Valeria H. Springer
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Received December 27, 2011
Revised February 9, 2012
Accepted February 27, 2012

Research Article

Micellar nanotubes dispersed electrokinetic chromatography for the simultaneous determination of antibiotics in bovine milk

A method to determine four antibiotics for veterinary use (ciprofloxacin, enrofloxacin, florfenicol, and chloramphenicol) of different families (fluoroquinolones and amphenicols) in bovine milk was developed. The determination of the analytes was carried out using micellar electrokinetic capillary chromatography (MEKC) with a common sodium borate-SDS buffer solution containing single-walled carbon nanotubes (SWCNTs). In this way, a great improvement in the electrophoretic resolution and the separation efficiency was achieved compared to MEKC. An online reverse electrode polarity-stacking mode (REPSM) was carried out to enhance sensitivity. This step was performed in only 2 min and it allowed a stacked percentage of 103. That means that all the amount of injected analytes is effectively stacked. When this stacking procedure was combined with an off-line preconcentration step, based on SPE, analytes could be detected in lower concentration than the established maximum residue limits (MRLs). The LODs for the four compounds were between 6.8 and 13.8 $\mu g\ L^{-1}$ and the RSD values were between 1.1% and 6.6%. The whole method was applied to spiked real samples with acceptable precision and satisfactory recoveries.

Keywords:
Antibiotics / Carbon nanotubes / Micellar electrokinetic chromatography / Sample stacking
DOI 10.1002/elps.201100713

1 Introduction

Antibiotics represent a group of medicaments widely used in human and veterinary medicine. In the current cattle practices, veterinary drugs are massively used mixed with feedstuff and drinking water. These drugs are used to prevent the outbreak of diseases due to bacterial infections, dehydration, or to loss during their transportation [1]. On the other hand, veterinary drugs (e.g., antibacterial substances, anthelmintics, anticoccidiostats, sedatives, and nonsteroidal anti-inflammatory drugs) are included as growth-promoting agents [1, 2].

Fluoroquinolones (FQs) and amphenicols (APhs) are commonly antibiotics used in veterinary medicine because they are effective against a wide range of Gram positive and negative bacteria. Ciprofloxacin (CIPRO) and enrofloxacin (ENRO) are synthetic antimicrobial agents with bactericidal action, used for the treatment of respiratory and gastrointestinal infections. Chloramphenicol (CLOR) is a broad-spectrum antibiotic, and florfenicol (FLOR) is a compound derivated from CLOR and has other structural features. It removes the ability to produce anemia in animal plasma unlike CLOR, and has been currently indicated for the treatment of bovine respiratory disease [3]. Due to the excessive use of these veterinary drugs, their residues can be found in food of animal origin, like milk and meat. Also, natural water reservoirs and soils may be contaminated with these residues from the cattle manures [4]. Antibiotics in food can cause allergic reactions due to hypersensitivity, and subsequent effects on the human immune system. With the purpose of ensure the safety of food for consumers, several control agencies such as the US Food and Drug Administration (FDA) [5], European Union (EU) [6, 7], and SENASA in Argentina [8] have set maximum residue limits (MRLs) for a wide range of antibiotics, including FQs and APhs. According to EU, these limits in milk samples have been established in 100 $\mu g\ kg^{-1}$ for CIPRO plus ENRO, and 50 $\mu g\ kg^{-1}$ for FLOR. CLOR is forbidden in this kind of sample.

Therefore, several methods have been developed to detect and quantify the residues of FQs and APhs in food of animal origin by using HPLC with different detections and diverse

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Abbreviations: **APh**, amphenicol; **CIPRO**, ciprofloxacin; **CLOR**, chloramphenicol; **CNT**, carbon nanotube; **ENRO**, enrofloxacin; **FLOR**, florfenicol; **FQ**, fluoroquinolone; **MiNDEKC**, micellar nanoparticle dispersed electrokinetic chromatography; **MRL**, maximum residue limit; **MWCNT**, multiwalled carbon nanotube; **PSP**, pseudostationary phase; **REPSM**, reverse electrode polarity-stacking mode; **SC**, surfactant-coated; **SWCNT**, single walled carbon nanotube

