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Fluoroquinolone antibiotic determination in bovine, ovine and caprine milk using solid-phase extraction and high-performance liquid chromatography-fluorescence detection with ionic liquids as mobile phase additives

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ABSTRACT

This paper describes the use of 1-ethyl-3-methylimidazolium tetrafluoroborate (EMIm-BF_4) as mobile phase additive for the analysis by high-performance liquid chromatography with fluorescence detection of a group of seven basic fluoroquinolone antibiotics (i.e. fleroxacin, ciprofloxacin, lomefloxacin, danofloxacin, enrofloxacin, sarafloxacin and difloxacin) in different milk samples. EMIm-BF_4 was found superior to 1-butyl-3-methylimidazolium tetrafluoroborate for the separation of the analytes from chromatographic interferences of the sample matrix. The optimized method was applied to the analysis of ovine, caprine and bovine milk, in the last case in either skimmed, semi-skimmed and full-cream milk after suitable acidic deproteination followed by a solid-phase extraction procedure. Recovery values between 73% and 113% were obtained for the three types of bovine milk samples, as well as for ovine and caprine milk (RSDs below 16% in all cases), which clearly demonstrates the applicability of the method to the three types of milk irrespective of the fat content of the samples. Limits of detection were in the range of 0.5–8.1 μg/L (approximately 0.5–25.9 μg/kg), well below the maximum residue limits established for these compounds by the current European legislation. A screening study of 24 different milk samples was also developed. In none of the samples, residues of the selected antibiotics were found.

1. Introduction

Milk, is a nutritious wholesome food widely consumed around the world. Among the different types of milk commercially available, bovine milk comprises the highest percentage of the milk commercialized worldwide, while ovine and caprine milk comprise the lowest amounts. Apart from their direct use in the diet, which is widely known, they are also important in the manufacture of milk derived products such as yogurts, butter, creams, etc. which are also extensively consumed by humans. In recent years increasing public concern about possible health risks of food consumption is being observed. In milk, for example, chemical residues of veterinary drugs like hormones, antimicrobials, etc., melamine and other chemical contaminants such as pesticides, dioxins, etc. have been found [1–4] with its subsequent concern of the population. Consequently, it is necessary to control/monitor residual levels of these compounds, in order to meet regulatory requirements and especially to protect the consumer and the environment.

Fluoroquinolone (FQ) antibiotics are synthetic antibacterial compounds widely used in human as well as in veterinary medicine for the treatment of digestive, urinary and pulmonary infections. FQs such as danofloxacin, (DANO), enrofloxacin (ENRO), ciprofloxacin (CIPRO, a metabolite of ENRO), sarafloxacin (SARA) and difloxacin (DIFLO, a metabolite of SARA) have been included in the EU Council Regulation 2377/90, which establishes their maximum residue limits (MRLs) in foodstuffs of animal origin [5]. In bovine, ovine and caprine milk, the only ones of them allowed are DANO, CIPRO and ENRO at concentrations below their MRLs which are 30 μg/kg for DANO, 100 μg/kg for CIPRO and ENRO (also 100 μg/kg for the sum of both of them since CIPRO is a metabolite of ENRO). These low MRLs require the development/application of highly sensitive and selective analytical methods for their monitorization.

Concerning the analysis of these antibiotics in milk, most of the applications deal with their analysis in full-cream bovine milk samples, however, no works have been published concerning the analysis of ovine milk or the simultaneous analysis of the three types of milk (bovine, caprine or ovine). In the case of caprine milk, only two works have been published but only CIPRO and ENRO were analyzed in both of them [3,6].

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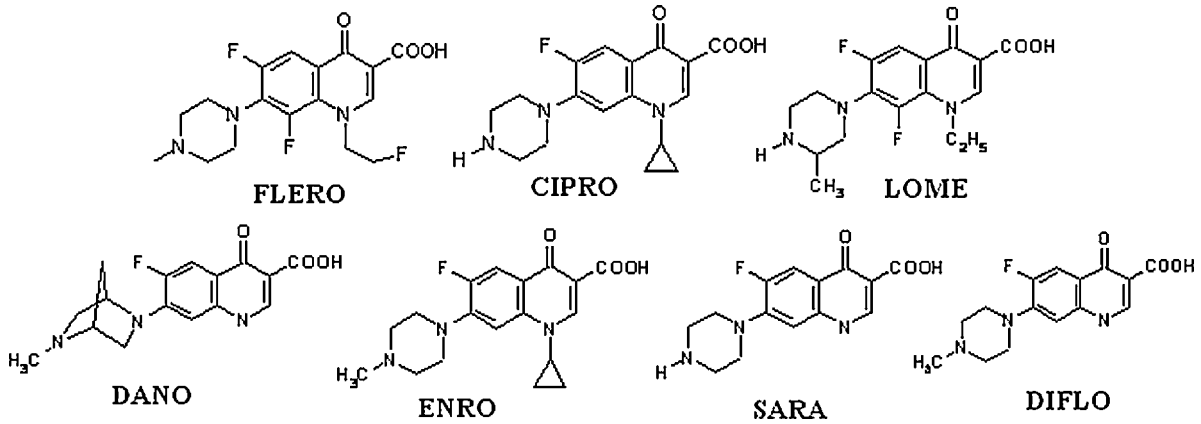

Fig. 1. Chemical structure of the studied FQ antibiotics.

