Valeria Springer[1,2]
Johan Jacksén[1]
Patrik Ek[1]
Adriana G. Lista[2]
Åsa Emmer[1]*

[1]Analytical Chemistry, Division of Applied Physical Chemistry, Department of Chemistry, School of Chemical Science and Engineering, KTH Royal Institute of Technology, Stockholm, Sweden
[2]Analytical Chemistry Section, INQUISUR (UNS-CONICET), Buenos Aires, Argentina

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Research Article

Determination of fluoroquinolones in bovine milk samples using a pipette-tip SPE step based on multiwalled carbon nanotubes prior to CE separation

A simple CE–UV method was developed for the simultaneous determination of ciprofloxacin, norfloxacin, and ofloxacin in milk samples. The optimum separation was obtained using a 20 mM ammonium dihydrogenphosphate solution with 2 mM cetyltrimethylammonium bromide at pH 3.0 as the BGE. Satisfactory resolution for structurally very similar analytes, like norfloxacin and ciprofloxacin, was achieved without including any organic solvent. Milk samples were prepared using a simple/extraction procedure based on acidic protein precipitation followed by an SPE step using only 5 mg of multiwalled carbon nanotubes as the sorbent material. The LODs for the three compounds were between 7.5 and 11.6 μg/L and the RSDs for the peak areas were between 2.6 and 4.9%. The complete method was applied to spiked real milk samples with satisfactory recoveries for all analytes (84–106%).

Keywords: CE / Fluoroquinolones / Multiwalled carbon nanotubes / SPE
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1 Introduction

Fluoroquinolones (FQs) are synthetic antibacterial agents of broad-spectrum activity against both Gram-positive and Gram-negative bacteria inhibiting the enzyme DNA gyrase. FQs are commonly used in human and veterinary medicine for the treatment of infectious diseases [1]. However, their use in veterinary practices differs significantly depending on animal species and geographical region [2]. In general, FQs are used for the treatment and prevention of diseases and as feed additives in food-producing animals [3, 4]. The massive use of FQs has become a serious problem since residues can be found in processed products and food of animal origin. These residues can be directly toxic, or can induce pathogens resistant to antibiotics and allergic hypersensitivity reactions in humans. Taking this into account, several international organizations, such as the EU (European Union) and the FDA (Food and Drug Administration) have established tolerance levels ranging from 30 to 1900 μg/kg for several FQ compounds in different samples in order to protect the human health [5, 6]. For example, according to Regulation (EU) 37/2010, maximum residue limits (MRLs) established in milk are 100 μg/kg for the sum of enrofloxacin and ciprofloxacin (CIP), 75 μg/kg for marbofloxacin, and 30 μg/kg for danofloxacin. Norfloxacin (NOR) and ofloxacin (OFL) are not allowed in milk produced for human consumption. Therefore, there is a substantial need for sensitive and selective analytical methods for the control of FQ levels to comply with the current legislation. So far, the determination of FQs has been performed using HPLC combined with UV, fluorescence, or MS detection [2, 7–12]. MS detection offers high sensitivity and selectivity to ensure the correct identification of these compounds in food samples [13–15]. However, some aspects must be considered for the compatibility of MS when coupled to separation techniques. In addition, MS detection is expensive and, thus, not fully available to all laboratories.

CE has also been proposed for analyzing FQ residues in milk samples since it offers advantages over other techniques, such as fast separations, high resolution power, and minimal consumption of samples and reagents [16–19]. Although excellent separation efficiency can be achieved, the main drawback of this technique is the limited sensitivity

Correspondence: Valeria Springer, Analytical Chemistry Section, INQUISUR (UNS-CONICET), Av. Alem 1253, B8000CPB Bahía Blanca, Buenos Aires, Argentina
E-mail: valeria.springer@uns.edu.ar
Fax: +54-291-4595160