Colour Online: See the article online to view Figs. 1 and 2 in colour.

modes of CE [3, 9–12]. CE is a powerful and useful technique in the separation and determination of antibiotics, including FQs and APhs [13, 14], since it offers numerous advantages over other techniques, such as faster separations, higher resolution power, and the use of smaller sample amounts. Micellar electrokinetic capillary chromatography (MEKC) is one of the electrophoretic modes most widely applied to determine a large variety of compounds including neutral species. In this modality, a pseudostationary phase (PSP) is added to the buffer to improve the separation and resolution of different compounds. In the last researches, different PSPs for MEKC have been proposed [15–17].

In recent years, there is an increasing interest in carbon nanostructures as PSPs in MEKC. Despite its hydrophobic nature, carbon nanotubes (CNTs) can be used as PSPs in aqueous medium when they are functionalized or dispersed in surfactants [18]. The electrophoresis mode that uses surfactant-coated carbon nanotubes (SC-CNTs) is named micellar nanoparticle dispersed electrokinetic chromatography (MiNDEKC). This mode allows the enhancement of resolution due to interaction between analytes and the surface of CNTs [19].

Although excellent separation efficiency is easily achieved, the main drawback of CE is the low sensitivity obtained when ultraviolet-visible detectors are used. As alternative analytical approaches to enhance sensitivity in CE-UV, several online preconcentration procedures based on electrophoretic techniques have been proposed [20, 21]. One of them is sweeping that has been developed for the online concentration of neutral analytes for MEKC separation [22]. On the other hand, several stacking techniques have been proposed to carry out the preconcentration of neutral and charged analytes inside the capillary before separation. Generally, in these techniques a large volume of the sample solution is introduced into the capillary by pressure or electrokinetic modes. The analytes in a long sample zone are focused into a narrow zone before separation. Reverse electrode polarity-stacking mode (REPSM) can be applied to obtain higher concentration efficiency when MEKC is used. Stacking is evaluated based on analyte retention factors and the nature of the PSPs. In this case, samples are prepared in low conductivity matrixes, which are injected as long plugs into the capillary and a voltage is applied. Therefore, the change in migration velocity is caused by the change in the electrical field strength between the sample solution and the separation zone or the change in the effective charge on the analytes [21]. To preconcentrate anions by REPSM, a negative voltage is first applied to remove the sample matrix. Then, the polarity is switched to positive when the current reaches 90–99% of the predetermined current to which the separation and detection of stacked zones are carried out. For the preconcentration of cations, an additive is used to reverse the EOF. This online preconcentration technique allows sensitivity to increase about 100-fold. Sometimes, it is necessary to include an off-line preconcentration step in order to improve the analytical sensitivity. Solid phase extraction (SPE) is one of the most used procedures to carry out both, the preconcentration and the clean-up of the samples [14, 23, 24].

In this work, a simple, selective, and sensitive method for identification and simultaneous quantification of CIPRO, ENRO, CLOR, and FLOR in different milk samples is developed. Taking into account the different chemical properties of the analytes, MEKC is applied in this work combined with single-walled carbon nanotubes (SWCNTs). So, the analytical procedure is based on the use of off-line SPE step prior to REPSM-MiNDEKC-UV analysis. Although there are many methods to determine FQs and APhs, the authors have not found in the literature works where these four analytes are simultaneously determined in bovine milk samples. To this reason, the relevance of this work lies in the novel strategy to simultaneous determination of all these antibiotics. Moreover, this method has enough sensitivity to detect and quantify the antibiotics bellow the established MRLs in milk samples without tedious treatment sample steps and in concentration levels comparable to those reached with more complex techniques.

Furthermore, the use of SWCNTs as PSPs improves significantly the resolution and selectivity of all these compounds by using this electrophoretic mode. By this way, this work presents an alternative to use of higher organic solvents quantities commonly added in MEKC as buffer modifiers.