In a previous work developed by our group [7], we proposed the determination of a group of seven FQ antibiotics (fleroxacin, FLERO, CIPRO, lomefloxacin, LOME, DANO, ENRO, SARA and DIFLO) in water samples by HPLC (high-performance liquid chromatography)-fluorescence detection (FL) using also ionic liquids as additives of the mobile phase (for structures see Fig. 1). FQs are basic compounds that are highly adsorbed onto HPLC silica columns and the use of ionic liquids decreased their adsorption onto the stationary phases and consequently, the useful life of the chromatographic column was increased. The comparison of different ionic liquids with the same counterion, like tetraethylammonium tetrafluoroborate (Et_4N-BF_4), 1-ethyl-3-methylimidazolium tetrafluoroborate (EMIm-BF_4), 1-butyl-3-methylimidazolium tetrafluoroborate (BMIm-BF_4), 1-hexyl-3-methylimidazolium tetrafluoroborate (HMIm-BF_4) and 1-methyl-3-octylimidazolium tetrafluoroborate (OMIm-BF_4) showed that the use of BMIm-BF_4 as additive of the mobile phase provided an effective separation of these compounds with high efficiency, relative low analysis time (14 min) and high sensitivity. In that work, also Oasis HLB cartridges of 500 mg were used as solid-phase extraction (SPE) materials for the first time for the extraction of these antibiotics, providing mean recovery values very close to 100%. Besides, very recently, the ionic liquid BMIm-BF_4 was also found clearly superior to the classical triethylamine (TEA) additive in terms of efficiency as well as peak shape enhancement of β-blockers which are also highly adsorbed onto C_{18} columns [8]. He et al. [9] also noted differences between TEA and ionic liquids in the separation of ephedrines and confirmed that ionic liquids are better additives. These two works as well as previous ones [10,11] have studied the effect of ionic liquids in the mobile phase but none of them have applied the method to the analysis of real samples.

In an attempt to extend and to demonstrate the application of the methodology previously developed by our group to the analysis of the seven FQs in milk samples maintaining at the same time high recovery values, we have studied its application to different types of these samples (ovine, caprine and bovine milk, in the last case in either skimmed, semi-skimmed and full-cream milk) using the ionic liquid EMIm-BF_4 as additive of the mobile phase instead of BMIm-BF_4 because of its higher ability to resolve interfering compounds from the milk samples. Concerning the current legislation, the EU has set MRLs in milk for CIPRO, ENRO y DANO but we have also included in this study other FQs like FLERO, LOME, SARA and DIFLO (metabolite of SARA) because FQs have such an exceptional efficiency that their illegal use in certain veterinary applications is very probable. In the specific case of DIFLO, EU regulation clearly establishes that it cannot be used in animals from which milk is produced for human consumption [5].

To the best of our knowledge, this is the first work published in which a unique method is applied to the analysis of FQs antibiotics in either bovine, ovine and caprine milk (also with recovery values higher than 73% in all cases) no matter the fat or protein content of the samples. It is also the first published application to the analysis of antibiotics in ovine milk and also the first application of an HPLC-FL method using ionic liquids as additives in the mobile phase to the simultaneous determination of these compounds in milk samples. Up to now, only one work (developed by our group) [7] has applied an HPLC method using ionic liquids as mobile phase additives to the analysis of real samples.

2. Experimental

2.1. Chemicals and samples

All chemicals used were of analytical-reagent grade. FLERO, CIPRO, LOME hydrochloride, DANO, ENRO, SARA hydrochloride and DIFLO hydrochloride were provided from Sigma–Aldrich (Madrid, Spain). Individual standard solutions (100 mg/L) were prepared in methanol and kept in the dark under refrigeration at 4 °C. Mixtures of pertinent concentrations were prepared by appropriate combination and dilution with methanol. The working solutions were prepared daily by dilution of these mixtures with the mobile phase.

Acetonitrile and methanol (HPLC grade) were from Scharlau (Sentmenat, Spain) and distilled water was deionized by using a Milli-Q gradient system A10 (Millipore, Bedford, MA, USA). Formic acid, trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany) and dehydrated ethylenediaminetetraacetic acid disodium salt (EDTA) was from Panreac (Barcelona, Spain). The ionic liquids were obtained from Fluka (Switzerland). EMIm-BF_4 and BMIm-BF_4 had purities higher than 97%.

