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Laser induced fluorescence coupled to capillary electrophoresis for the determination of fluoroquinolones in foods of animal origin using molecularly imprinted polymers☆

Manuel Lombardo-Agüí, Ana M. García-Campaña*, Laura Gámiz-Gracia, Carmen Cruces Blanco

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Campus Fuentenueva, s/n, E-18071 Granada, Spain

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ABSTRACT

A method for the simultaneous determination of four fluoroquinolones of veterinary use (ciprofloxacin, danofloxacin, enrofloxacin and sarafloxacin) in two complex matrixes, such as bovine raw milk and pig kidney, has been established and validated. The method is based on the use of capillary electrophoresis (CE) coupled with a very sensitive detection mode, such as laser induced fluorescence (LIF) detection, due to the fact the all the compounds selected show native fluorescence. In order to achieve high selectivity in the sample treatment procedure, a commercially available molecularly imprinted polymer has been used for the solid phase extraction of the analytes. Once the retention and elution processes were optimized, the final extract was analyzed by CE-LIF using a 325 nm He–Cd laser. Optimum separation was obtained in a 70 cm × 75 μm capillary using a 125 mM phosphoric acid solution at pH 2.8 with 36% methanol as background electrolyte. The method provided very low detection limits, ranging from 0.17 to 0.98 μg/kg for milk and 1.10 to 10.5 μg/kg for kidney, with acceptable precision and satisfactory recoveries.

1. Introduction

Fluoroquinolones (FQs) belong to an important group of antibiotics, derivatives of quinolones, widely used in veterinary and human medicine for the treatment of a wide variety of infections [1,2]. They are effective against a wide range of gram positive and negative bacteria. Their activity is based on the inhibition of bacterial DNA, which prevent bacterial multiplication. In the last years the extensive use of FQs in veterinary has promoted the persistence of these residues in foods derived from animals, which can represent an increment in adverse reactions for human health. Consequently the European Union has established maximum residues limits (MRLs) for several FQs in foodstuffs of animal origin through the Council Regulation 2377/90//EEC [3]. As an example, these limits have been established in 30 μg/kg for danofloxacin, and 100 μg/kg for the sum of enrofloxacin and ciprofloxacin in bovine milk, while 200 μg/kg for danofloxacin, ciprofloxacin and enrofloxacin in pig kidney.

Quinolones have been mainly analyzed by HPLC and CE. Some recent reviews summarize some of the reported analytical methods [4–6]. The chemical structure of quinolones, with a carboxylic group (acidic quinolones) and in most cases with an amino group as well (zwitterionic quinolones), allows their separation by CE usually in the capillary zone electrophoresis (CZE) mode, being UV–vis the preferred detection mode [7–14], although fluorescence [15], mass spectrometry (MS) [16–18], amperometry [19] and electrochemiluminescence detection [20] have also been reported. Those methods have been described for the analysis of FQs in different matrices, mainly animal tissues [8,9,11,16,17,19] and in less proportion in milk [13,18,20], biological fluids [7,15] or environmental waters [15]. Laser induced fluorescence (LIF) detection has also been proposed as a very sensitive method for the determination of ofloxacin and its metabolites in urine [21], moxifloxacin [22], ciprofloxacin (CIPRO) and its metabolite [23] in plasma, enrofloxacin (ENRO) and CIPRO in chicken muscle [24], and ofloxacin, lomefloxacin, norfloxacin, danofloxacin (DANO), ENRO and sarafloxacin (SARA) in environmental waters [25].

Taking into account the low MRLs established for food samples, a sample pretreatment is mandatory, being solid phase extraction (SPE) in the off-line mode the preferred sample preparation technique [4,6,11,18,26], although the in-line coupling of SPE sorbents [16] and microwave-assisted extraction [27] have also been proposed. Most of the methods reported for the analysis of FQs in foods involve the use of different SPE cartridges, such as Oasis HLB [11], Strata X [26], ENV+ Isolute or Oasis MAX [11]. Also, a tandem of MAX and HLB cartridges was used to ensure a convenient preconcen-

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* Corresponding author. Tel.: +34 958 242385; fax: +34 958 249510.
E-mail address: amgarcia@ugr.es (A.M. García-Campaña).