2 Materials and methods

2.1 Chemicals and samples

All reagents were of analytical reagent grade and ultra pure water (>18 MΩ cm^{-1}) was used.

FLOR, CLOR, CIPRO, and ENRO were purchased from Sigma–Aldrich (Buenos Aires, Argentina). Individual standard solutions (100 mg L^{-1}) were prepared in ultra pure water and kept in the dark at 4°C. These solutions were stable for at least 2 months. The standard working solutions were daily prepared by appropriate dilutions of stock solutions.

SWCNTs with average external diameters of 10–30 nm and purity >95% were provided by Bayer (Buenos Aires, Argentina). A SDS solution with 2-butanol (Anedra, Buenos Aires, Argentina) was used to prepare the dispersion of SWCNTs.

The electrophoretic buffer was daily prepared with sodium borate (Baker, Chemical Center, Buenos Aires, Argentina), hydrochloric acid (Merck, Buenos Aires, Argentina), SDS (Cicarelli, Buenos Aires, Argentina), and ethanol (Baker).

Three different types of bovine milk samples were used. Two of them were purchased from a supermarket and the other was collected from a local farm (raw milk).

2.2 Instrumentation

Beckman Coulter (Palo Alto, CA, USA) capillary electrophoresis instrument MDQ equipped with a diode array detector was used. The capillaries were also from Beckman System. Control and data processing was carried out with 32 Karat software.

An ultrasonic bath Cole Parmer (Chicago, USA) (50W – 60Hz) was used to disperse the SWCNTs.

To treat the milk samples, a centrifuge Rolco (Buenos Aires, Argentina) (4000 rpm) was used.

2.3 SPE procedure

The SPE procedure was carried out with a homemade column containing 100 mg of C18, placed in a continuous system. At the end of the tube, a cellulose frit was used to hold the adsorbent material in the column. The column was activated with methanol and water and then dried with air before introducing the samples. Then 300 μL of methanol was selected as eluent.

2.4 CE analysis

The separation was carried out in a fused-silica capillary (52.5 cm effective length, 75 μm id and 375 μm od) with a positive power supply of 20 kV at 25°C. A mixture of 25 mM sodium borate, 50 mM SDS, 6% v/v ethanol, 2 mg L^{-1} SC-SWCNTs (adjusted at pH 9.3 with 0.4 M HCl) was used as running buffer solution. The capillary was daily conditioned by flushing 0.1 M NaOH (5 min), ultrapure water (3 min), and buffer solution (5 min). The hydrodynamic injection mode was used applying 0.5 psi during 10 s. The absorbance values for FLOR were measured at 200 nm and for CLOR, CIPRO and ENRO at 282 nm.

2.5 Reversed electrode polarity stacking mode

The capillary was first filled with the electrophoretic buffer containing 25 mM sodium borate, 100 mM SDS, 6% v/v ethanol, and 2 mg L^{-1} SC-SWCNTs (adjusted at pH 9.3). Then, the sample injections were performed in hydrodynamic mode for 60 s at 1.0 psi and the online preconcentration was carried out applying reverse polarity during 60 s at 20 kV.

2.6 Sample preparation

Bovine milk samples were spiked with each studied antibiotics and then homogenized on a vortex mixer for 30 s.

Taking into account the procedures reported in literature [24], milk samples were treated with 3 M HCl in order to promote protein precipitation before the extraction procedure. The raw milk samples need a filtration step before adding HCl. For this purpose a common cellulose filter was employed. After the proteins precipitation, the mixture was centrifuged at 4000 rpm for 20 min. The supernatant was collected and filtered through a 0.45 μm membrane filter. Before the sample was loaded into the C18 column, the pH must be adjusted. So, a pH range between 2 and 7 was evaluated, and the most efficient retention of the analyte was achieved at pH 4.

3 Results and discussions

3.1 Optimization of CE analysis

In order to find out the best conditions for the correct separation of four studied analytes, different buffer solutions were tested.