Pasteurized bovine milk samples (skimmed, semi-skimmed and full cream) were purchased from local supermarkets of Tenerife (Canary Islands, Spain) while raw ovine and bovine milk and pasteurized and raw caprine milk were kindly supplied by Quesería El Faro from Lanzarote (Canary Islands, Spain). The samples were spiked with the selected antibiotics at several concentrations (see Section 3) in order to identify and quantify the analytes in the real samples.

2.2. HPLC-FL

A Waters HPLC system (Waters, Milford, MA, USA) was used consisting in two pumps (Model 510) controlled by a Waters automated gradient controller (Model 680), an injector (Rheodyne Model 7125 with a 20 μL loop) and a fluorescence detector (Waters Model 2475). For data storage and evaluation the Millenium32 software (Waters) and a personal computer were used. The analytical column was a

Nova-Pak C_{18} (150 mm × 3.9 mm, 4 μm) while the precolumn was a Guard-Pak C_{18} 4 μm both from Waters. The optimum mobile phase consisted of 3 mM EMIm-BF_4 and 10 mM ammonium acetate (pH 3.0 adjusted with acetic acid) and acetonitrile (87:13, v/v). Mobile phase was pumped at 1 mL/min at ambient temperature. The fluorescence detector was set at an excitation wavelength of 280 nm and an emission wavelength of 450 nm.

2.3. SPE procedure

Aliquots of 2 mL of milk were spiked with the selected antibiotics. Then, 6 mL of 20% (w/v) TCA in methanol were added. The mixture was sonicated for 30 min, kept in dark for 15 min more and centrifuged at 4500 rpm for 15 min. The supernatant was filtered through a Chromafil Xtra PET-45/25 filter (pore size 0.45 μm, Macherey-Nagel, Düren, Germany) and evaporated at 40 °C and 180 mbar using a Rotavapor R-200 (from Büchi Labortechnik, Flawil, Switzerland). The residue was dissolved with 50 mL of water containing 11.24 g/L EDTA (0.562 g of EDTA in 50 mL water). The pH was adjusted to 4.0 with 1 M NaOH and the sample was filtered through a Chromafil PET-45/25 filter again. Afterwards, the solution was subjected to a SPE procedure performed using a Vac-Master manifold from IST (Hengoed, UK). The sample was slowly passed through an Oasis HLB SPE cartridge (6 mL, 500 mg) from Waters previously activated with 5 mL methanol followed by 5 mL of water purified with a Milli-Q system (Millipore). After loading the sample into the SPE cartridge, it was dried under vacuum of −10 mmHg (1 mmHg = 133.322 Pa) for 20 min. The retained antibiotics were eluted with 10 mL methanol containing 1.5% (w/v) acetic acid. The organic solvent was then evaporated to dryness at 40 °C and 200 mbar. The residue was dissolved in 1 mL of mobile phase, filtered through 0.20 μm filter (Chromafil PET-20/25) and directly injected into the HPLC instrument (20 μL).

3. Results and discussion

3.1. Selection of the ionic liquid as mobile phase additive

As it has been mentioned in the introduction, in a previous work developed by our group [7], we proposed the determination of a group of seven FQ antibiotics in water samples by HPLC-FL using a mobile phase composed of 5 mM BMIm-BF_4, 10 mM ammonium acetate at pH 3.0 and 13% (v/v) acetonitrile (mobile phase flow of 1.0 mL/min, ambient temperature separation and 20 μL injection). The use of the ionic liquid BMIm-BF_4 as additive of the mobile phase decreased the adsorption of these basic compounds onto the stationary phases and consequently, the useful life of the chromatographic column was increased. A considerable reduction of the analysis time (from 34 min with TEA in the mobile phase to 14 min) as well as an increase in the peak efficiencies were achieved. In that work, also Oasis HLB cartridges of 500 mg were used as SPE materials for the first time for the extraction of these antibiotics from different water samples.

In an attempt to extend the application of the proposed method to the analysis of these antibiotics in milk samples (which are samples of great interest for the analysis of veterinary drug residues), several milk samples were subjected to the previously developed SPE procedure after deproteination with an acid. Preliminary studies were developed (all of them at ambient temperature), which will be later described. In these preliminary studies, when the milk samples extracts were injected in the HPLC system, a strong chromatographic interference from the sample matrix was found, especially for full-cream bovine milk. Fig. 2A shows the overlap of the peak corresponding to FLERO with the chromatographic interference. Based on previous experience and taking into account that the addition of BMIm-BF_4 was not able to resolve the problem,