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tration step and a very clean extract [18]. These alternatives have proved to be suitable for the determination of quinolone residues by CE or HPLC in food of animal origin such as meat [11,9,28], milk [18,29–31] or eggs [32].

Others materials with highest selectivity, such as molecularly imprinted polymers (MIPs) can provide cleaner sample extract and easier process than usual SPE sorbents. MIPs are synthetic materials with artificially generated recognition sites able to specifically capture target molecules. The use of MIPs as selective sorbent materials allows performing a customized sample treatment step prior to the final determination. Thus, the strong retention between the MIP and its target molecules makes it ideal for the selective extraction of compounds at trace levels, being of special interest when the sample is complex. Several reviews show the characteristics of these materials and their applications in analytical chemistry [33–37]. In the last years MIPs have been employed successfully in the extraction of different antibiotics, such as β-lactam antibiotics in environmental water [38] or sulfamethazine in milk [39]. Some MIPs have been synthesized in several laboratories using different templates for the determination of FQs. CIPRO has been used as template for the selective analysis of FQs in soils [40] or baby food [41]. Also, an ENRO-imprinted polymer was applied to the selective extraction of this FQ and CIPRO from urine and pig liver [42]. A novel highly selective sample cleanup procedure combining molecular imprinting and matrix solid phase dispersion (MI-MSPD) was developed for the simultaneous isolation of ofloxacin, pefloxacin, norflorxacin, CIPRO, and ENRO in chicken eggs and swine tissues [43] and ofloxacin-imprinted polymers were prepared in water-containing systems and used as SPE sorbents for the selective extraction of nine quinolones from urine samples [44] and six quinolones in serum samples [45].

In this paper we propose a useful alternative to quantify very low concentrations of four FQs of veterinary use (CIPRO, DANO, ENRO and SARA) using the very recently commercially available MIPs as sorbent for SPE (MISPE) in the treatment of complex matrices, such as bovine raw milk and pig kidney. The use of CE coupled to LIF detection is also proposed as a way of improving sensitivity. Extraction using MIPs has been optimized in order to improve analytes recoveries. The obtained results demonstrate the possibilities of CE-LIF for the quantification of residues of these compounds in foodstuffs of animal origin at the concentrations required by their MRLs.

2. Experimental

2.1. Chemicals

All reagents were of analytical reagent grade. Solvents were HPLC grade and FQs were analytical standard grade. Ultrapure water (Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout the work. Methanol (MeOH), acetonitrile (ACN), sodium hydroxide, ammonium hydroxide (30%), sodium dihydrogen phosphate monohydrate and phosphoric acid (85%) were obtained from Panreac-Química (Barcelona, Spain). Acetic acid (96%) was purchased from Merck (Darmstadt, Germany). CIPRO, DANO, ENRO, and SARA were obtained from Sigma (St. Louis, MO, USA). Chemical structures of FQs includes in this study are shown in Fig. 1.

A vacuum manifold system from Supelco (Bellefonte, USA) was used for SPE procedure. Extraction cartridges containing molecularly imprinted polymer (SupelMIP FQs SPE Column, 25 mg, 3 mL) supplied by Supelco (Bellefonte, PA, USA) were used for extraction and cleanup process. Sterile syringe filters with 0.45 μm polyethersulfone membrane (VWR, Barcelona, Spain) were use for kidney extract filtration after solid–liquid extraction and 13 mm filters with 0.2 μm Nylon membrane (Bulk Acrodisc®, Pall Corp., MI, USA), were used for filtration of the final extracts before CE analysis.

2.2. Preparation of solutions

Background electrolyte (BGE) consisted on 125 mM phosphoric acid solution adjusted to pH 2.8 with 4 M sodium hydroxide, containing 36% methanol. For milk sample treatment, 10 mM ammonium acetate buffer (pH 5) was prepared from acetic acid by using 3% ammonium hydroxide solution to adjust the pH. The 50 mM phosphate buffer (pH 7.4) was prepared from NaH_2PO_4, adjusting the pH using 4 M sodium hydroxide solution.

Stock standard solutions (100 mg/L) of each FQ were prepared by dissolving the appropriate amount of each analyte in ACN, and were stored in the dark at 4 °C. Under such conditions, they were stable for at least 1 month. A 1 mg/L working mixed-standard solution of CIPRO, ENRO and SARA was prepared daily in ACN by diluting the stock solutions. An independent solution was prepared for DANO under the same conditions.