Abbreviations: ACN, acetonitrile; **CIP**, ciprofloxacin; **CNT**, carbon nanotube; **CTAB**, cetyltrimethylammonium bromide; **FA**, formic acid; **FQ**, fluoroquinolone; **HCl**, hydrochloride acid; **MRL**, maximum residue limit; **MWCNT**, multiwalled carbon nanotube; **NOR**, norfloxacin; **OFL**, ofloxacin

*Additional corresponding author: Professor Åsa Emmer, E-mail: aae@kth.se

obtained when UV detectors are used. To overcome this, several on-line/off-line preconcentration steps can be included to determine low concentrations of analytes in complex samples, such as biological, food, or environmental samples. Liquid–liquid extraction, SPE, cloud-point extraction, and dispersive liquid–liquid microextraction are the commonly employed methods for sample pretreatment [20–24]. SPE is one of the most used procedures to carry out both the preconcentration of analytes and the clean-up of samples, especially in food analysis [17, 25]. Furthermore, miniaturized SPE is promoted as an important tool for cleaning and preconcentration procedures that employ small amounts of sorbent material and minimal sample and reagent consumption [26].

In recent years, carbon nanotubes (CNTs) have been proposed as sorbent material for sample preconcentration devices in SPE procedures. Because of their unique physical and chemical properties, such as high adsorption capacity, good thermal stability, and wide pH range of application, these nanoparticles show excellent potential as a sorbent material [27–29].

In this work, we have developed a simple, selective, and sensitive method for the simultaneous separation and quantification of CIP, NOR, and OFL in milk samples by CE–UV. Moreover, a simple, fast, and cheap miniaturized SPE procedure based on nonfunctionalized multiwalled CNTs (MWCNTs) was performed as a clean-up and preconcentration step prior to analysis. To the best of our knowledge, this is the first time that CIP and NOR peaks are completely resolved in CE analysis using a very simple composition of BGE solution without including any organic solvents. In addition, this is the first time that a MWCNTs-based tip device is used for the extraction of these FQs.

2 Materials and methods

2.1 Chemicals

CIP, NOR, and OFL (Supporting Information Fig. S1) were purchased from Parafarm (Buenos Aires, Argentina); ammonium formate, 2-propanol, and Tween 20 were obtained from Sigma–Aldrich (Stockholm, Sweden). Ammonium dihydrogenphosphate, potassium dihydrogenphosphate, formic acid (FA), hydrochloride acid (HCl), methanol as well as cetyltrimethylammonium bromide (CTAB) were acquired from Merck (Buenos Aires, Argentina). Acetonitrile (ACN), ethanol, and Triton X-100 were obtained from Fluka (Stockholm, Sweden). Nonfunctionalized MWCNTs with external diameters of 40–60 nm and purity >95% were provided by Bayer (Buenos Aires, Argentina). Individual stock solutions of FQs were prepared in 50:50 ACN/ultrapure water (Synergy 185 system, Millipore, Bedford, USA) and kept in the dark at 4°C. These solutions were stable for at least one month. The standard working solutions were prepared daily by appropriate dilutions of stock solutions in ultrapure water.

2.2 Instrumentation

A Hewlett Packard HP ^{3D}CE system (Palo Alto, CA, USA) was used for the initial experiments regarding optimization of the BGE composition and the SPE procedure. Also, a Beckman Coulter (Palo Alto, CA, USA) CE instrument MDQ P/ACE equipped with a diode array detector was used for evaluating the analytical performance of the proposed method and the analysis of real samples. The capillaries were from CM Scientific (Silsden, UK) using the HP instrument and from Beckman Coulter using the Beckman instrument. Control and data processing was carried out with Chemstation software (HP instrument) and 32 Karat software (Beckman instrument). To treat the milk samples, a Rolco centrifuge (Buenos Aires, Argentina; 4000 rpm) was used and a magnetic stirrer (IKA® C-MAG HS 4) was employed for the assistance of the analyte extraction.