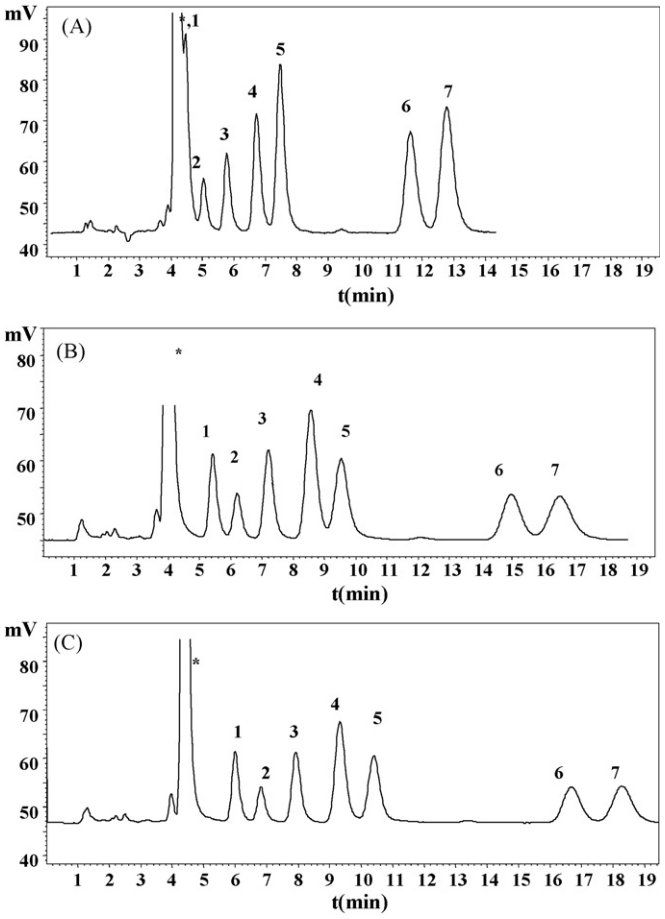

Fig. 2. Effect of different ionic liquids on the separation of the studied antibiotics in spiked full-cream bovine milk. Mobile phase: 10 mmol L^{-1} ammonium acetate at pH 3.0 with 13% (v/v) acetonitrile and (A) 5 mM BMIm-BF_4; (B) 3 mM EMIm-BF_4; (C) 5 mM EMIm-BF_4. Flow rate: 1 mL/min. Detection: λ_{exc} = 280 nm and λ_{em} = 450 nm. Peak identification: *, interference; 1, FLERO; 2, CIPRO; 3, LOME; 4, DANO; 5, ENRO; 6, SARA; 7, DIFLO.

we decided to test the performance of another ionic liquid EMIm-BF_4, at different concentrations (always below 10 mM) in the same mobile phase as well as different flow rates. In our previous work, the use of this ionic liquid also provided a good separation of the selected analytes but with a higher analysis time. A concentration of 6 mM in the same mobile phase and a flow rate of 1.0 mL/min provided a complete separation of the antibiotics as well as a complete resolution of FLERO with the matrix interference. However, at this concentration the analysis time was found to be 22 min. When this concentration was gradually reduced down to 3 mM, the analysis time was reduced maintaining at the same time a complete resolution for all the analytes (analysis time of approximately 17.5 min). Fig. 2B and C also shows the effect of the addition of different amounts of EMIm-BF_4 to the mobile phase. Therefore, since the addition of this ionic liquid solved the problem, a mobile phase composed of 3 mM EMIm-BF_4 (Fig. 2B) was used for subsequent experiments. The use of this ionic liquid will also prevent components of the sample matrix to be adsorbed by the stationary phase and thus increase the useful life of the column.

Table 1 shows calibration data, limits of detection (LODs, calculated as three times the signal-to-noise ratio), limits of quantification (LOQs, calculated as 10 times the signal-to-noise ratio), as well as the result of a repeatability study consisting in the three consecutive injections of a standard mixture of the seven FQs in three different days. As it can be seen in the table, relative standard deviation values for the retention times and peak areas were below 0.27%

Table 1
Intraday and interday precision, calibration data, LODs and LOQs of the method for standard solutions.

Peak	Antibiotic	Intraday precision (n = 3, % RSD)		Day-to-day precision (n = 9)		Range of concentration tested (mg/L)	Slope (n = 7)	Intercept (n = 7)	R^2	$S_{y/x}$	LOD[a] (μg/L)	LOQ[b] (μg/L)
		t_R	Areas	t_R	Areas							
1	Fleroxacin	0.12	4.0	1.2	8.4	0.01–0.28	$(2.34 \pm 0.26) \times 10^6$	$(-2.41 \pm 4.18) \times 10^4$	0.991	2.54×10^4	3.7	12.3
2	Ciprofloxacin	0.14	1.5	1.1	7.3	0.02–0.44	$(1.26 \pm 0.95) \times 10^6$	$(-0.92 \pm 2.23) \times 10^4$	0.996	1.49×10^4	6.0	20.1
3	Lomefloxacin	0.12	1.1	1.2	9.7	0.02–0.48	$(1.64 \pm 0.11) \times 10^6$	$(-2.44 \pm 3.09) \times 10^4$	0.997	1.92×10^4	6.7	22.2
4	Danofloxacin	0.20	1.8	1.2	8.6	0.01–0.06	$(1.51 \pm 0.12) \times 10^7$	$(-2.79 \pm 4.15) \times 10^4$	0.995	2.35×10^4	0.5	1.7
5	Enrofloxacin	0.19	2.2	1.5	8.8	0.01–0.17	$(5.07 \pm 0.58) \times 10^6$	$(-3.66 \pm 6.66) \times 10^4$	0.990	4.14×10^4	2.6	8.7
6	Sarafloxacin	0.27	1.8	1.7	7.3	0.02–0.40	$(9.34 \pm 0.77) \times 10^5$	$(-0.94 \pm 2.14) \times 10^4$	0.995	1.35×10^4	5.6	18.7
7	Difloxacin	0.26	1.1	1.8	9.5	0.01–0.18	$(2.00 \pm 0.12) \times 10^6$	$(-1.63 \pm 1.72) \times 10^4$	0.997	1.06×10^4	2.8	9.3

