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Development of a novel strategy for preconcentration of antibiotic residues in milk and their quantitation by capillary electrophoresis

Luciana Vera-Candioti[a], Alejandro C. Olivieri[b], Héctor C. Goicoechea[a,*]

[a] Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ), Cátedra de Química Analítica I, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Santa Fe, S3000ZAA, Argentina
[b] Departamento de Química Analítica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario and Instituto de Química Rosario (IQUIR-CONICET), Suipacha 531, Rosario, S2002LRK, Argentina

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ABSTRACT

A novel analytical method based on capillary zone electrophoresis coupled with diode array detection is developed and validated for the identification and simultaneous quantitation of four antibiotics in bovine raw milk. The studied antibiotics belong to different groups: β-lactams, tetracyclines, quinolones, amphenicols and sulfonamides. An experimental design including both a factorial and a central composite design allowed a reduction in the number of optimization experiments. The multiple response criterion was successfully used to optimize the separation between chloramphenicol, ciprofloxacin, ampicillin, tetracycline and sulfamethoxazol, allowing the reduction of the analysis time with excellent peak resolutions and low capillary current. Different strategies for preconcentration and extraction of the studied antibiotics were applied, in order to remove potential interferences from the sample and to increase the sensitivity. Milk samples were prepared by a clean-up/extraction procedure based on protein precipitation with trichloroacetic acid followed by liquid–liquid extraction with dichloromethane combined with solid-phase extraction, and injection into the electrophoretic system hydrodynamically. The limits of detection and quantification (below 30 and 100 $\mu g\,L^{-1}$, respectively) were in all cases lower than the maximum residue limits tolerated for these compounds in milk. Accuracy was evaluated by computing recoveries for the target antibiotics which were between 93.08% and 102.89%.

1. Introduction

The term antibiotic (ATB) is normally reserved for a wide range of chemical substances, synthetic, semi-synthetic or produced by microorganisms, which kill or inhibit the growth of other microorganisms [1]. In veterinary medicine, antibiotics are widely used in food-producing animals for the treatment and prevention of diseases and as feed additives to increase the animal mass [2–4]. However, the inappropriate use of ATB may result in drug residues being present in milk, especially if the label directions are not followed [2,5].

Chloramphenicol (CLOR) is a broad-spectrum antibiotic, which has been found to produce aplastic anemia in a small percentage of humans exposed to this drug [5]. The use of CLOR in food-producing animals is prohibited, and its use in humans is reserved to treat serious infections, when no other alternative is available [6,7]. Ciprofloxacin (CPF) is a synthetic antimicrobial agent with bactericidal action, developed exclusively for use in veterinary practice for the treatment of respiratory and gastrointestinal infections [8,9]. Ampicillin (AMP) is extensively used in veterinary medicine. The presence of β-lactam residues in milk may be responsible for allergic reactions in certain individuals [5,10]. Tetracycline (TC) is widely used for the treatment of bovine mastitis and is added at sub-therapeutic levels to cattle feed for prophylaxis and to promote growth of livestock [5,11,12]. Finally, sulfamethoxazole (SMX) is an antibacterial and anti-infective drug, commonly used for the treatment of diseases in medicine and veterinary medicine, such as gastrointestinal and respiratory affections [13]. The structure, pK_a values and maximum residues limits (MRLs) of the ATBs analyzed in this work are included in Table 1.

Because a large amount of milk is consumed all over the world, mainly due to the fact that it is a good source of calcium and proteins, appropriate quality control programs are especially important to maintain its maximum health benefits [14]. To limit human exposure to ATBs, several control authorities such as the European Community (EC), the US Food and Drug Administration (FDA) and the SENASA in Argentina, have set MRLs of these drugs in both milk and dairy products [3,15,16]. MRL is based on the amount of a substance that can be ingested on a daily basis over a lifetime without appreciable health risks. It is therefore important to

* Corresponding author. Tel.: +54 342 457 5205; fax: +54 342 457 5205.
E-mail address: hgoico@fbcb.unl.edu.ar (H.C. Goicoechea).

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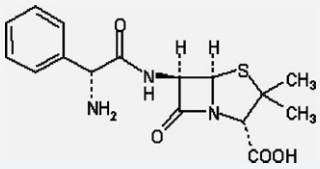
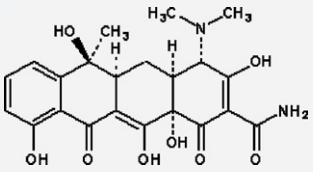
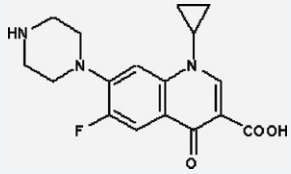
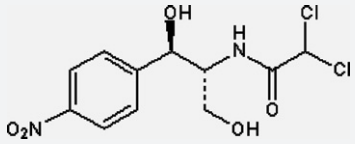
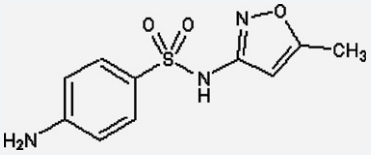
Table 1
Structural formulae, pK_a values and maximum residue limits (MRLs) of the five analyzed antibiotics.