2.3 Miniaturized SPE procedure

The SPE procedure was carried out using 5 mg of nonfunctionalized MWCNTs as sorbent material placed into a 1000 μL pipette tip (Supporting Information Fig. S2). Glass wool frits were used to keep the adsorbent material inside the tip. The SPE sorbent material was activated with successive rinsing steps of methanol and water and then dried with air before introducing the sample. In all cases, an aliquot of defined volume was aspirated into the conditioned MWCNTs tip and dispensed back into the same vial, and this is referred to as an aspirating/dispensing cycle. The tip was positioned inside the sample solution and the extraction procedure was assisted by magnetic stirring at room temperature. Before elution, the tip was rinsed with water using the same aspirating/dispensing cycles described above and dried by vacuum. Then, 500 μL of methanol containing 2% FA was aspired into the tip in the elution step. Five consecutive extraction cycles were performed. The eluting solvent was evaporated to dryness at 45°C and finally reconstituted with 100 μL of water before CE–UV analysis.

2.4 CE analysis

The separation was carried out in a fused-silica capillary. The dimensions of capillaries were 70 cm effective length, 84 cm total length, 50 μm id, and 375 μm od (using the Beckman Coulter Instrument) and 71 cm effective length, 95 cm total length, 50 μm id, and 375 μm od (using the HP instrument). A negative voltage of 25 kV was applied at 20°C. A mixture of 20 mM ammonium dihydrogenphosphate and 2 mM CTAB at pH 3.0 was used as the BGE. At the beginning of each day, the capillary was conditioned by flushing 0.1 M NaOH (5 min), ultrapure water (3 min), and buffer solution (5 min). Between runs, the capillary was rinsed with buffer solution for 5 min. The hydrodynamic injection mode was used

applying 0.5 psi for 15 s. All electropherograms were recorded at 280 nm.

2.5 Milk sample preparation

Commercially available full-cream, infant formula, and skimmed milk samples were obtained from a local supermarket and selected for analysis. Thus, samples with different contents of fat were considered. Furthermore, infant formula milk was included because it contains extra components in the formula, such as B1, B3, B6 vitamins and minerals among other components that could have an effect on the FQ analysis (additional details in Supporting Information Table S1). The samples were initially analyzed to verify the absence of the studied analytes. Then, 15 mL of milk samples were spiked with each antibiotic, homogenized by shaking (30 s), and left to equilibrate for 20 min. Considering the procedure reported by Springer and Lista [17], milk samples were treated with 2 mL of 3 M HCl in order to promote protein precipitation before the extraction procedure. After protein precipitation, the mixture was centrifuged at 4000 rpm for 30 min, and the supernatant was collected in a centrifuge tube. Before performing the SPE procedure the pH was adjusted with 0.1 M NaOH since the most efficient retention of the analytes was achieved at pH 6. The extracts were centrifuged again for 10 min at 4000 rpm and clear supernatant solutions were obtained. These supernatant extracts were subjected to the SPE procedure described in Section 2.3 before CE–UV analysis (Section 2.4).

3 Results and discussion

3.1 Optimization of SPE procedure

Considering the low concentration of OFL, NOR, and CIP in milk and the generally limited sensitivity of CE–UV analysis, a preconcentration step is necessary prior to CE analysis. In this case, SPE using nonfunctionalized MWCNTs is proposed for sample pretreatment. The interaction of CNTs with the aromatic ring of organic substances through π–π interactions has previously been described [30]. Taking into account the chemical structure of the target analytes, MWCNTs were selected as sorbent material for the SPE procedure.

A simple device including a minimal amount of MWCNTs inside a pipette tip was developed and a dynamic extraction procedure was carried out. This tip-based SPE procedure allows a significant reduction in conditioning/washing/elution volumes. In order to evaluate the SPE performance, the amount of sorbent material, the number of extraction cycles, extraction temperature, the eluent solvent, the effect of pH on the samples, and eluent were considered. All experiments were performed using a standard solution (200 μg/L of each analyte in aqueous solution). Analyte peak areas obtained during the CE analysis (at 280 nm) were used for evaluation of the extraction performance.