As phosphate and citrate buffers can catalyze the hydrolysis of CLOR the use of borate buffer is recommended [25]. On the other hand, FLOR does not have functional groups, which are ionized between pH 2 and pH 12, so this antibiotic is uncharged within this pH range. Consequently, CZE could not be used to carry out the separation and determination of FLOR. For this reason, MEKC was applied. A buffer solution containing sodium borate and SDS was evaluated at different concentration levels and pH.

Different concentrations of sodium borate solutions (20–50 mM) were tested to optimize the separation. The shorter migration times and the best shape of the peaks were obtained with 25 mM sodium borate in the buffer solution.

The influence of SDS on the separation was studied analyzing a concentration range (25–75 mM) of this buffer component. Evaluating the increment in the peak area and the shape of the peak for each analyte, a 50 mM SDS was used in the buffer.

A pH range between 8 and 10 was tested. The best resolution and peak areas were obtained at pH 9.3.

With these run buffer conditions, the resolution for FLOR and CLOR was not enough and the ENRO signal was not satisfactory. For this reason, the addition of CNTs into the buffer solution was evaluated.

Multiwalled carbon nanotubes (MWCNTs) and SWCNTs were tested in this case. The dispersion of these nanoparticles is a critical point when they were used as a PSP. The presence of nanotube aggregates can result in an unstable baseline, irreproducible migration times, and clogging of the capillary. The dispersion of nanotubes was carried out according to the literature [26]. In this step, CNTs were dispersed in a 17.5 mM SDS solution containing 10% v/v of 2-butanol by using an ultrasonic bath (20 min, 50 W–60 Hz). In this way, a 10 mg L^{-1} SC-CNTs solution was obtained ant it was stable for a week. Different volumes of this solution were added to the electrophoretic buffer in order to improve the electrochromatographic separation and the stability of the baseline. When 2.0 mg L^{-1} of SC-MWCNTs were added in the buffer, there was no improvement on the resolution of the peaks and the baseline was unstable. The use of 2.0 mg L^{-1} of SC-SWCNTs as PSP improved the sensitivity and the resolution of the electrophoretic separation. It suggests that the interaction between the analytes and this carbon nanostructure-based PSP introduce changes in the electrophoretic mobility, which produces an improvement in the separation of the analytes. Therefore, three concentrations of SWCNTs in the buffer were tested. As can be seen in Fig. 1,

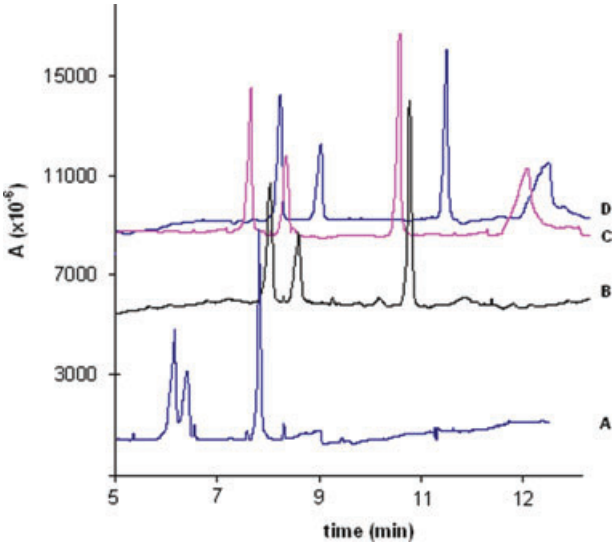

Figure 1. Comparison between electropherograms of standard solution antibiotics (20 mg L^{-1} of each drug). (A) Without SC-SWCNTs in the buffer, (B) with SC-SWCNTs (1 mg L^{-1}), (C) with SC-SWCNTs (2 mg L^{-1}), (D) with SC-SWCNTs (2.5 mg L^{-1}). Peaks: (1) FLOR, (2) CLOR, (3) CIPRO, (4) ENRO. The experimental conditions are mentioned in Section 3.1.

a concentration of 2.0 mg L^{-1} SC-SWCNTs was the optimum. Besides, variations on current intensity were negligible with and without carbon nanostructures in the buffer solution. In addition, the quantity of carbon nanotubes added does not alter the electrophoretic system (current and baseline stability). To improve the peak shapes, different percentages of ethanol (3–10% v/v) were added to the buffer in order to modify the interaction between the analytes and the PSP. In this way, well-defined peaks were observed when 6% v/v of ethanol was added to the buffer.