[a] LOD calculated as three times the signal-to-noise ratio.
[b] LOQ calculated as 10 times the signal-to-noise ratio.

and 4.0% for the intraday precision, respectively, and below 1.8% and 9.7% for the interday precision, respectively. Good correlation coefficients were obtained in all cases, with LODs between 0.500 and 6.67 μg/L. The linear range selected in this work was found appropriate since real milk samples with a high content of antibiotics rarely occur. For the calibration, seven different concentration levels were injected in quintuplicate in the HPLC system.

3.2. SPE procedure

Preceding methods dealing the SPE of antibiotics from milk samples involve a previous deproteination step with an acid (i.e. TCA, TFA, etc.) and a subsequent extraction with SPE cartridges [6,12,13]. Since Oasis HLB cartridges of 500 mg have not been used till now for their extraction from milk samples, preliminary experiments were developed using the optimized SPE procedure with this cartridges after different deproteination steps with either TCA and TFA. These cartridges were also used in our previous work for the extraction of the selected antibiotics from water samples obtaining high recovery values (close to 100%) [7]. These preliminary experiments were developed using 2 mL of spiked full-cream bovine milk treated with different amounts of TFA in MeOH. After deproteination, evaporation was carried out and the residue redissolved in 50 mL water containing 11.24 g/L of EDTA. pH was adjusted to 4.0 and the solution was passed through the SPE cartridge. Addition of EDTA to the liquid sample to be extracted by SPE is necessary since the formation of complexes between these FQs and EDTA clearly improve their extraction [7]. In this case, due to the competition of EDTA with the calcium present in the milk samples, special care was taken to ensure that the amount of EDTA was enough to quantitatively complex the seven FQs.

Concerning the efficiency of the extraction, for the addition of 6 mL of 25% (w/v) of TFA in MeOH, for example, mean recoveries were in the range between 52.2% for SARA and 62.2% for CIPRO. An increase in the amount of EDTA added or any other change in the SPE procedure (elution solvent volume, etc.) did not improve the recovery values. When TFA was changed for TCA and increase in the recovery values were obtained. The addition of 6 mL of 20% (w/v) of TCA provided mean recovery values between 96% for LOME and 113% for FLERO. The method was later applied to the extraction of skimmed and semi-skimmed bovine milk, finding very similar recovery values. According to the manufacturer skimmed, semi-skimmed and full-cream milk contained approximately 30 g/L of proteins and 3.6, 1.6, and 0.3 g/L of fat, respectively. When the method was applied to the extraction of caprine and ovine milk, it was also found that the recovery values were close to the previous ones, slightly lower in the case of ovine milk. In all cases, the use of these SPE cartridges provided very clean chromatograms for fluorescence detection. Figs. 3–5 show the HPLC-FL separations obtained for full-cream bovine milk, caprine and ovine milk, respectively, as well as for spiked samples (concentration range in the samples between 18 and 60 μg/L) of the same type. Similar chromatograms to the ones of Fig. 3 were obtained for skimmed and semi-skimmed bovine milk.

In order to demonstrate the applicability of the method to the extraction of these FQs from the three types of bovine milk no matter the fat content, as well as for ovine and caprine milk, a recovery study was developed at two concentration levels for each type of milk (five extraction in each case, n = 5). Results as well as the spiking levels for each analyte are shown in Table 2. As it can be seen in the table, recovery values higher than 73% were obtained for the three types of bovine milk samples, as well as for ovine and caprine milk at the different spiking levels (concentration range in the samples between 18 and 480 μg/L). Recoveries for bovine milk samples ranged between 94 and 113%, which represents LODs in the range 0.5–6.8 μg/L. For caprine milk, recoveries of 79–92%