3.1.1 pH of sample solution

It is well known that the pH value of the solution can affect extraction of the analytes as the dissociation equilibrium is affected together with the solubility of the acidic/basic analytes. Therefore, the extractions were performed under different pH conditions ranging from pH 3 to 8 (by adding appropriate volumes of 0.1 M HCl or 0.1 M NaOH to the solution). An increase in peak-area response was observed when the pH was increased from 3 to 6 and the response decreased slightly above 6. In view of that, sample solution pH was adjusted to 6 because at this pH value, all compounds could exist as near neutral molecules and should be easily extracted from the solution. According to the literature, the pK_{a1} and pK_{a2} values are between 5.86 and 8.62 for all analytes [31].

3.1.2 Amount of sorbent material

As the efficiency of extraction in SPE is influenced by the interaction between analytes and MWCNTs, the amount of sorbent material placed into the tip must be evaluated [26]. It is preferred to employ a minimal quantity, while maintaining satisfactory extraction for all analytes. Thus, it is possible to reduce the sample and reagents consumption, and minimal amounts of residue are generated. Varying amounts of MWCNTs (3–8 mg) in the miniaturized SPE device were evaluated by extracting 15 mL of aqueous solution (pH 6) containing 200 μg/L of each antibiotic. It was observed that satisfactory extraction (decided based on peak-area response) for the three antibiotics was achieved when 5 mg of MWCNTs were employed without improvement of the extraction performance when larger quantities were used. Also, glass wool and acetate wool were proposed for packing the sorbent material into the tip, and it was observed that glass wool presents better operational resistance than acetate wool during the dynamic SPE procedure. Glass wool frits stay unchanged inside the tip throughout the whole SPE procedure.

3.1.3 Sample volume and extraction temperature

The initial sample volume placed into the vial was set at 15 mL and then a sample aliquot was aspired and dispensed from the SPE tip into the vial during the extraction procedure. The sample aliquot volumes tested were between 500 and 1000 μL. It was observed that lower initial sample volumes and aliquots volume generally resulted in smaller peak areas in the CE analysis, and a decrease in peak area was also noted when larger sample volumes were used. One possible reason for this phenomenon might be saturation of the MWCNTs by large samples so the MWCNTs no longer can retain additional analytes, while only 5 mg of sorbent material was packed into the device. Thus, 15 mL was selected as the initial sample volume for the extraction, and a sample aliquot volume of 1000 μL was chosen.

The temperature was varied between 20 and 40°C, while the extraction was assisted by magnetic stirring. No improvement of the analyte extraction was observed at higher

temperatures than 20°C and, therefore, all further experiments were performed at room temperature.

3.1.4 Optimization of dynamic extraction and eluting solvent

The number of rinsing sets necessary for FQ extraction was studied and optimized. As mentioned in Section 2.3, one cycle was considered to be complete when a determined volume of solution was aspired and dispensed from the tip. Different cycle schemes were tested for the activation of MWCNTs and the loading of samples. Methanol was used for the activation of this sorbent material according to the consulted literature [30]. Thus, 500 μL of both methanol and water were employed for the activation protocol, including four cycles with methanol first, followed by three cycles with water. For sample loading, a 1000 μL volume was withdrawn from the initial sample solution of 15 mL into the tip, testing 10, 20, or 30 cycles, and it was concluded that adequate extraction (determined by peak-area response) for the FQs was accomplished when 20 cycles were used. This number of aspirating/dispensing cycles was used in subsequent experiments giving an extraction time of approximately 2 min. Before the elution of analytes, the tip was washed with 500 μL of water performing two consecutive cycles.

Furthermore, the composition and volume of the eluent were also optimized. Taking into account the polarity of the FQs and the sorbent material, methanol was selected as eluent. However, the inclusion of FA was found to increase the analyte desorption, and to yield high recoveries for all three FQs. The content of FA was evaluated in a range between 1 and 10% v/v and the results showed that methanol with a 2% v/v of FA gave the highest peak area response, so it was used as eluent. In addition, different volumes of eluent were tested (500–1000 μL) aiming for the use of minimal amounts of organic solvent, still with satisfactory desorption for all analytes. Finally, 500 μL of methanol containing 2% v/v of FA was decided the optimum volume of eluent, and the analytes were desorbed employing five cycles (complete SPE protocol summarized in Supporting Information Table S2). The eluent was allowed to dry at 45°C and the FQs reconstituted in 100 μL of ultrapure water prior to CE analysis.