Analyte	Groups	MRL ($\mu g\ L^{-1}$)	pK_a	Structure
AMP	β-Lactams	4 [3,15]	2.8 7.2	
TC	Tetracycline	100 [3,15,16]	3.32 7.78 9.58	
CLOR	Amphenicols	not permitted [3,15]	5.5	
CPF	Quinolones	100–300 [3,15]	3.01 6.14 8.70 10.58	
SMX	Sulfonamides	100 [3,15,16]	1.85 5.60	

develop analytical methodologies for determining ATBs in milk at levels below the MRL, in order to ensure high quality of milk for human consumption.

Immunological or microbial inhibition screening tests are usually employed to determine if ATB residues are present in milk [17]. Screening test cannot identify which ATB is present in a milk sample, while the presence of high somatic cell counts may result in false positives. Several analytical methods have already been described for the simultaneous determination of antibiotic residues in milk. Liquid chromatography (LC) [4,7,10,11,14,18–21], and gas chromatography (GC) [22,23] coupled with different detection systems are the techniques most commonly used for this purpose.

On the other hand, bioanalytical methods based on the use of immunosensors are being increasingly used in the last years [24–26]. Problems encountered by these methods include lack of the required selectivity for complex mixtures, or only semi-quantitative analytical features.

Capillary electrophoresis (CE) is a separative analytical technique which is widely accepted due to its ability to simultaneously determine different analytes with both high efficiency and resolution, low consumption of samples and electrolytes, and short analysis times. The physicochemical properties of CLOR, CPF, AMP, TC and SMX, their ionizable nature, multiple ionization sites and different water solubilities, make these compounds highly suitable for electrophoretic analysis.

In recent years, several methods have been published using capillary zone electrophoresis (CZE) for the determination of quinolones [8,9] and tetracyclines [12]. Other authors used micellar electrokinetic capillary chromatography (MEKC) for the separation of tetracyclines [27,28], different β-lactams [1], penicillins [29] and sulfonamides [30,31]. The use of non-aqueous solvents has been shown to offer advantages over aqueous systems to separate tetracyclines [32]. Castro-Puyana et al. summarized the latest advances in the development of analytical methodologies applied to the analysis of ATB by CE and MEKC [33]. However, most of them focus on single ATB groups.

Nevertheless, a small number of applications has been presented describing the simultaneous determination of different groups of antibiotics [5,34,35]. It is noteworthy that, to the best of our knowledge, only a single method has been focused on the determination of ATB residues in milk [5]. It should be remarked that quality control using the same method for the determination of several kinds of compounds without the need of changing solvents, analytical columns and procedures, is highly advantageous.

The detection of several ATB residues in milk is a difficult task, because the analyte is immersed in a large volume of an aqueous matrix which consists of highly concentrated proteins, lipoproteins, lipids, vitamins, salts and numerous compounds that may be chemically similar to the analyte of interest. Moreover, the analytes are often present at low concentration in these samples. Due to this complex biological matrix, the recovery is dramatically affected. In this case, it is essential to have an effective clean-up step to remove interferences, proteins and particulate matter. The commonly utilized techniques for extraction and clean-up of ATBs from milk involve: ultrafiltration [4,11] and protein precipitation using trichloroacetic acid [4,5,14,32], organic solvents such as methanol

[18] or acetonitrile [10,19,21], or treatment with sodium tungstate in sulfuric acid [36]. These procedures can be followed by an extraction step carried out by liquid–liquid extraction (LLE) [7,9,19] or solid-phase extraction (SPE) [5,8–11,14,21,27] that can be regarded as a chromatographic approach to increase the detection sensitivity in CE.

In the present paper, we propose a selective and sensitive method based on the use of CE coupled to UV detection for the identification and simultaneous quantitation of CLOR, CPF, AMP, TC and SMX in bovine raw milk. The multiple response criterion was successfully used to optimize the separation of the five ATBs, allowing the reduction of the analysis time, with excellent peak resolutions and low capillary current. Furthermore, a simple procedure for preconcentration and extraction of these ATBs was developed, achieving reduced limits of detection for most of them.

2. Experimental

2.1. Instrumentation

All experiments were carried out on a capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), equipped with a diode array detector. Separation was carried out in an uncoated fused-silica capillary of 60 cm total length (effective length 51.5 cm), with an inner diameter of 75 μm (MicroSolv Technology Corporation, Eatontown, NJ, USA) in a normal mode, applying a voltage of 30 kV, with a typical current of about 93 μA. The cartridge was maintained at 16.0 °C. The wavelength used for recording the electropherograms was 200 nm. Injection was made from the positive electrode of the capillary hydrodynamically applying 50 mbar of pressure for 20 s.

The pHs of the background electrolyte (BGE) solutions were adjusted by a pHmeter (HANNA Instrument). All solutions were degassed in an ultrasonic bath Cole Palmer 8891 (Cole Palmer, Illinois, USA) and filtered through 0.45 μm nylon membrane (Sartorius, Germany) before use. Solid-phase extraction cartridges (Oasis HLB 1 cm^3/30 mg and Sep-Pack C-18, 1 cm^3/100 mg) were purchased from Waters Corporation (Milford, Massachusetts).

2.2. Software

In all cases, electropherograms were recorded in random order with respect to analyte concentrations using the software provided with the HP ChemStation (Agilent Technologies). Experimental design, data analysis and desirability function calculations were performed by using the software Stat-Ease Design-Expert trial Version 7.0.3 (Stat-Ease Inc., Minneapolis).