2.3. Instrumentation

CE experiments were carried out on an HP^{3D} CE instrument (Agilent Technologies, Waldbronn, Germany) coupled to a LIF detector (Zetalif Evolution model LIF UV-01, Picometrics S.A., Ramonville, France). Data were collected using the software provided with the HP ChemStation version A.09.01. Fused-silica capillaries (75 μm I.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

SPE was carried out on a Visiprep™ DL vacuum manifold (Supelco) for 12 cartridges. A pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit, a Universal 320R centrifuge (Hettich, Zentrifugen) and a vortex-2 Genie (Scientific Industries, Bohemia (NY, USA) were also used.

2.4. Electrophoretic procedure

Before the first use, the new capillary (70 cm × 75 μm I.D., effective length 55 cm) was conditioned by rinsing with 1 M NaOH at 60 °C for 15 min, then with water at room temperature for 10 min, and finally with the BGE for 25 min. Every day the capillary was prewashed with 0.1 M NaOH for 8 min, with water for 1.5 min and with BGE for 15 min. After each run, the capillary was washed with 0.1 M NaOH for 2 min, water for 1 min and BGE for 2 min, to maintain an adequate repeatability of run-to-run injections. At the end of the day, the capillary was cleaned with water for 4 min and finally flushed with air for 4 min. In all cases, a N_2 pressure of 1 bar was applied.

Half an hour before starting a series, the laser was shut on. The electrophoretic separation was achieved using the described BGE, and applying a voltage of 26 kV (normal mode). Sample was injected by hydrodynamic injection using a pressure of 50 mbar for 10 s. The temperature of the capillary (35 cm thermostatized plus 35 cm at room temperature) was kept constant at 15 °C and the room temperature at a value of ca. 20 °C.

2.5. Sample treatment

The method has been applied to two types of foodstuffs of different origin: bovine raw milk and pig kidney.

2.5.1. Preparation of milk samples

Samples of 10 mL of bovine raw milk (obtained from a local farm) were spiked at different concentration levels using the working standard solutions. After spiking and homogenizing in vortex, samples were diluted with 10 mL of 10 mM ammonium acetate buffer

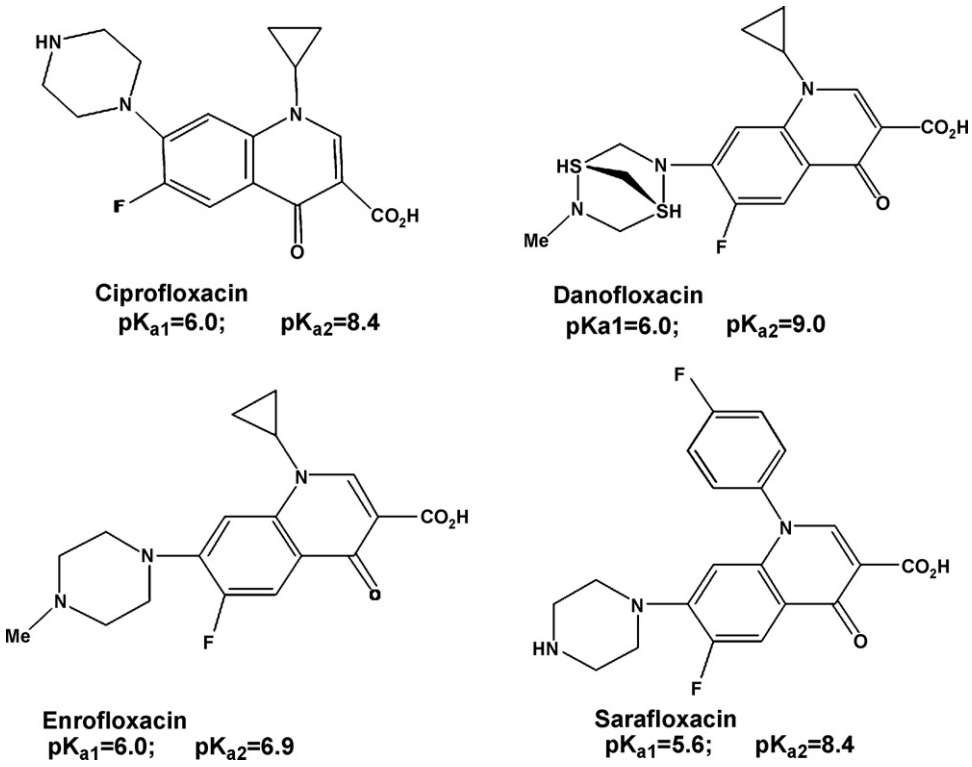

Fig. 1. Structures and pK_a values of the fluoroquinolones under study.