3.2 Optimization of CE analysis

According to several previous studies [32–34] regarding the determination of FQs, the use of high concentrations (over 40% v/v) of organic solvent in the buffer solution is necessary for the separation of the structurally very similar analytes NOR and CIP. Taking this into account, the primary aim of this work was to develop a method using a BGE with a simple composition for the separation of OFL, CIP, and NOR. In addition, this BGE was considered for a possible subsequent final identification of these compounds by CE–MS. Therefore, certain aspects of the CE separation must be taken into account to be compatible with the further MS analysis because commonly used buffer ions and surfactants can suppress the MS signals of the analytes in, for example, MALDI-TOF-MS analysis [35]. Thence, several BGEs with the addition of organic solvents or surfactants were investigated. As the initial step, CE–UV analyses were performed to find optimal parameters for the BGE and possible additives. For this purpose, ammonium formate, ammonium dihydrogenphosphate, and potassium dihydrogenphosphate were proposed as BGEs, and the concentration for all solutions was fixed at 20 mM. Considering that the studied FQs have a carboxylic and a piperazinyl group including additional amino groups, the pH was adjusted at 3.0 in order to protonate the analytes [31]. Because it was not possible to accomplish the complete resolution of the analyte peaks when only using simple BGE solutions, surfactants such as Tween 20, Triton X-100, and CTAB and organic solvents (ACN, ethanol, 2-propanol, and methanol) were evaluated as additives. From the first set of experiments, it was seen that CIP and NOR were not completely separated when 2-propanol or ethanol (5–20% v/v) were added. Also, ACN and methanol were tested in the same percentage range, and only a minor enhancement of resolution along with an unstable baseline was obtained when percentages above 10% v/v were used. Triton X-100 in the BGE was discarded due to unsatisfactory peak shapes and baselines when tested in a concentration range between 0.05 and 0.2% w/v. Furthermore, Tween 20 and CTAB were tested in a concentration range between 0.05 and 0.1% w/v and 1 and 20 mM, respectively (a comparison of the electropherograms is shown in Supporting Information Fig. S3). From these experiments, satisfactory resolution was obtained when 2 mM CTAB was included in the separation solution instead of Tween 20. Finally, a BGE containing 20 mM ammonium dihydrogenphosphate with 2 mM CTAB at pH 3.0 was selected since acceptable separation for these three analytes could be achieved. In order to obtain the best resolution and the shortest analysis time possible, the voltage applied as well as the separation temperature were evaluated. The separation voltage was changed between 15 and 25 kV applying reverse polarity, and the temperature was varied from 15 to 25°C. From these conditions, optimal separations in terms of resolution and analysis time were obtained using 20°C and 25 kV. In addition, different injection times and a range of injection pressures were evaluated taking into account the effect of these variables on the sensitivity and the resolution of peaks. Thus, the samples were injected in the hydrodynamic mode, and injection time and pressure were tested between 5 and 20 s and 0.1 and 0.5 psi, respectively. The optimal results were obtained when the samples were injected applying 0.5 psi for 15 s.

Finally, Fig. 1 shows the electropherogram obtained under the optimal conditions for FQ separation carried out when a different CE instrument was used (Beckman Coulter MDQ P/ACE). It demonstrates the minimal effect of different instruments and analysis days on the separation selectivity and resolution (see Supporting Information Fig. S3C for a comparison of the electropherograms). Hence, it was