2.3. Reagents

All the reagents were of analytical grade. Ultra-pure water was obtained from an ultra-pure water purification system from Millipore (Bedford, MA, USA) and was used in all the CE experiments. Sodium phosphate, sodium borate, sodium hydroxide, trichloroacetic acid (TCA), ethylic ether, dichloromethane, acetonitrile (ACN), methanol (MeOH) and ethanol (EtOH) LC grade were obtained from Merck (Darmstadt, Germany). Chloramphenicol, ampicillin and tetracycline were obtained from Sigma–Aldrich (Steinheim, Germany). Ciprofloxacin and sulfamethoxazol were obtained from Merck (Darmstadt, Germany).

Stock standard solutions of the individual drugs were prepared at a concentration of 2 g L^{-1} by dissolving the accurately weighed amount in ultra-pure water, except TC that was dissolved in MeOH. These solutions were preserved at 4 °C in the darkness during the experiments. Working standard solutions of all analytes were prepared everyday by diluting the stock standard solutions in a mixture of ACN–ultra-pure water (1:1, v/v). The background electrolyte and washing solutions were prepared at the beginning of the day. All solutions were filtered through a 0.45 μm nylon membrane (Sartorius-Germany) and degassed before use.

2.4. Electrophoretic conditions

A careful activation and conditioning of the capillary was essential in order to obtain reproducible results and to remove substances adsorbed to the capillary wall. Thus, at the beginning of every working day the capillary was rinsed with: 0.1 mol L^{-1} sodium hydroxide (10 min), ultra-pure water (10 min) and 0.020 mol L^{-1} sodium borate solution pH 9.51 (10 min) which is the BGE. Between runs the capillary was successively flushed with 0.1 mol L^{-1} sodium hydroxide, ultra-pure water and BGE for 3 min each. At the end of the day the capillary was washed with 0.1 mol L^{-1} sodium hydroxide (5 min), ultra-pure water (5 min) and then air-dried for 3 min.

2.5. Extraction procedure

Before the extraction procedure, milk samples were treated as follows: 100 mL aliquots of commercially available bovine milk were spiked with different aliquots of stock standard solution of all the ATBs. Samples were shaken on a vortex mixer for 30 s and then allowed to stand at 4 °C in the dark, for at least 20 min, to enable sufficient equilibrium with the milk matrix. Then, samples were centrifuged at 4000 rpm during 20 min and the solid fat was separated from the aqueous portion with a spatula.

After the above procedure, 10 mL of saturated trichloroacetic acid solution was added in order to promote protein precipitation. The mixture was immediately shaken vigorously for 1 min and then centrifuged at 4000 rpm for 15 min. The supernatant was transferred into a 250-mL separatory funnel and the analytes were extracted with three successive aliquots of 20.00 mL of dichloromethane. The organic extracts were combined and transferred to a clean tube and evaporated to dryness at 45 °C in a hot plate. Finally, the residue was reconstituted in 2 mL of a mixture of EtOH–ultra-pure water (1:1, v/v), adjusted to pH 5.00 with HCl 0.1 mol L^{-1} and filtered through a syringe filter of 0.45 μm pore size.

Finally, the extract was passed through the HLB cartridge, which was previously conditioned with 2 mL of ultra-pure water, 2 mL of MeOH and 2 mL of a mixture of EtOH–ultra-pure water (1:1, v/v) to ensure a reproducible retention. The cartridge was then washed with 2 mL of ultra-pure water and the analytes were eluted with two portions of 150 μL of ACN–ultra-pure water (1:1, v/v). The eluate was filtered through a PTFE-syringe filter of 0.2 μm pore size and then injected into the CE system. Fig. 1 shows the sequence of the sample clean-up and extraction of antibiotics. The extraction procedure was developed based on previous literature works [5,7–9,14,36].

2.6. Statistical methods for optimization

In order to evaluate the main factors affecting the efficiency of separation, i.e. concentration and pH of the BGE, separation voltage and temperature, a Packett–Burman design with 12 experiments was performed. The factors showing significant effects were then considered within a central composite design consisting of 30 experiments, in order to find optimum factor levels for all the response signals by optimizing an objective function. Finally, the multiple response criterion using the desirability function was successfully used to optimize the resolution between the five ATBs, analysis time and current [37].

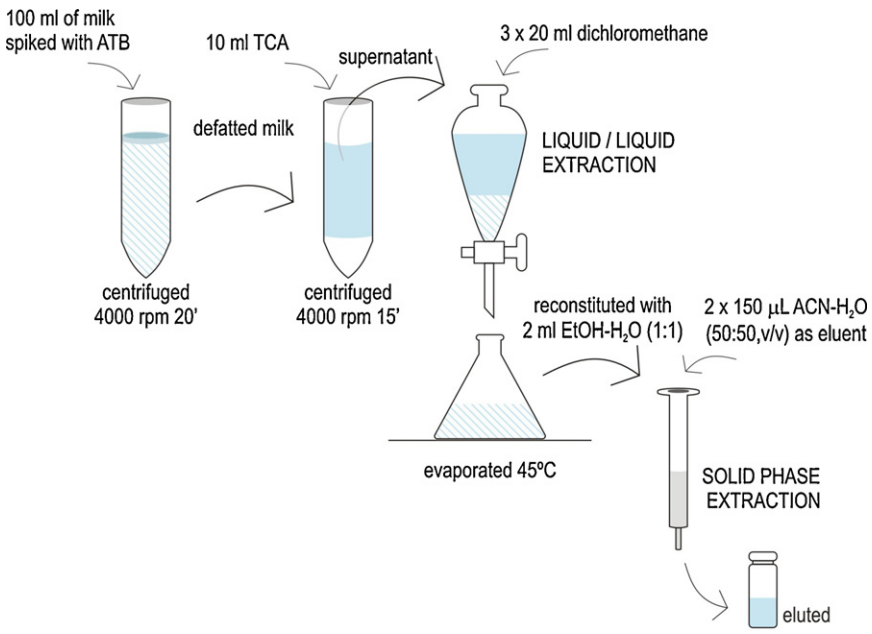


Fig. 1. The sequence of sample clean-up and extraction of antibiotics from milk samples.