pH 5.0, shaken for 5 min and then centrifuged at 5000 rpm. The pH of the supernatant was adjusted to 7.0 with 3% ammonium hydroxide solution, and ammonium acetate buffer was added to obtain a final volume of 25 mL; the pH of the final solution was checked to be 7.0. SupelMIP FQ SPE Column was used for the selective SPE procedure. For the cartridge conditioning, 1 mL of methanol, 2 mL of water and 0.5 mL of 10 mM ammonium acetate buffer were successively applied. An aliquot of 1 mL of the final solution was charged into the preconditioned cartridge at a flow rate of approximately 0.2 mL/min. After that, the cartridge was washed with 3 mL of water and 1 mL of ACN, both of them at a flow rate lower than 0.5 mL/min. After each washing step, vacuum (400 mbar) was applied for 2 min. The elution was achieved using 3 mL methanol/H_2O (50/50, v/v) with 3% ammonium hydroxide. This extract was evaporated to dryness at 35 °C under a stream of nitrogen and the residue was redissolved in 400 μL of ACN/BGE (25/75, v/v), filtered and analysed by the proposed CE-LIF method.

2.5.2. Preparation of kidney samples

Pig kidney was purchased from a local market. Kidney was crushed and homogenized and portions of 2 g were spiked at different concentration levels of FQs using the working standard solutions. Solvent was evaporated under N_2 stream, and the kidney sample was homogenized again. For sample pretreatment, this portion was mixed with 30 mL of 50 mM NaH_2PO_4 pH 7.4, shacken for 5 min and centrifuged for 5 min at 5000 rpm.

Table 1
MISPE protocols for the extraction of quinolones after the sample pretreatment. Comparison between the commercially proposed and optimized protocols.

	Commercially proposed protocol	Optimized protocol in this work
1. Condition/equilibrate	1 mL methanol 2 mL ultra pure water.	1 mL methanol 2 mL ultra pure water 0.5 mL extraction buffer[a]
2. Load	1 mL of sample supernatant	1 mL of sample supernatant
3. Wash in the described order*	3 mL ultra pure water 1 mL ACN 1 mL 15% ACN in ultra pure water[b]. 1 mL 0.5% acetic acid in ACN −1 mL 0.1% ammonia in ultra pure water *A strong vacuum was applied through the cartridge for 2 min between each wash step.	3 mL ultra pure water 1 mL ACN *Vacuum was applied through the cartridge for 2 min between each wash step.
4. Elute	Elute FQs with 1 mL 2% ammonium hydroxide in methanol	Elute FQs with 3 mL 3% ammonium hydroxide in methanol/water (50/50)
5. Evaporate/reconstitute	The SPE eluent was evaporated gently under nitrogen at 35 °C and reconstituted in 150 μL 50% ACN in 0.1% formic acid prior to analysis.	The SPE eluent was evaporated gently under nitrogen at 35 °C and reconstituted in 400 μL ACN/BGE (25/75) prior to analysis.

[a] 10 mM ammonium acetate buffer pH 5.0 for milk samples and 50 mM phosphate buffer pH 7.4 for kidney samples.
[b] Only for milk samples.

Table 2
Calibration curves, statistical parameters, LODs and LOQs of the proposed method.