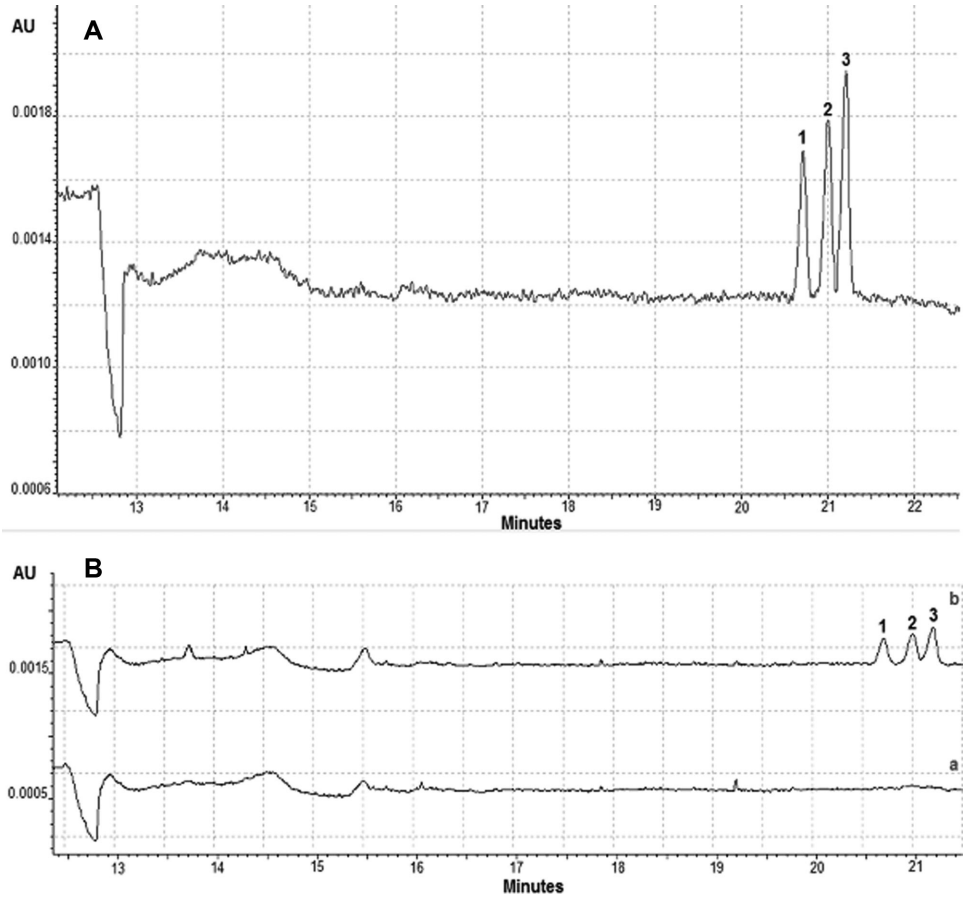

Figure 1. (A) Electropherogram of a standard solution containing 20 mg/L of each analyte. (B) Analysis of a real milk sample using the optimized SPE–CE–UV method. (a) Electropherogram of a full-cream milk sample. (b) Electropherogram of a full-cream milk sample spiked with 100 μg/L of each antibiotic. Peaks: (1) OFL, (2) CIP, (3) NOR. Electrolyte solution: 20 mM ammonium dihydrogenphosphate and 2 mM CTAB (pH 3.0). CE instrument: Beckman Coulter MDQ P/ACE (Palo Alto, CA, USA). Optimal experimental conditions are given in Section 3.2.

possible to reproduce the analytical methodology maintaining the reproducibility of the test results, which demonstrates the robustness of the proposed method.

3.3 Analytical parameters and analysis of real samples

Under the optimal conditions mentioned in Section 3.3, a highly efficient separation and enrichment have been achieved for the quantitative analysis of OFL, CIP, and NOR. The linearity, LODs, and peak area reproducibility for the proposed method were investigated using the optimal conditions and the results are listed in Table 1 (for additional details, see Supporting Information Table S3). Six points were included in the calibration graph and each one corresponds to the average of three individual measurements. The LODs were calculated as three times $S_{y/x}$/slope [36] of the calibration graph and they meet the general MRLs established by EU for FQs in milk samples. Also, the LOD values are comparable with those obtained in previous studies by using other preconcentration techniques and CE, LC, and HPLC [8,16,19,37–39]. In most cases, even a slightly better sensitivity was obtained by using this straightforward sample pretreatment procedure, and a simple BGE system for the CE–UV determination (see Supporting Information Table S4).