In order to obtain the best resolution and the shorter analysis time, the voltage applied as well as the cartridge temperature were studied. The temperature was changed between 15 and 30°C, while the applied voltage was varied between 15 and 25 kV. The values that provided an adequate separation in terms of resolution and analysis time were 25°C and 20 kV.

3.2 Stacking procedure

The conductivity of the sample solution should be less than the one of the buffer solution when this kind of online preconcentration technique is used. With this purpose, the analytes were dissolved in both water and 1 mM sodium borate solution. The used run electrophoretic buffer was the mentioned in Section 3.1. The higher sensitivity was achieved by dissolving the analytes in 1 mM sodium borate solution. Moreover, it was proved experimentally that the sample plug was not affected by the Joule heating.

In order to obtain the best preconcentration results, the sample injection time, the applied pressure, and the composition of the buffer were tested. Different injection times and a range of pressure were selected taking into account the effect of these variables on the sensitivity and resolution of the peaks. The optimum results were obtained when the samples were injected during 60 s at 1.0 psi.

Initially, the separation buffer was used to carry out the stacking step of the analytes when the reverse polarity was applied, but the resolution of the peaks was reduced. So, an increment in the concentration of surfactant (50, 60, 75, and 100 mM) into the initial buffer was evaluated to improve this analytical parameter and to increase the sensitivity. The best results were obtained with 100 mM of SDS. In fact, this concentration of SDS together with 2 mg L^{-1} SC-SWCNTs allows a more effective preconcentration of analytes and the obtention of well-resolved peaks. This is possible because the interaction between analytes–SC-SWCNTs and SDS micelles is facilitated.

The reversal time for eliminating the matrix of the sample was 1 min.

This online preconcentration step allowed a stacked percentage of 103, calculated as Quirino et al. recommend [20]. The sensitivity enhancement factor was calculated taking into account the peak heights in stacking and regular injections. Also, this value indicated that all the analytes that are brought to the concentration boundary are effectively stacked.

3.3 Off-line preconcentration step

As the concentration of these antibiotics in milk samples is low, a continuous flow system was developed to carry out an off-line SPE pretreatment. By this way, it was possible to preconcentrate the analytes and to clean up the samples. A column with 100 mg of C18 was selected. The precondition of the column was done with 5 mL of methanol, 5 mL of water, and air for 3 min at 2.60 mL min^{-1}. Then, 5 mL of sample was passed through the column. Next, the column was washed with 5 mL of water and then an air stream was pumped through the column to remove the water. Acetonitrile, methanol, and different aqueous mixtures of them were studied as eluent solution. The best results were obtained by using methanol, taking into account the polarity of the analytes and the sorbent material. So, different volumes of methanol were tested (200, 300, and 500 μL). Finally, 300 μL of methanol was the optimum volume of the eluent. Before the CE analysis, the eluate was evaporated to dryness and the residue was reconstituted in 200 μL of 1 mM sodium borate solution.

3.4 Analytical parameters and analysis of real samples

Under the optimal conditions mentioned above, high efficient separation and enrichment have been achieved for the quantitative analysis of FLOR, CLOR, CIPRO, and ENRO.

The analytical figures of merit of the method are shown in Table 1. Each point of the calibration graph corresponds to the average of three individual measurements. The LODs calculated as three times $S_{y/x}$/slope [27] of the calibration

Table 1. Analytical parameters of the proposed method

Antibiotic	Slope ± SD	Intercept ± SD	R^2	Linear range (μg L^{-1})	LOD [a] (μg L^{-1})	RSD [b] (%)
CIPRO	20.9 ± 0.33	762.6 ± 95.9	0.999	20 – 500	13.8	1.1
ENRO	35.2 ± 1.00	767.2 ± 161.7	0.998	20 – 300	13.9	4.1
FLOR	161.2 ± 0.94	5136.5 ± 434.1	0.999	10 – 500	8.1	2.4
CLOR	263.3 ± 10.1	−2012.8 ± 599.9	0.997	10 – 100	6.8	6.6

a) LOD calculated as 3 × $S_{y/x}$/slope.
b) RSD of peak area for five measurements (corresponding to standard of 50 μg L^{-1}).