	Bovine raw milk				Pig kidney			
	CIPRO	DANO	ENRO	SARA	CIPRO	DANO	ENRO	SARA
Linear dynamic range (μg/kg)	1.78–75	0.55–30	1.14–75	3.26–75	19.5–400	3.5–200	10.4–400	35–400
Slope	13.2	75.4	30.0	9.3	2.1	14.8	4.9	1.5
Intercept	93.3	75.6	11.1	17.0	67.2	−93.5	−116.0	21.2
R^2	0.991	0.994	0.992	0.994	0.990	0.990	0.983	0.990
LOD (μg/kg)	0.53	0.17	0.34	0.98	5.9	1.1	3.12	10.5
LOQ (μg/kg)	1.78	0.55	1.14	3.26	19.5	3.5	10.4	35.0
MRLs (μg/kg)[a]	100[b]	30	100[b]	Not established	200[b]	200	200[b]	Not established

[a] MRLs established by the European Regulation (EEC) No 2377/90.
[b] Quantified as the sum of CIPRO and ENRO.

An aliquot of 2 mL of the resulting supernatant was filtered and 1 mL of this final solution was charged into the SupelMIP FQ cartridge, previously preconditioned at the same way that was indicated before, at a flow rate of approximately 0.2 mL/min, and submitted to the elution process above described for milk samples.

3. Results and discussion

3.1. Optimization of the CE-LIF experimental conditions

As it was previously reported, and according with the pK_a values of the different analytes indicated in Fig. 1, the most adequate CE separation of quinolones is commonly achieved with basic buffers [46]. However, fluorescence intensities of the selected FQs are very low at alkaline conditions, so for this reason, and taking into account previous research [25], an acidic electrophoretic buffer at pH 2.8 was chosen. Nevertheless, an exact adjustment of the buffer pH is not so critical, because the electrophoretic mobilities of the quinolones hardly change with pH values between 2.0 and 4.0 [7,46,47]. At this pH, citric and phosphoric acid solutions were tested, obtaining the best result for the later. Phosphoric acid concentration for the BGE was modified between 100 and 150 mM (adjusting the pH with 1 M NaOH solution and the best results in terms of resolution, migration time and current intensity were found for a concentration of 125 mM. The effect of different modifiers in the BGE (ACN, MeOH and ethanol) was studied. MeOH provided the best results and therefore its influence was studied in the range of 5–40%. The migration times decreased when increasing the MeOH percentage, obtaining the best resolution and a shorter analysis time with a percentage of 36% of MeOH in the BGE, in agreement with the results found in the literature [48]. Then, the separation voltage was varied from 20 to 28 kV. A voltage of 26 kV was selected as optimum, as it provided the best compromise between resolution, migration time and electric current. The effect of the temperature on the separation was tested in the range of 15–30 °C, selecting 15 °C as optimum, as this temperature provided the best resolution and no significant differences in peak areas were observed.

3.2. Optimization of MISPE procedure

The use of MIPs can simplify the extraction method of these complex matrixes, providing a greater selectivity and a lower sample manipulation. Initially, the protocols proposed by Supelco for the SupelMIP FQs SPE Columns for the analysis of SARA, ENRO, CIPRO, norfloxacin, lomefloxacin, and ofloxacin in milk and kidney samples [49], were tested for our analytes, but we tried to simplify them in order to reduce the extraction steps, considering the intrinsic selectivity of the LIF detection.

3.2.1. Milk samples

Based on the commercially proposed protocol [49], a re-optimization of the MISPE was carried out in order to adapt it to the CE-LIF method. A comparison between both protocols is shown in Table 1. A significant increase in the recovery percentages, above 10% for SARA, ENRO and CIPRO and above 35% for DANO, was observed when a final additional step was added in the conditioning, consisting of 0.5 mL of 10 mM ammonium acetate buffer (pH 5) for milk samples and 50 mM phosphate buffer (pH 7.4) for kidney samples.

As can be seen in Table 1, the commercially proposed protocol suggested five washing steps with different solvents after loading of 1 mL of the supernatant from the sample pretreatment. However, we observed that satisfactory recoveries (even better than those reported by the fabricant) were obtained with only the first and second washing steps. The elution step proposed in the commercial protocol did not produce a complete elution of the analytes, so different aqueous mixtures of methanol (25–90%) were studied in presence of a 2% ammonium hydroxide solution, selecting a methanol/water mixture (50/50; v/v) as optimum. Different percentages (1–5%) of ammonium hydroxide solution in the mixture were studied and the best results were obtained using 3%. Finally, different elution volumes (1–4 mL) were tested in order to increase the recovery percentages. The highest recoveries were obtained with a volume of 3 mL. This final extract was evaporated under gentle nitrogen stream and the residue was recomposed in 400 μL of ACN/BGE (25/75) solution [25]. This procedure do not involve any preconcentration because it is not necessary due to the high sensitivity of the propose method.