3. Results and discussion

3.1. CE optimization

3.1.1. Screening phase

For finding out the best conditions for the correct separation of the five studied analytes, two electrolytes were evaluated (borate and phosphate) at different concentration levels and pHs. Moreover, MEKC was tested by adding different surfactants to the BGE. The evaluation consisted in analyzing the stock standard solution in all conditions. In each case, the number, shape and resolution of the electrophoretic peaks were evaluated. Consequently, a borate buffer 0.020 mol L^{-1} (pH 9.20) was selected, as it provided a good separation and higher sensitivity for all four analytes.

Stacking was performed by dissolving the sample in a low conductivity matrix (such as ACN:water) and injecting sample solutions for a much longer time compared to the usual hydrodynamic injection (50 mbar, 3 s). Sample solutions were introduced at 50 mbar during different intervals of time. Focusing occurs at the interface between the low conductivity matrix and the BGE, due to the abrupt change in the electrophoretic velocity. A limitation in normal stacking mode (NSM) is the short optimum sample plug length that can be injected into the capillary, without loss of separation efficiency or resolution. Hence, the optimum plug length was assessed by injecting at different intervals and inspecting the peak shapes. A length corresponding to 20 s injection was chosen, since it generated a better peak shape compared to longer injection times. Concentration factors of around 10 are usually obtained with NSM, improving the limit of detection by an order of magnitude.

In order to achieve a good separation between CLOR, CPF, AMP, TC and SMX in a short analysis time and without generation of Joule's effect, electrophoretic conditions were evaluated and optimized. An experimental Plackett–Burman design was built for the evaluation of the main factors affecting the peak resolution between ATBs, analysis time and capillary current. The resolution can be defined according to Eq. (1):

$$R = 2\frac{(tm_2 - tm_1)}{(w_1 + w_2)} \quad (1)$$

where tm_1 and tm_2 are the migration times, and w_1 and w_2 are the electrophoretic peak widths. When the resolution is higher than 1.5, two species are considered to be resolved at the baseline [38].

The analyzed factors were: concentration and pH of the BGE, separation voltage and temperature. These factors were evaluated at two levels each. The evaluation consisted in analyzing a stock standard solution containing the five ATBs (2.50 mg L^{-1}) in all the conditions. In each case, the peak resolution between ATBs, the analysis time and capillary current were evaluated.

An ANOVA test was applied to the experimental data. As a conclusion of this analysis, all factors evaluated were shown to be significant ($p < 0.05$) (Table 2) and were considered in the further optimization analysis.

3.1.2. Response surface method

Once the conditions that ensure the analyte separation were established, an optimization procedure was applied in order to find out the exact values of the most important factors for a correct separation and a rapid analysis.

A central composite design was used, consisting of 30 experiments which corresponded to combinations of the selected independent variables in the following ranges: BGE concentration 18–28 mmol L^{-1}, pH 8.00–10.00, temperature 16–32 °C and separation voltage 15–30 kV. These ranges were selected based on prior knowledge about the system under study and were limited by the physical constraints of the instrument and buffer systems. On the other hand, the injection mode was hydrodynamic, applying 50 mbar during 20 s, and the detection wavelength was set at 200 nm. All experiments were performed in random order to minimize the effects of uncontrolled factors that may introduce a bias on the measurements.

The evaluation consisted in analyzing a stock standard solution containing the five ATBs (2.00 mg L^{-1}) in all the conditions. In each case, the peak resolution between ATBs, the analysis time and capillary current were evaluated for all the 30 experiments fitting polynomial models. The model coefficients were computed by backward multiple regression and validated by the analysis of variance (ANOVA) [39]. Resolutions 1 and 2 were adjusted using cubic models, while resolution 3, analysis time and current were adjusted by quadratic models. Finally, resolution 4 required a linear model

Table 2
Probability values obtained when applying ANOVA to all the responses studied with the Plackett–Burman design built for factor selection.

Response	Probability value[a,b]				
	Model[c]	BGE concentration (mol L^{-1})	pH	Separation voltage (kV)	Temperature (°C)
Resolution 1 (CPF/CLOR)	0.0189	0.0195 (+)	0.0222 (+)	0.1000	0.0213 (+)
Resolution 2 (CLOR/AMP)	0.0376	0.1000	0.0352 (+)	0.0225 (−)	0.0392 (−)
Resolution 3 (AMP/TC)	0.0039	0.0090 (+)	0.1000	0.0118 (−)	0.0139 (+)
Resolution 4 (TC/SMX)	0.0050	0.2331	0.1329	0.0405 (−)	0.0844
Analysis time (min)	0.0004	0.1279	0.1415	0.0001 (−)	0.0462 (−)
Current (μA)	0.0042	0.0063 (+)	0.0445 (−)	0.0029 (+)	0.0049 (+)

[a] Considered significant when $p < 0.05$.
[b] Signs between parenthesis correspond to the effects on the variables.
[c] The quoted value is a parameter indicating if the linear model was properly selected.