Table 2. Comparison between the proposed method and different methodologies to determine APhs and FQs in milk samples

Analytes	Milk sample pretreatment	Analysis	LODs	Ref.
Chloramphenicol, gentamicin, streptomycin, tylosin, and tetracycline	Protein denaturation with diethyl ether. The extracted was filtered and stored at −20°C before analysis	Suspension array technology (Bio-Plex ™), using a secondary antibody biotin	25 μg L^{-1} (chloramphenicol)	[28]
Chloramphenicol, ampicillin, tetracycline, sulfamethoxazole, and ciprofloxacin	Protein precipitation with trichloroacetic acid solution Liquid–liquid extraction (LLE) and evaporated to dryness SPE step	CE-UV	30 μg L^{-1} (chloramphenicol) and 12 μg L^{-1} (ciprofloxacin)	[13]
Enrofloxacin, ofloxacin, pefloxacin, and difloxacin	Deproteination with 10% $HClO_4$ solution. The protein was removed after centrifugation for 3 min	HPLC-ECL	20 μg L^{-1} (enrofloxacin)	[12]
Sulfametoxazole, oxytetracyclin, sulfaquinoxaline, sulfamethazin, tetracycline, minocycline, chloramphenicol, and chlortetracycline	Protein precipitation with 30% v/v TCA–methanol LLE with buffer, pH 4. SPE	HPLC-DAD	20 μg L^{-1} (chloramphenicol)	[29]
Chloramphenicol, tetracyclin, amoxicillin, ampicillin, cloxacillin, and penicillin	Deproteination with 20% TCA SPE with three C18 cartridges	CE-UV HPLC-UV	720 μg L^{-1} (chloramphenicol) 450 μg L^{-1} (chloramphenicol)	[14]
Ciprofloxacin, enrofloxacin, sarafloxacin, oxolonic acid, and flumequine	Deproteination with 5% (w/v) metaphosphoric acid Online column clean up	LC-FD	17 μg L^{-1} (ciprofloxacin) and 18 μg L^{-1} (enrofloxacin)	[11]
Ciprofloxacin, enrofloxacin, florfenicol, chloramphenicol	Protein precipitation with 3 M HCl solution SPE with C18 column	CE-UV	13.8 μg L^{-1} (ciprofloxacin) 13.9 μg L^{-1} (enrofloxacin) 8.1 μg L^{-1} (florfenicol) 6.8 μg L^{-1} (chloramphenicol)	This method

graph were below the MRLs established by EU for these antibiotics and they were in agreement with those obtained in other previous works for milk samples [13, 14] applying CE-UV analysis. In addition, the obtained results can be compared with those shown in the literature when more complex methodologies were used, as can be seen in Table 2. The RSD values in Table 1 are lower than those obtained without SC-SWCNTs as additive in the electrophoretic buffer solution. It demonstrates that the use of SC-SWCNTs as PSP improves the repeatability of the method.

Different bovine milk samples were analyzed to check the applicability of the proposed method to determine these antibiotics. For this purpose, raw milk, full cream, and skimmed milk were analyzed. Preliminary analysis of samples showed that they were free of the selected antibiotics. So, a recovery study was carried out at two concentration levels for FQs and three for APHs. Table 3 shows the obtained recovery values when the proposed method was applied to real samples. As can be seen, these recoveries varied between 80% and 109%, which were acceptable for these milk samples and the analytes