3.2.2. Kidney samples

As in the case of milk samples, the commercially proposed protocol for kidney samples (see Table 1) was tested. However the same optimized MISPE protocol for milk samples was applied to kidney samples. Only the sample pretreatment was different in this case (the sample was mixed with 30 mL of 50 mM NaH_2PO_4 (pH 7.4), shaken for 5 min and centrifuged for 5 min at 5000 rpm) and the buffer in the conditioning step was the above described. Better results were obtained compared to the commercially proposed protocol. Using this procedure, no interferences comigrating with the analytes were observed in the electropherogram.

3.3. Method validation

3.3.1. Calibration curves, detection and quantification limits

Matrix-calibration curves for cow raw milk and pig kidney samples free of analytes and spiked with different concentration levels were established, considering peak areas as analytical signal.

Cow raw milk samples were spiked at 1, 5, 10, 20 and 30 μg/kg for DANO and 5, 15, 25, 50 and 75 μg/kg for the rest of FQs, and pig kidney samples were spiked at 10, 50, 100, 150 and 200 μg/kg for DANO and 50, 100, 200, 300 and 400 μg/kg for the rest of FQs. Each

Table 3
Precision study.

	Bovine raw milk				Pig kidney			
	CIPRO	DANO	ENRO	SARA	CIPRO	DANO	ENRO	SARA
Intraday RSD (%) (n = 9)								
Level 1[a]	4.9	4.7	7.5	5.9	10.0	7.8	5.9	7.0
Level 2[b]	3.2	3.1	5.5	4.7	7.3	8.5	10.5	8.5
Mig. time (min)	22.5	22.9	23.2	23.6	22.6	23.1	23.4	23.7
RSD (%) ($n = 18$)	1.7	1.7	2.0	1.7	4.8	4.7	4.6	4.6
Interday RSD (%) (n = 15)								
Level 1[a]	5.3	8.3	11.6	8.7	13.9	11.6	12.8	12.2
Level 2[b]	7.7	10.9	8.8	6.9	12.5	12.9	10.1	12.5
Mig. time (min)	24.1	24.5	24.9	25.2	23.1	23.6	23.9	24.2
RSD (%) ($n = 30$)	4.4	4.3	4.3	4.2	3.1	3.0	3.0	3.2

[a] Level 1: 5 μg/kg for DANO and 15 μg/kg for the rest in milk samples; 50 μg/kg for DANO and 100 μg/kg for the rest in kidney samples.
[b] Level 2: 20 μg/kg for DANO and 50 μg/kg for the rest in milk samples; 150 μg/kg for DANO and 300 μg/kg for the rest in kidney samples.

Table 4
Recovery study ($n = 15$).

	Bovine raw milk				Pig kidney			
	CIPRO	DANO	ENRO	SARA	CIPRO	DANO	ENRO	SARA
Level 1[a]	91.1	93.8	86.5	95.2	98.6	91.4	85.5	96.4
RSD (%)	3.3	1.7	4.9	3.2	9.1	5.6	10.1	8.2
Level 2[b]	88.1	94.0	85.2	86.2	97.3	89.7	87.2	97.5
RSD (%)	5.8	4.5	3.8	3.5	7.0	6.3	7.8	9.5

[a] Level 1: 5 μg/kg for DANO and 15 μg/kg for the rest in milk samples; 50 μg/kg for DANO and 100 μg/kg for the rest in kidney samples.
[b] Level 2: 20 μg/kg for DANO and 50 μg/kg for the rest in milk samples; 150 μg/kg for DANO and 300 μg/kg for the rest in kidney samples.

concentration level was prepared by triplicate, and all spiked samples were submitted to the SPE procedure. A blank sample was also analysed, and no peaks were found comigrating with the analytes. Limits of detection (LODs) and limits of quantification (LOQs) were considered as the minimum analyte concentration yielding an S/N ratio equal to 3 and 10, respectively. The statistic parameters calculated by least-square regression, LODs and LOQs of the method for milk and kidney samples are shown in Table 2. As can be seen, very low LODs were obtained in all cases, allowing the quantification of the analytes at concentrations much lower than the established MRLs for these samples, without any preconcentration step.