Table 3
Criteria for the optimization of the individual responses.

Responses	Criteria	Lower limits	Upper limits	Importance
$Resolution_{CLOR/CPF}$	Maximize	0.5	1.30	5
$Resolution_{CPF/AMP}$	Target = 1.50	1.02	3.04	3
$Resolution_{AMP/TC}$	Target = 1.50	1.00	2.38	3
$Resolution_{TC/SMX}$	Target = 1.50	1.00	3.47	3
Current (μA)	In range	54.50	100.00	3
Analysis time (min)	Minimize	5.69	15.00	5

to be fitted. It is apparent that different models were obtained, due to the fact that each response has it own pattern as a function of the studied factors.

3.1.3. Multiresponse optimization

Six responses were simultaneously optimized by using the desirability function [37]. The desirability function includes the researcher's priorities and desires on building the optimization procedure. Its application involves creating a function for each individual response (d_i) and finally obtaining a global function D that should be maximized choosing the best conditions of the designed variables. The latter function varies from 0 (totally undesirable value) to 1 (all responses are in a desirable range simultaneously), and can be defined by Eq. (2):

$$D = (d_1^{r1} \times d_2^{r2} \times, \ldots, \times d_m^{rm})^{1/\sum r_j} = \left(\prod_{j=1}^{m} d_j^{rl}\right)^{1/\sum r_j} \quad (2)$$

where $d_1, \ldots, d_m$ correspond to the individual desirability functions for each response being optimized, m is the number of responses, and r is the relative importance of each response with respect to the remaining ones.

Table 3 shows the criteria which were followed for the optimization of the individual responses, the lower and upper limits and the importance assigned to each response, giving more importance to the smallest resolution ($resolution_{CLOR/CPF}$) and to the analysis time.

Under the above mentioned optimization criteria, the experimental conditions corresponding to a maximum in the desirability function (D = 0.619) are: BGE concentration 19.35 mol L^{-1}, pH 9.50, temperature 16 °C, separation voltage 30 kV. The sample was hydrodynamically injected applying 50 mbar for 20 s and the detection was at 200 nm. The suggested values during the optimization procedure were experimentally corroborated, and the corresponding electropherogram is shown in Fig. 2. Using these CZE conditions and according to the structure and pK_a values of the ATBs, they were negatively charged, but migrated toward the detection window in less than 8 min due to the electroosmotic flow (EOF).

3.2. Method performance

The instrumental method was validated by using univariate methodology, based on peak areas at a fixed wavelength (200 nm for all the five analytes). The analytical figures of merit were calculated by using ACN–ultra-pure water (1:1, v/v) as solvent.

3.2.1. Linearity and figures of merit

In order to verify the method linearity within a concentration range 0.25–4.50 mg L^{-1} of CLOR, CPF, AMP, TC and SMX, three replicates were prepared at six concentration levels by dilution of known amounts of each analyte standard solution in ACN–ultra-pure water (1:1, v/v) and subjected to the analytical procedure.

For computing figures of merit under univariate calibration, peak areas of the ATBs were plotted against nominal concentrations (expressed in mg L^{-1}) fitting the line by the least-squares method. The values of limit of quantification (LOQ) and limit of detection (LOD) were computed according to IUPAC recommendations (Eqs. (3) and (4)) [40].

$$\mathrm{LOD} = \frac{3.3 S_{y/x}}{b} \quad (3)$$

$$\mathrm{LOQ} = \frac{10 S_{y/x}}{b} \quad (4)$$

where $S_{y/x}$ is the residual standard error of the calibration curves and b is the slope.

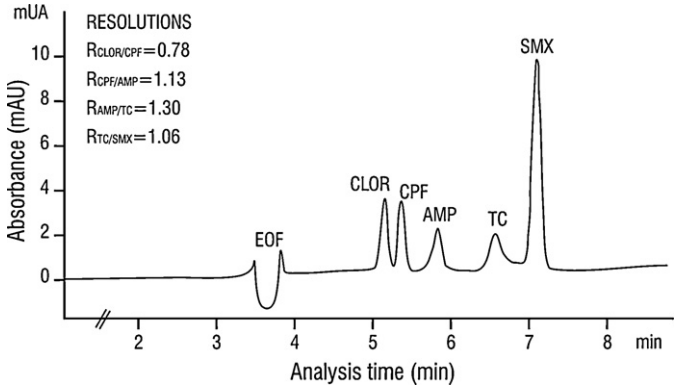


Fig. 2. Electropherogram corresponding to standard solution of five ATBs (2.00 mg L^{-1} in ACN–ultra-pure water) under the optimized experimental conditions: 19.35 mol L^{-1} sodium borate, pH 9.50, temperature 16 °C, separation voltage 30 kV, hydrodynamic injection (applying 50 mbar 20 s) and detection at 200 nm (EOF: electroosmotic flow).

Table 4
Analytical figures of merit for the determination of antibiotics in a mixture of ACN–ultra-pure water by CE–UV.

