ciprofloxacin, danofloxacin, and enrofloxacin together with the lowest retention times for flumequine and oxolinic acid were obtained using the gradient conditions corresponding to chromatogram g. The separation of the eight quinolones was achieved in about 19 min. These conditions were used for the development of the chromatographic separation and have been detailed under the procedure section.

Postcolumn Derivatization Variables. The study of the flow rate of the derivatizing reagent solution showed that 0.6 mL min^{-1} provided the optimum terbium(III) solution column effluent ratio when 1 mL min^{-1} was used for the chromatographic separation. Flow rates higher than 1.2 mL min^{-1} caused overpressure at the mixing point and backflowing into the chromatographic column. The length of the reactor L_1 was evaluated to allow an adequate residence time for the derivatization reaction. Dispersion phenomena, which are present in flow systems and can decrease the separation efficiency, were observable using reactors longer than 50 cm. This length was chosen as the optimum value, which is long enough for the relatively fast rate for the terbium chelate formation as previously described (*17*). Also, this relatively short reaction coil does not significantly decrease the sample throughput. An ammonium acetate buffer solution was chosen to increase the pH of the chromatographic eluent to achieve the optimum luminescence signal without terbium hydroxide precipitation. There was a remarkable decrease in the peak area when the pH was higher than 6.0. The influence of terbium(III) concentration was evaluated for each quinolone−terbium chelate; in most cases, the area was independent of terbium concentration from 10 mM. Only marbofloxacin showed higher luminescence intensity at lower terbium concentrations. **Figure 3** depicts a chromatogram obtained for a standard mixture of quinolones, including their corresponding chemical structures, carried out under optimum conditions using the LC-FL method. The variables related to the time-resolved detection mode, such as delay, gate, and cycle times, were studied in the ranges of 0.012−30, 0.001−30, and 5−30000 ms, respectively. The optimum values found for these variables were 0.03, 2, and 10 ms for delay, gate, and cycle times, respectively.

Analytical Features. Calibration graphs were run under the optimum experimental conditions by using an external calibration method. **Table 3** shows the retention time for each quinolone, the calibration parameters, and the detection limits (LODs) obtained. The r values obtained indicate a good correlation of experimental data to the corresponding calibration curve. The LODs, which were calculated following IUPAC recommendations (*26*), were lower in all instances using the LC-FL method. The high detection limits obtained for marbofloxacin are due to the relatively low fluorescence intensity of this compound, which explains why this antibiotic has not been previously determined by monitoring its fluorescence. The precision of the proposed methods (relative standard deviation)

was evaluated using standards ($n = 10$) at two analyte concentration levels (50 and 500 ng mL^{-1}). **Table 4** shows the results obtained, which were in the ranges of 3.7−7.3 and 1.9−6.8% for the LC-TR and LC-FL methods, respectively. The sample throughput under the optimum working conditions for the LC methods was approximately 3 h^{-1}, calculated without taking into account the sample preparation.

Applications. The LC-FL method was applied to the determination of enrofloxacin, ciprofloxacin, danofloxacin, and flumequine, which can be found in milk, in three different samples: whole, semiskimmed, and skimmed milk. The sample treatment consisted of only a deproteinization step in all instances. LC-FL was the approach chosen because the LC-TR method did not afford an adequate limit of quantification for flumequine determination in milk samples taking into account its relatively low MRL (50 μg kg^{-1}) (*1*). Although marbofloxacin can be used in animal species producing milk, the determination of this antibiotic cannot be done at 0.5, 1, or 1.5 MRL. A similar behavior has also been observed in a method described for the separation of five fluoroquinolones (*8*) in which, although a SPE step was used, the quantification of marbofloxacin could not be done at 0.5 MRL. The other quinolones assayed, such as oxolinic acid, sarafloxacin, and difloxacin, are not allowed in milk-producing species and, therefore, their determination was not carried out in these samples. These compounds were completely resolved from the fluoroquinolones of interest, and they did not interfere with their determination. **Table 5** shows the MRLs, the decision limits (CCα), and the detection capabilities (CCβ) for ciprofloxacin, danofloxacin, enrofloxacin, and flumequine achieved for whole bovine milk, calculated following EC recommendations (*2*). CCα indicates the limit at and above which it can be concluded with an error probability of α that a sample is noncompliant. CCβ indicates the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β. The values of α and β are 5%. In this case, because a permitted level has been established for each analyte in the samples, the calculation of CCα was done by analyzing 20 blank sample aliquots fortified at the MRL level. CCα was calculated as the MRL plus 1.64 times the standard deviation for the analyzed samples. From a strict point of view, the same procedure should be repeated for the calculation of CCβ with samples spiked at the CCα level (*2*). However, due to the proximity between MRL and CCα, a previously reported simplification (*7*) was done by assuming that standard deviations at both levels were the same. Therefore, CCβ was calculated as CCα plus 1.64 times the standard deviation at MRL. The recovery study was performed by preparing three sets, of six samples each, and adding the analytes at different concentration levels. The absence of matrix effects allowed the use of an external calibration method. **Table 6** summarizes the concentrations found, the standard deviation for each set of samples, and the recoveries obtained. As can be seen, the recovery values were in the range of 93.3−106.0%.

The usefulness of TSL as a detection system for the simultaneous determination of eight quinolones is described for the first time. The comparative study carried out using LC techniques with FL and TR detection shows that LC with postcolumn derivatization and FL detection mode is a selective and sensitive option to determine ciprofloxacin, danofloxacin, enrofloxacin, and flumequine in milk samples in the presence of other quinolones. The relatively low quantification limits obtained, which are lower than the MRLs permitted by EU legislation, allow the direct injection of the sample extracts after their deproteinization, without any preconcentration step. The relatively long emission wavelength of the terbium chelate can help to reduce the interferences from some components of the sample matrix. Thus, the use of SPE to clean up the samples is avoided by this approach, and the sample throughput of the system can be enhanced. Although a derivatization step is included, this is automatically performed, which does not affect the expeditiousness of both LC-FL and LC-TR methods. The LC-FL method has been validated to check its usefulness for the analysis of different milk samples with satisfactory results.

ABBREVIATIONS USED

LC, liquid chromatography; RP-LC, reverse phase LC; FL, fluorescence; TR, time-resolved; LOD, limit of detection; EC, European Community; SPE, solid-phase extraction; MRL, maximum residue limit; TSL, terbium-sensitized luminescence; CCα, decision limit; CCβ, detection capability; HPLC, high-performance liquid chromatography; HAc, acetic acid; NH_4-Ac, ammonium acetate; IUPAC, International Union of Pure and Applied Chemistry; EU, European Union; SDS, solvent delivery system; HPP, high-pressure quaternary gradient pump; HPIV, high-pressure injection valve; LPP, low-pressure pump; LD, luminescence detector; PC, personal computer; DR, derivatizing reagent; ACN, acetonitrile; SD, standard deviation; RSD, relative standard deviation.

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