3.3.2. Precision study

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision).

Repeatability was assessed by means of repetitive application of the whole procedure to three samples (experimental replicates) spiked at two concentration levels: 5 and 20 μg/kg for DANO; and 15 and 50 μg/kg for the rest of FQs for milk samples, and 50 and 150 μg/kg for DANO; and 100 and 300 μg/kg for the rest of FQs for kidney samples. Each one was injected by triplicate (instrumental replicates) on the same day. Intermediate precision was assessed with a similar procedure, with five samples analysed in different days. The results, expressed as RSD of peak areas and migration times, are given in Table 3. As can be observed, acceptable precisions were obtained in all cases.

3.3.3. Recovery studies

In order to check the trueness of the proposed methodology, recovery experiments were carried out in cow raw milk and pig kidney samples.

Samples were spiked with a mixture of the four FQs at two levels, similar to those used in the precision study. Each level was prepared by quintuplicate, submitted to the proposed method and injected by triplicate. Blank samples were also analysed, and none

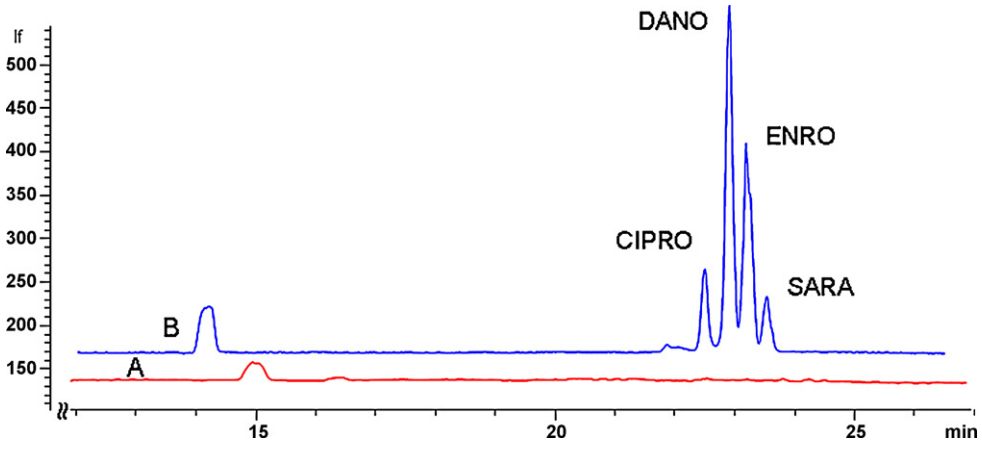

Fig. 2. Electropherograms at optimum conditions of (A) blank sample of pig kidney; (B) spiked pig kidney sample (300 μg/kg for CIPRO, ENRO and SARA and 150 μg/kg for DANO).

of them gave a positive result for FQ residues. The results are shown in Table 4. Typical electropherograms of blank and spiked samples are shown in Fig. 2 for kidney and very similar results were obtained for milk. As can be seen, a very clean extract was obtained using this MISPE procedure in such complex matrix, being a very selective and efficient analyte extraction method. This advantage, combined with the highly sensitivity of the LIF detection provided a very useful method for the analysis of this four FQs in this kind of samples of animal origin. The proposed method provided also satisfactory results in terms of trueness and precision, so the accuracy for the analysis of these samples was demonstrated.

4. Conclusions

A simple, selective and sensitive strategy for the determination of four FQs in two complex matrixes has been developed, showing the usefulness of MIPs as a powerful tool for extraction and sample cleanup. The MISPE procedure has been reduced, compared with the commercially proposed, without any decreases in the recovery percentages. Additionally, LIF is proposed as a very attractive detection technique in CE, showing very high sensitivity and selectivity. LOQs were much lower than the MRLs established by EU, without any preconcentration step, and could be easily improved by just increasing the sample volume loaded into the MIP cartridge or by decreasing the final recomposition volume after the elution process. The recoveries and precisions obtained are good enough, and show the suitability of this procedure for the monitoring of FQs residues in foodstuffs of animal origin. This study shows interesting perspectives of the application of MIPs for the monitoring of these compounds in other samples.

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