Figures of merit	CLOR	CPF	AMP	TC	SMX
Linearity (mg L^{-1})	0.28–4.54	0.26–4.16	0.25–4.00	0.28–4.48	0.25–4.00
R^2 adjusted	0.995	0.997	0.991	0.994	0.994
p-Value (lack of fit test)[a]	0.086	0.061	0.073	0.058	0.114
LOD (mg L^{-1})	0.29	0.17	0.33	0.24	0.20
LOQ (mg L^{-1})	0.89	0.51	1.01	0.72	0.61
MRL (mg L^{-1}) [3,15,16]	Not permitted	0.10	0.04	0.10	0.10

[a] The probability value of the lack of fit test should be greater than 0.05.

The results are summarized in Table 4 and the following observation can be made: (a) the coefficients of determination (R^2 adjusted) were higher than 0.99, (b) the ANOVA test of lack of fit allows one to conclude that linearity is fulfilled within the studied range, and (c) the LODs and the LOQs obtained by direct sample injection are higher than the MRLs established for CLOR, CPF, AMP, TC and SMX in milk samples. As will be shown below, the developed method is not sensitive enough to determine concentrations below the permissible MRL of each ATB in milk samples. Thus, it is apparent that the method requires to develop a strategy to increase the concentration of the analytes before injection and hence to improve the sensitivity of the assay.

3.2.2. Preconcentration methods

In order to reach the MRLs established for all ATBs, different strategies were applied to extract and preconcentrate the analytes. They are described in Table 5.

Electrophoretic-based methods, such as NSM and large volume sample stacking (LVSS) were applied to increase the analyte concentration prior to their separation. Although the maximum improvement in sensitivity was obtained with LVSS, because this method was carried out by filling the complete capillary volume, we chose the NSM method for injecting the sample, in order to avoid the elimination of CLOR from the capillary. Moreover, when injecting real samples by LVSS, the high milk conductivity interfered with the elimination of the matrix, making it impossible the application of this methodology.

On account of the complexity of the milk samples, which contain large concentrations of fat and proteins, pretreatment was performed before carrying out the CE separation. LLE and chromatographic-based methods, such as SPE were used to purify and concentrate the analyte.

Two different reversed-phase sorbents were tested, including silica-based C18 and polymeric HLB. Recoveries from C18 cartridges were not satisfactory, mainly for CPF and TC. This fact can be explained considering that unlike silica-based C18 sorbent, Oasis HLB provides excellent recoveries, with no breakthrough and no undesirable secondary retention mechanisms. In addition, Oasis HLB has a larger capacity than C18 and is capable to retain both non-polar and very polar compounds. Additionally, TC's are known to interact not only with surface silanols, but also with metals in silica-based sorbents, troubles that are not present with Oasis HLB copolymer. Finally, low recoveries were obtained on C18 cartridges due to either breakthrough or interaction with silanols groups, while only the Oasis HLB extraction cartridge gave high recoveries for all analytes. Therefore, HLB cartridges were used for subsequent extractions. Using an elution plug consisting of a mixture of ACN and ultra-pure water (1:1, v/v), ATBs were efficiently eluted from the HLB sorbent.

Before conducting the SPE procedures to milk samples, we applied a protein precipitation step with TCA. This precipitating agent was chosen because it removes many interferences and has a low dilution effect.

Table 5
Different strategies applied in the present work to extract and preconcentrate ATBs.

Preconcentration method	Conditions	Conclusions
1- NSM	ATBs standard solution was injected applying 50 mbar for 40 s	• The LODs obtained for all ATB were upper than the MRLs established for these ATBs
2- LVSS	ATBs standard solution was injected applying 50 mbar for 360 s and stacking at −20 kV until 95% original current	• The sensitivity was improved due to the stacking process • The EOF pushed the CLOR out of the capillary
3- LVSS in milk samples	Milk samples was injected applying 50 mbar for 360 s and stacking at −20 kV until 95% original current	• The high conductivity of the matrix interfering with sample stacking and matrix elimination • Numerous substances were concentrated simultaneously with target analytes and this finally leads to bad separations
4- LLE	ATBs standard solution were separately extracted with ethilic ether and dichloromethane	• Inefficient extraction in LLE process due to strong emulsification of the milk • Numerous peaks caused for extraction solvent interfering with the target analytes resolution
5- SPE	ATBs standard solution were separately extracted with C-18 and Oasis HLB cartridges	• Low recoveries for all ATBs using C-18 cartridges • Better recoveries mainly for CPF, AMP y SMX using HLB cartridges
6- Protein precipitation (TCA) + SPE-HLB + NSM	Milk samples fortified with ATBs were precipitated with TCA and extracted with Oasis HLB cartridges	• Milk matrix interferences • Changes in the migration times between runs • Losses of resolution due to peak overlapping • TCA decreases the extraction efficiency
7- Preconcentration method applied	Milk samples fortified with ATBs was defatted, deproteinized and extracted firstly with dichloromethane and then with Oasis HLB cartridges	• The LODs are low enough to determine concentrations of CPF, AMP and SMX lower than the permissible MRLs for these ATBs. The LOD of CLOR was 0.03 mg L^{-1}

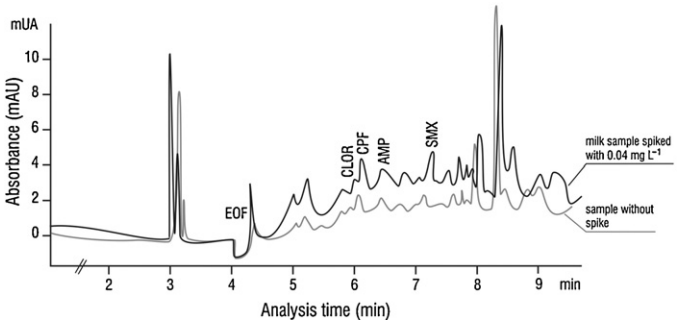


Fig. 3. Comparison between electropherogram of milk sample spiked with 0.04 mg L^{-1} of five ATB and electropherogram of the blank samples, precipitated with TCA and extracted with Oasis HLB cartridges, under the optimized experimental conditions.