Table 3. Analysis of spiked milk samples using the proposed method

Added concentration ($\mu g\ L^{-1}$)	Sample					
	Raw milk		Full-cream milk		Skimmed milk	
	Recovery (%)[a)]	RSD (%)	Recovery (%)[a)]	RSD (%)	Recovery (%)[a)]	RSD (%)
25						
FLOR	109	2.8	104	3.2	88	2.5
CLOR	90	4.0	91	4.8	80	0.7
50						
CIPRO	94	0.7	91	2.4	84	2.8
ENRO	103	0.1	**100**	2.6	102	1.9
FLOR	91	2.8	97	1.1	98	1.6
CLOR	98	1.2	94	1.9	105	1.0
100						
CIPRO	101	1.8	98	1.0	96	1.8
ENRO	107	2.5	106	1.2	104	0.9
FLOR	96	2.4	97	1.2	95	1.1
CLOR	96	2.0	99	0.8	95	1.2

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

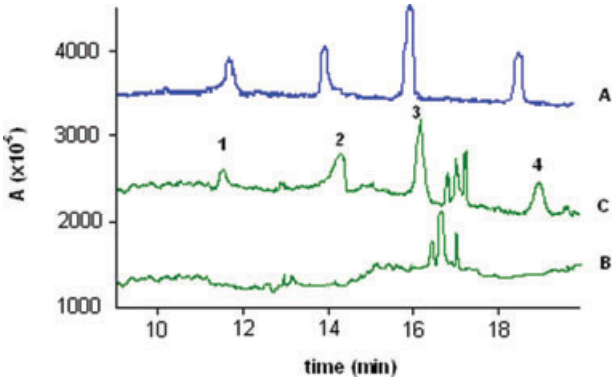

Figure 2. (A) Electropherogram of standard solution (50 $\mu g\ L^{-1}$ of each antibiotic), (B) Electropherogram of full-cream milk sample, (C) Full-cream milk sample spiked with 50 $\mu g\ L^{-1}$ of each antibiotic. The experimental conditions are: MiNDEKC buffer: 20 mmol L^{-1} sodium borate–50 mmol L^{-1} SDS–6% (v/v) ethanol, pH = 9.3, 2.0 mg L^{-1} SC-SWCNTs, 20 kV, 25°C. The optimal experimental conditions for the stacking step are mentioned in Section 3.2. Peaks: (1) FLOR, (2) CLOR, (3) CIPRO, (4) ENRO.

investigated. Also, they are comparable with those obtained in previous works by using other preconcentration techniques and CE and even by HPLC (Table 2). Moreover, these results demonstrated the reliability of the method. Figure 2 shows a typical electropherogram obtained for the analysis of a blank sample and a spiked milk sample containing 50 $\mu g\ L^{-1}$ of each antibiotic. It can be seen that the migration time of the matrix constituents slightly changes when the analytes are added. Probably the presence of analytes modifies the interaction between the PSP and the other milk constituents varying the migration time. In spite of it the milk constituents do not interfere with the migration times of the analytes.

4 Concluding remarks

The proposed method allows the simultaneous determination of two different antibiotic families, fluoroquinolones and amphenicols, in milk samples. The quantification of the four analytes was carried out using MiNDEKC with REPSM as online preconcentration strategy and a previous SPE preconcentration step. In this way, the obtained LODs were much lower than the MRLs established by the EU.

The use of SC-SWCNTs in the buffer allowed a great improvement of resolution and sensitivity of the electrochromatographic separation and the migration time was not significantly increased. In addition, the electrophoretic buffer is simple and only a small amount of CNTs is introduced in the electrophoretic system. Moreover, it was observed the analytical potential of surfactant-coated single-walled CNTs as PSP in MiNDEKC, which represents an alternative to conventional use of organic modified-MEKC commonly pointed in the literature.

On the other hand, the pretreatment of the milk samples is very simple, only a separation of the proteins and a filtration step were needed.

The method was applied to real samples and the obtained results were satisfactory, taking into account the recoveries percentages and the RSD% values. Therefore, the proposed method represents an alternative to analyze different milk samples in which all these four different compounds would be simultaneously present.

Financial support from the Universidad Nacional del Sur is gratefully acknowledged. V.S. is grateful to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

The authors have declared no conflict of interest.

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