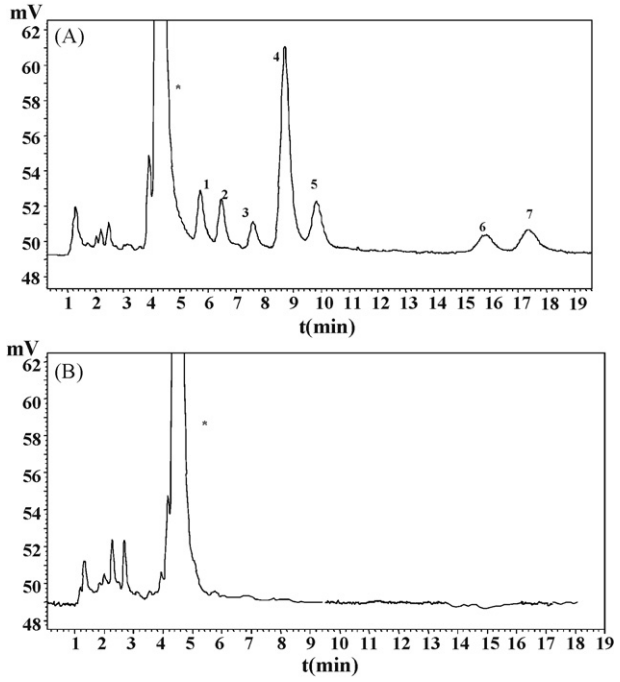

Fig. 3. LC-FL chromatograms of (A) a full-cream bovine milk sample spiked with the FQs (concentration: 0.04 mg/L of FLERO, 0.04 mg/L of CIPRO, 0.04 mg/L of LOME, 0.02 mg/L of DANO, 0.02 mg/L of ENRO, 0.06 mg/L of SARA and 0.04 mg/L of DIFLO); (B) a non spiked full-cream bovine milk sample. Mobile phase: 3 mM EMIm-BF_4, 10 mM ammonium acetate at pH 3.0 with 13% (v/v) acetonitrile. Flow rate: 1 mL/min. Detection: λ_{exc} = 280 nm and λ_{em} = 450 nm. Peak identification: *, interference; 1, FLERO; 2, CIPRO; 3, LOME; 4, DANO; 5, ENRO; 6, SARA; 7, DIFLO.

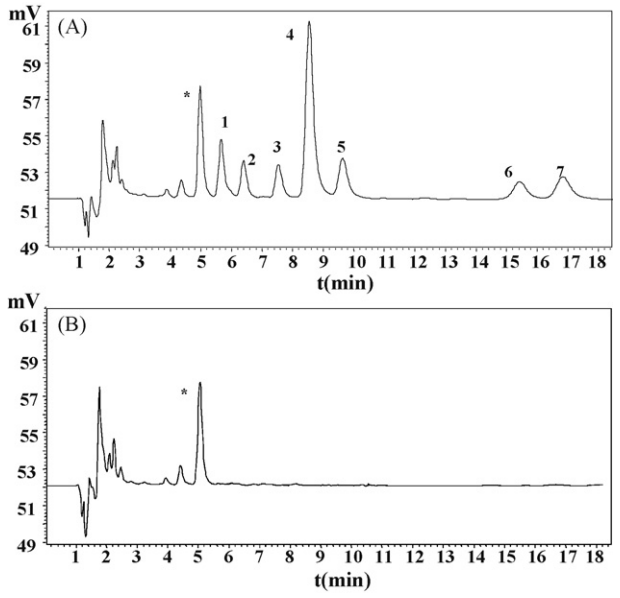

Fig. 4. LC-FL chromatograms of (A) a caprine milk sample spiked with the FQs (concentration: 0.04 mg/L of FLERO, 0.04 mg/L of CIPRO, 0.04 mg/L of LOME, 0.02 mg/L of DANO, 0.02 mg/L of ENRO, 0.06 mg/L of SARA and 0.04 mg/L DIFLO); (B) a non spiked caprine milk sample. Mobile phase: 3 mM EMIm-BF_4, 10 mM ammonium acetate at pH 3.0 with 13% (v/v) acetonitrile. Flow rate: 1 mL/min. Detection: λ_{exc} = 280 nm and λ_{em} = 450 nm. Peak identification: *, interference; 1, FLERO; 2, CIPRO; 3, LOME; 4, DANO; 5, ENRO; 6, SARA; 7, DIFLO.

Table 2
Mean recovery percentages (n = 5), RSD (between parenthesis), spiking level (between parenthesis), LOD and LOQ values of method.