The electropherogram of the SPE eluate of spiked milk samples (Fig. 3) showed the presence of non-identified endogenous peaks at different migration times with high signals, which interfere with the detection and quantitation of all ATBs. Moreover, the repeatability was poor, and changes in the migration times between runs and peak broadening were observed, making this methodology unsuitable. Furthermore, the peak corresponding to TC could not be detected, probably due to the presence of sample matrix components interfering in the extraction procedure.

Consequently, in order to remove potentially interfering compounds from the matrix sample, and also to increase the concentration of the analytes, we combined two methodologies of ATB extraction: LLE with dichloromethane and SPE with Oasis HLB cartridges. Furthermore, fat was eliminated by centrifugation, and proteins were removed by precipitation using trichloroacetic acid before LLE.

Subsequently, the method was validated using milk samples fortified with several levels of ATBs stock standard solution and subjected to the entire extraction procedure.

3.2.3. Validation of the method

The whole analytical method was validated in terms of linearity, LOD, LOQ, selectivity, repeatability and accuracy by carrying out recovery studies.

Firstly, and in order to evaluate matrix effects, two calibration curves were built using ACN–ultra-pure water (1:1, v/v) and bovine raw milk as matrices. Four replicates were prepared at four concentration levels by addition of known amounts of ATB standard solutions to both matrices over the concentration range 20–100 μg L^{-1} and subjected to the analytical procedure.

For computing figures of merit under univariate calibration, ATB peak areas were plotted against nominal concentrations (expressed in μg L^{-1}) and the lines were fitted by least-squares. The ANOVA test for lack of fit allowed us to conclude that linearity is fulfilled within the studied range antibiotics for both curves (Table 6).

A comparison between the calibration graphs was performed using a Student's *t*-test with a confidence level of 95% [41]. The performed tests show that significant differences exist between the slopes, a fact which demonstrates that the milk matrix affects the analytical signal for the target antibiotics (Table 6).

Therefore, the milk matrix produces systematic errors for all analytes, making it impossible to directly use the standard calibration graphs for the quantitation of CLOR, CPF, AMP and SMX using ACN–ultra-pure water (1:1, v/v) as solvent. Hence, whenever CLOR, CPF, AMP and SMX have to be quantited in milk samples, the standard addition method should be used. However, it is important to note that according to the results presented in Table 6, the proposed method is sensitive enough for the analysis of CPF, AMP and SMX in milk, because the obtained values of LOQ were below the MRLs

Table 6
Detailed evaluation of the comparison between the calibration graphs obtained using ACN–ultra-pure water (1:1, v/v) as solvent, and the matrix calibration curve using bovine raw milk fortified before extraction procedure. The test was performed using a Student's *t*-test with a confidence level of 95%.

Matrix	CLOR		CPF		AMP		SMX	
	Ultra-pure water–ACN	Milk	Ultra-pure water–ACN	Milk	Ultra-pure water–ACN	Milk	Ultra-pure water–ACN	Milk
Intercept[a]	−4 (2)	−0.4 (0.9)	−2 (2)	−0.6 (0.3)	−6 (1)	0.01 (0.4)	−14 (3)	−3 (2)
Slope[a]	0.42 (0.02)	0.16 (0.01)	0.39 (0.03)	0.156 (0.005)	0.57 (0.02)	0.160 (0.007)	1.45 (0.05)	0.62 (0.02)
R^2 adjusted	0.942	0.894	0.938	0.984	0.987	0.975	0.987	0.981
p-Value (lack of fit test)[b]	0.855	0.785	0.425	0.360	0.316	0.480	0.097	0.749
LOD (μg L^{-1})		29		12		13		14
LOQ (μg L^{-1})		105		35		38		41
MRL (μg L^{-1})		Not permitted		100		40		100
$t_{computed}$	9.27		8.77		23.78		15.68	
t_{table}	2.11		2.14		2.16		2.17	

[a] Values between parenthesis correspond to the standard deviation.
[b] The probability value of the lack of fit test should be larger than 0.05.

Table 7
Recovery for the test samples obtained by standard addition method.

Added ($\mu g\,L^{-1}$)	Recovery ($\mu g\,L^{-1}$)[a]			
Sample	CLOR (20.00)	CPF (21.00)	AMP (19.00)	SMX (23.00)
1	20.02 (100.11)	21.45 (102.16)	20.79 (109.45)	19.74 (85.82)
2	20.02 (100.11)	20.18 (96.11)	17.06 (89.77)	18.13 (78.81)
3	18.76 (93.79)	17.64 (84.00)	19.55 (102.89)	20.06 (87.22)
4	17.49 (87.46)	18.91 (90.05)	20.79 (109.45)	20.70 (90.02)
Mean recovery (%)	95.37	93.08	102.89	85.47
RSD (%)	6.35	8.40	9.02	5.58

[a] Recovery (%) between parentheses.

established for these ATBs. The method is also useful to quantitate the presence of CLOR above 105 $\mu g\,L^{-1}$.