Three commercial milk samples were analyzed, full-cream, skimmed, and infant formula milk, to evaluate the applicability of the proposed method to determine these FQs. After ensuring that the samples were free of the selected antibiotics, a recovery study was carried out at two concentration levels (50 and 100 μg/L). Table 2 shows the obtained recovery values when the complete proposed method (including SPE step) was applied to the real samples. As can be seen, these recoveries varied between 84 and 106%, which were acceptable for these milk samples and the analytes investigated. In addition, the recovery and RSD values for the peak area are comparable with the experimental data previously reported in the literature in which real occurrence of CIP in bovine milk is demonstrated after mastitis treatment [40]. The slightly lower recovery values of OFL at 50 μg/L in infant formula and full-cream milk samples could be explained considering the high total fat content of both samples (≥3.0%) and the lipophilicity of this compound [41]. All the obtained results demonstrated the reliability of the method. Finally, Fig. 1B shows a typical electropherogram obtained for the analysis of a milk sample and a spiked milk sample containing 100 μg/L of each antibiotic. As can be seen, there are no interfering peaks from the matrix components that could affect the determination of these FQs (for additional details, see Supporting Information Fig. S4).

Table 1. Analytical parameters of the miniaturized SPE–CE–UV method

Antibiotic	Slope	Intercept	R^2	Linear Range (μg/L)	LOD[a] (μg/L)	RSD [b] (%)
OFL	26.0 ± 0.5	1353.6 ± 80.2	0.998	30–250	11.6	3.6
CIP	38.1 ± 0.5	219.3 ± 80.2	0.999	20–250	8.1	4.9
NOR	37.6 ± 0.4	663.2 ± 72.6	0.999	20–250	7.5	2.6

a) LOD calculated as three times $S_{y/x}$/slope [36].
b) RSD of peak area for five measurements (corresponding to a standard of 100 μg/L).

Table 2. Analysis of spiked milk samples using the proposed method

	Sample					
	Infant milk		Full-cream milk		Skimmed milk	
Added Concentration (μg/L)	Recovery (%)[a]	RSD (%)	Recovery (%)[a]	RSD (%)	Recovery (%)[a]	RSD (%)
50						
OFL	86	6.2	84	6.4	92	4.4
CIP	106	6.4	104	1.7	94	5.4
NOR	105	2.8	99	6.2	99	3.4
100						
OFL	93	5.0	97	2.1	103	5.4
CIP	91	5.7	96	5.9	98	1.9
NOR	99	4.0	105	5.3	102	2.3

a) Mean of three measurements ($n = 3$).

4 Concluding remarks

The proposed method allows the simultaneous determination of three FQ compounds in milk samples at low concentration levels. Separation between CIP and NOR was demonstrated with a common electrolyte solution without the inclusion of any organic solvent, and employing minimal amounts of surfactant. The obtained LODs meet the general requirements according to the MRLs established by the EU for FQs in milk. Therefore, the proposed method represents a straightforward alternative for the analysis of CIP, NOR, and OFL in different milk samples compared to results obtained using more complex analytical methodologies.

Moreover, the pretreatment of the samples is very simple, only the elimination of the proteins and fat followed by centrifugation steps were needed. The preconcentration of analytes was done with a simple, fast, and cheap miniaturized SPE procedure, with a small amount of MWCNTs in the pipette tip and low volume consumption of reagents and samples. In addition, this is the first time that a pipette-tip-based miniaturized SPE with nonfunctionalized MWCNTs as sorbent material has been used for the preconcentration of these FQs.

Finally, the developed method employing a BGE with a simple composition could represent a useful strategy for the future off-line coupling between CE and MALDI-MS that allows the complete analysis and identification of OFL, CIP, and NOR.

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