Peak	Antibiotic	Bovine milk						LOD method (μg/L)	LOQ method (μg/L)
		Full-cream milk		Semi-skimmed milk		Skimmed milk			
		Level 1, recovery[a] (%RSD) (added mg/L)	Level 2, recovery[a] (%RSD) (added mg/L)	Level 1, recovery[a] (%RSD) (added mg/L)	Level 2, recovery[a] (%RSD) (added mg/L)	Level 1, recovery[a] (%RSD) (added mg/L)	Level 2, recovery[a] (%RSD) (added mg/L)		
1	Fleroxacin	113 (9) (0.18)	102 (2) (0.04)	104 (9) (0.15)	98 (9) (0.04)	104 (5) (0.18)	102 (11) (0.04)	3.6	11.8
2	Ciprofloxacin	108 (9) (0.12)	96 (5) (0.04)	98 (5) (0.12)	97 (3) (0.04)	97 (5) (0.12)	96 (8) (0.04)	6.1	20.3
3	Lomefloxacin	107 (11) (0.36)	92 (10) (0.04)	99 (7) (0.36)	95 (8) (0.04)	99 (9) (0.36)	95 (8) (0.04)	6.8	22.6
4	Danofloxacin	106 (8) (0.04)	96 (9) (0.02)	99 (6) (0.04)	104 (8) (0.02)	97 (10) (0.04)	96 (5) (0.02)	0.5	1.7
5	Enrofloxacin	102 (8) (0.12)	96 (3) (0.02)	100 (6) (0.12)	104 (3) (0.02)	96 (7) (0.12)	102 (6) (0.02)	2.6	8.7
6	Sarafloxacin	107 (8) (0.48)	96 (5) (0.06)	100 (8) (0.48)	94 (10) (0.06)	98 (6) (0.48)	101 (6) (0.06)	5.6	18.8
7	Difloxacin	107 (13) (0.24)	100 (3) (0.04)	100 (7) (0.24)	106 (4) (0.04)	99 (6) (0.24)	98 (7) (0.04)	2.8	9.1
Peak	Antibiotic	Caprine milk				Ovine milk		LOD method (μg/L)	LOQ method (μg/L)
		Level 1, recovery[a] (%RSD) (added mg/L)	Level 2, recovery[a] (%RSD) (added mg/L)	LOD method (μg/L)	LOQ method (μg/L)	Level 1, recovery[a] (%RSD) (added mg/L)	Level 2, recovery[a] (%RSD) (added mg/L)		
1	Fleroxacin	91 (7) (0.18)	92 (6) (0.04)	4.1	13.5	83 (5) (0.18)	85 (11) (0.04)	4.4	14.7
2	Ciprofloxacin	85 (8) (0.12)	79 (7) (0.04)	7.3	24.5	76 (7) (0.12)	80 (9) (0.04)	7.7	25.8
3	Lomefloxacin	86 (9) (0.36)	87 (9) (0.04)	7.7	25.7	79 (8) (0.36)	87 (11) (0.04)	8.1	26.8
4	Danofloxacin	89 (8) (0.04)	81 (12) (0.02)	0.6	1.9	80 (9) (0.04)	84 (15) (0.02)	0.6	2.0
5	Enrofloxacin	82 (7) (0.12)	86 (16) (0.02)	3.1	10.4	77 (8) (0.12)	79 (14) (0.02)	3.3	11.2
6	Sarafloxacin	84 (6) (0.48)	85 (6) (0.06)	6.6	22.1	73 (11) (0.48)	86 (11) (0.06)	7.0	23.4
7	Difloxacin	82 (6) (0.24)	83 (13) (0.04)	3.3	11.0	74 (13) (0.24)	80 (11) (0.04)	3.6	12.1

[a] Mean of five extractions (n = 5).

Table 3
Characteristics of the milk samples analyzed in this work.

Sample name	Type of milk	Content of proteins (g/100 mL)	Content of fat (g/100 mL)	Content of calcium (g/100 mL)	FQs found
1	Full cream pasteurized bovine milk	3.00	3.60	120	None
2	Full cream pasteurized bovine milk	3.00	3.60	120	None
3	Full cream pasteurized bovine milk	3.10	3.60	120	None
4	Full cream pasteurized bovine milk	3.10	3.60	120	None
5	Full cream pasteurized bovine milk	3.10	3.00	120	None
6	Semi-skimmed pasteurized bovine milk	3.00	1.50	120	None
7	Semi-skimmed pasteurized bovine milk	3.00	1.60	120	None
8	Semi-skimmed pasteurized bovine milk	3.15	1.55	120	None
9	Semi-skimmed pasteurized bovine milk	3.10	1.55	130	None
10	Skimmed pasteurized bovine milk	3.10	0.30	120	None
11	Skimmed pasteurized bovine milk	3.00	0.30	120	None
12	Skimmed pasteurized bovine milk	3.20	0.30	120	None
13	Skimmed pasteurized bovine milk	3.20	0.30	120	None
14	Raw bovine milk (local farmer)	–	–	–	None
15	Raw caprine milk (local farmer)	–	–	–	None
16	Raw caprine milk (local farmer)	–	–	–	None
17	Raw caprine milk (local farmer)	–	–	–	None
18	Raw caprine milk (local farmer)	–	–	–	None
19	Raw caprine milk (local farmer)	–	–	–	None
20	Raw caprine milk (local farmer)	–	–	–	None
21	Raw caprine milk (local farmer)	–	–	–	None
22	Raw caprine milk (local farmer)	–	–	–	None
23	Pasteurized caprine milk (local farmer)	–	–	–	None
24	Raw ovine milk (local farmer)	–	–	–	None

–, data not available.

and LODs between 0.6 and 7.7 μg/L were obtained, while for ovine milk, recoveries were in the range 73–87% and LODs between 0.6 and 8.1 μg/L. RSD values were below 16% in all cases. These LODs are also between 0.5 and 25.9 μg/kg of milk, which are well below the MRLs established by the UE (30–100 μg/kg). Therefore, the SPE procedure is highly suitable for the quantitative extraction of these antibiotics from any type of bovine, caprine and ovine milk sample. It should also be remarked that it is well known that ovine milk has a higher fat content than caprine or bovine milk. The fact the method is able to extract these compounds from all types of milk samples, is clearly a good advantage of the use of this method.