As a consequence, accuracy was evaluated computing recoveries by using the standard addition method, in which the sample is used for performing the calibration. Known amounts of CLOR, CPF, AMP and SMX stock standard solutions were added to 100 mL of commercial milk in order to reach the following concentration levels: 20, 40, 60 and 100 $\mu g\,L^{-1}$. These latter concentrations are lower than the MRLs of each antibiotic. The mixtures were then analyzed by the extraction procedures cited in Section 2.5. The extracts were injected into the electrophoretic systems under the optimized conditions.

The analyte concentrations in fortified samples were computed using the curve. In the absence of absolute systematic errors the negative intercept on the concentration axis corresponds to the absolute value of the sample concentration. These concentration values were then compared with the theoretical amount added. As can be appreciated in Table 7, recoveries for the target antibiotics were ranged between 93.08% and 102.89%, figures that can be considered as excellent in view of the complexity of the sample and the low concentrations being analyzed.

Precision of the method was determined according to Decision 2002/657/EC [42] by performing the test on three sets of blank raw milk samples (six replicates each), fortified with CLOR, CPF, AMP and SMX at concentration of 0.5, 1.0 and 1.5 times the MRL. Since no reported MLR value is available for CLOR, it was arbitrarily taken as the lowest MLR (the one corresponding to AMP). Precision was calculated in terms of repeatability (RSD %), the variability of independent test results obtained with the same method on identical test items, in the same laboratory, by the same operator, and using the same equipment. Results are shown in Table 8.

Regulation 401/2006/EC [43] issued that the permitted experimental RSD for each concentration value must be below twice-fold the value derived by Horwitz equation. The equation is:

$$\mathrm{RSD} = 2^{(1-0.5\ \log\ C)} \quad (5)$$

where C is the mass fraction expressed as $mg\,g^{-1}$.

As can be seen in Table 8, the RSDs are lower than the calculated repeatability by Horwitz equation; these results indicate that the method satisfies the minimum performance criteria established by the above mentioned regulation.

Table 8
Repeatability for the determination of CLOR, CPF, AMP and SMX in spiked milk samples.

Fortification level	Repeatability (RSD %)[a]			
	CLOR	CPF	AMP	SMX
0.5 MRL	11.5 (20)	12.2 (18)	16.4 (20)	9.4 (18)
1.0 MRL	9.2 (18)	10.3 (16)	13.6 (18)	10.3 (16)
1.5 MRL	9.7 (17)	9.0 (15)	9.4 (17)	6.8 (15)

[a] Values between parenthesis correspond to twice-fold the value derived by Horwitz equation (RSD %).

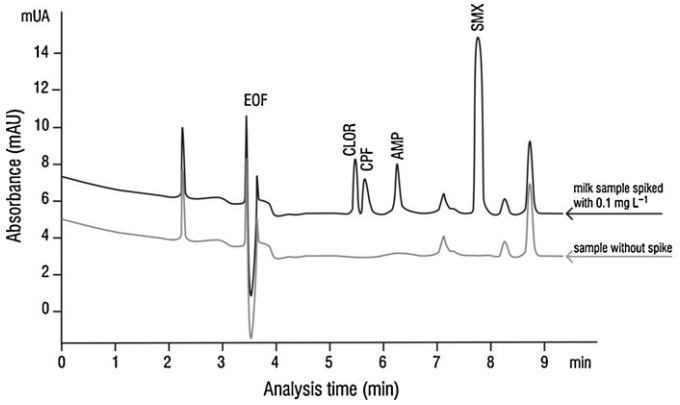


Fig. 4. Comparison between electropherogram of milk sample spiked with 0.1 $mg\,L^{-1}$ of each ATB and electropherogram of the blank samples after extraction procedure, under the optimized experimental conditions.

The selectivity indicates the ability of the method to accurately measure the analyte response in the presence of potentially interfering sample components. With the aim of verifying that CLOR, CPF, AMP and SMX peaks correspond to the pure compounds, a comparison between the electropherogram of a milk sample spiked with 0.1 $mg\,L^{-1}$ of each ATB and the electropherogram of the blank sample after the extraction procedure was performed. As a result, the separation of ATBs from blank peaks (peaks due compounds present in the sample matrix) was satisfactory. A clean baseline was observed throughout the run, making it easy the peak integration (Fig. 4).

4. Conclusions

The antibiotics CLOR, CPF, AMP and SMX can be determined in milk samples by using a CE method with a high efficiency and in a short analysis time (*ca.* 8 min). The detailed study carried out to select and optimize the significant variables that affect the resolution between the ATBs and the analysis time by means of experimental design appears to be a suitable alternative to the traditional univariate optimization.

Preconcentration strategies, based on combining electrophoretic and chromatographic methods, can be implemented with the aim of increasing the sensitivity of the CE technique, in order to detect residues of CLOR, CPF, AMP and SMX in milk.

We have demonstrated that CE–UV, combined with an effective sample clean-up, is a good alternative for the detection of CLOR, CPF, AMP and SMX in milk samples. The whole method is simple, accurate, selective, inexpensive and fast. Furthermore, it is sensitive enough for the analysis of CPF, AMP and SMX in milk, because the values of LODs obtained were below the MRLs established for these ATBs. Moreover, the sample components did not interfere with the signal of the target analytes. The method is also useful to quantify the presence of CLOR above 105 $\mu g\,L^{-1}$. Finally, it should be pointed

out that the standard addition method is necessary to get optimum quantitative values.

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