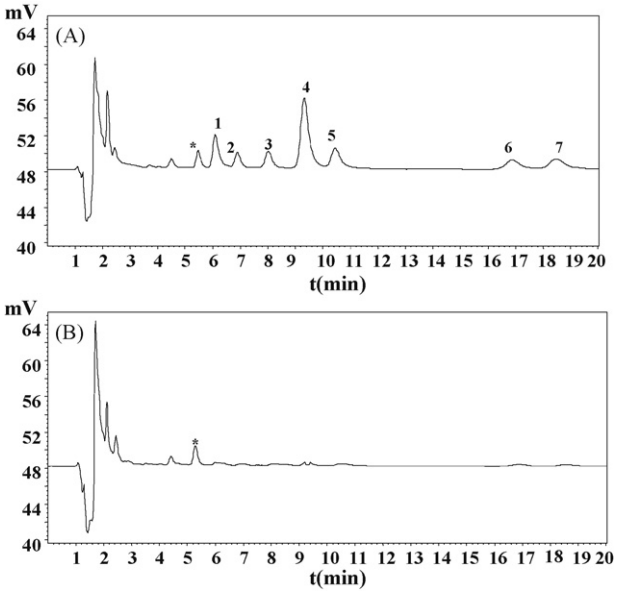

Fig. 5. LC-FL chromatograms of (A) a ovine milk sample spiked with the FQs (concentration: 0.04 mg/L of FLERO, 0.04 mg/L of CIPRO, 0.04 mg/L of LOME, 0.02 mg/L of DANO, 0.02 mg/L of ENRO, 0.06 mg/L of SARA and 0.04 mg/L DIFLO); (B) a non spiked ovine milk sample. Mobile phase: 3 mM EMIm-BF_4, 10 mM ammonium acetate at pH 3.0 with 13% (v/v) acetonitrile. Flow rate: 1 mL/min. Detection: λ_{exc} = 280 nm and λ_{em} = 450 nm. Peak identification: *, interference; 1, FLERO; 2, CIPRO; 3, LOME; 4, DANO; 5, ENRO; 6, SARA; 7, DIFLO.

The LODs values obtained in this work are also similar to the ones obtained by HPLC-MS [14] or HPLC-FL [3,15] for bovine milk and to the ones obtained by HPLC-FL [16] for CIPRO, ENRO, DANO and SARA in powdered infant formula. There are also similar to the ones obtained by HPLC-diode array detection (DAD) [6] and HPLC-FL [3] for CIPRO and ENRO in caprine milk. Concerning ovine milk, as previously indicated, no works have been found concerning the analysis of these FQs in milk samples. The recovery values obtained in this work are also very similar to the ones obtained in previous works in which a SPE procedure (with different cartridges than the ones used in this work) has been developed for similar FQs in bovine milk using CE-MS [17] and HPLC-MS [18] and with different detection systems (UV, FL and MS) [19] and also for ENRO and CIPRO in goat milk [3–6].

3.3. Milk sample analysis

In order to apply the proposed method to the analysis of the selected antibiotics in milk samples, a screening study was developed. For this purpose, different bovine milk samples currently commercialized in Spain as well as caprine and ovine milk from local farmers were analyzed following the proposed procedure. Table 3 compiles the information of the different samples (a total of 24) as well as the results obtained, concerning their content in the different antibiotics. In all cases, no residues of the selected antibiotics were found. Clean chromatograms as the ones obtained in Figs. 3–5B were obtained in all cases.

4. Conclusions

A sensitive and selective SPE–HPLC-FL method using ionic liquids as mobile phase additives has been described for the determination of seven FQs antibiotics in bovine, ovine and caprine milk samples below the MRLs legislated by the EU. The use of a concentration of 3 mM of EMIm-BF_4 in the mobile phase allowed a complete separation of the selected antibiotics in less than 18 min (no chromatographic interferences were found under these conditions). The screening of 24 milk samples some of them currently consumed in the Spanish market and other from local farmers, revealed the absence of the analyzed compounds in such samples.

The use of Oasis HLB cartridges of 500 mg allowed the application of the method to any type of bovine, caprine and ovine milk no matter their fat or protein content. The use of ionic liquids as mobile phase additives has greatly help to modify HPLC selectivity, especially important for the analysis of basic analytes also in highly complex samples like milk.

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