EVALUATION OF DIFFERENT STRATEGIES TO EXTRACT AND PRECONCENTRATE PENICILLINS PRESENT IN MILK PRIOR DETERMINATION BY CAPILLARY ELECTROPHORESIS

Evaluación de diferentes estrategias para extraer y preconcentrar penicilinas presentes en leche previa determinación por Electroforesis Capilar

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ABSTRACT

One of the main problems concerning the determination of residues of penicillins (PENs) in complex matrix, as milk samples of animal origin, is the sample treatment. The aim of this work was to evaluate the influence of different background electrolyte (BGE) composition and pH, found in the literature, in the determination of PENs by capillary electrophoresis (CE) and, different sample treatments for the determination of PENs in bovine milk samples by CE. Off-line preconcentration, as classical solid-phase extraction (SPE) and QuEChERS (namely quick, easy, cheap, effective, rugged and safe) methodology and in-line preconcentration strategies, as large volume sample stacking (LVSS) were applied. In general, the milk sample treatment included protein precipitation prior to preconcentration. For this purpose, hydrochloric acid (HCl) and trichloroacetic acid (TCA) were mainly used. To evaluate the SPE steps, several commercial sorbents (Oasis HLB, Bond Elut C18 and Strata-X) were studied. In order to provide useful information enabling the determination of these analytes in routine laboratories, the strengths and weaknesses of each of the sample treatments tested are presented. Protein precipitation with HCl followed by SPE or QuEChERS procedure proved to be efficient for removing matrix interferences, showing higher selectivity than other procedures evaluated.

Key words: Capillary electrophoresis, penicillins, milk, QuEChERS, SPE.

RESUMEN

El tratamiento de muestra es uno de los principales problemas relacionado con la determinación de residuos de penicilinas (PENs) en una matriz compleja, como leche de origen animal. El objetivo de este trabajo fue evaluar la influencia de la composición y pH de diferentes electrolitos de fondo o BGE (por sus siglas en inglés: background electrolyte) encontrados en la literatura para la determinación de PENs por electroforesis capilar (CE) y diferentes tratamientos de muestras para la determinación de PENs en muestras de leche bovina por CE. Para ello se aplicó una estrategia de preconcentración en línea, llamada *large volume sample stacking (LVSS)*, y preconcentración fuera de línea, como extracción en fase sólida (SPE) y la metodología QuEChERS (llamada así por sus siglas en inglés: Quick, Easy, Cheap, Effective, Rugged y Safe). En general, el tratamiento de la muestra de leche incluyó la precipitación de proteínas previo a la preconcentración. Para este propósito fueron empleados principalmente, ácido clorhídrico (HCl) y ácido tricloroacético (TCA). Para evaluar la SPE, se estudiaron varios sorbentes comerciales (Oasis HLB, Bond Elut C18 y Strata-X). En este trabajo se presentan las debilidades y fortalezas de cada uno de los tratamientos de muestras estudiados, con el fin de suministrar información útil para la determinación de estos analitos en laboratorios de rutina. La precipitación de proteínas con HCl seguido de SPE o QuEChERS resultaron ser los procedimientos más eficientes para la eliminación de interferentes de matriz demostrando mayor selectividad en comparación con otros procedimientos evaluados.

Palabras clave: Electroforesis capilar, penicilinas, leche, QuEChERS, SPE.

INTRODUCTION

Nowadays, foodstuffs is produced and distributed in a global market which requires stringent legislation and regulation for food quality and safety in order to protect consumers and ensure fair trade. Regulatory agency as European Food Safety Authority (EFSA) and Food and Drugs Administration (FDA) require the availability of analysis methods in order to provide the data for risk assessment, the establishment of maximum residue limits (MRLs) and the development and execution of monitoring plans.

Regulatory requirements for veterinary drug in food, as penicillins (PENs) residues in milk, are fairly stringent. Rapid screening tests are used to determine whether to accept or reject tanker loads of milk. For this reason, there is a need for sensitive confirmatory tests that can be used to assess accuracy of the screening tests. Also, the MRLs of these substances in foodstuffs of animal origin are established in 2377/90/EEC regulation, being 4 µg L^{-1} for amoxicillin (AMX), ampicillin (AMP), and penicillin G (PEN G), and 30 µg L^{-1} for cloxacillin (CLX) and oxacillin (OXA) [9].

Analytical methods for detecting PENs and their levels in milk samples have been widely developed in recent years. Liquid chromatography is the technique most commonly used for this purpose; however, capillary electrophoresis (CE) is becoming a useful alternative technique in this field. 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 physico-chemical properties of PENs, their ionizable nature and multiple ionization sites, make these compounds highly suitable for electrophoresis determination.

The determination of PENs by CE is mainly included in two different working modes: (i) capillary zone electrophoresis (CZE), and (ii) micellar electrokinetic chromatography (MEKC). Despite of the variety of buffers used to determination of PENs, in the literature there is a lack of systematic study on the influence of different background electrolyte (BGE) composition in the separation of these analytes.

In the food analysis field, there are few CE methods for the determination of PENs in milk [5, 23, 25, 28, 31] and animal tissues [6, 13, 14]. This may be due to it is quite difficult to measure PENs in food because of the complexity of the biological matrix and the extremely low concentration of these compounds. Most of the works related with the separation of penicillin mixtures are applied to the determination of these compounds in commercial pharmaceutical products [4, 8, 11, 12, 16, 17, 19-22, 27, 30]. Other examples are related with the determination of PENs in matrices of environmental impact as water [1, 6, 18], or in biological fluid samples [3, 10, 24].

In a complex matrix as milk, the sample treatment is still the major bottleneck in the analytical procedure. The determination of PENs in milk is a difficult task, due to its high protein and fat content, which often interfere in analytical procedures. Moreover, the analytes are often present at low concentration in these samples. In this case, it is essential to have an effective extraction and clean up steps to improve the selectivity of sample treatment and preconcentration step to improve the sensitivity of the method.

Different strategies for the extraction and preconcentration of PENs in milk samples have been used, such as liquid-liquid extraction (LLE) [26] and solid-phase extraction (SPE) [5, 23, 28]. The use of off-line SPE is probably the most widely used sample pretreatment procedure prior to CE determination. So far, different sorbents, such as C18, hydrophilic-lipophilic balance (HLB) and alumina N have been used.

Other strategies involving QuEChERS —which stands for Quick, Easy, Cheap, Effective, Rugged and Safe— and dispersive extraction by QuEChERS in MSPD format have also been used to treat milk samples containing antibiotics. QuEChERS methodology has some advantages over SPE and other traditional extraction methods such as operational simplicity and effective cleanup of complex samples.

Recently, other strategies involving QuEChERS –which stands for Quick, Easy, Cheap, Effective, Rugged and Safe– and dispersive extraction by QuEChERS in MSPD format with Strata X sorbent [15] have also been used to determine PENs in milk samples by high performance liquid chromatography (HPLC). QuEChERS was introduced by Anastassiades et al. [2] in 2003 and was promising to provide a fast and reliable way to determine the target antibiotics in milk. The original procedure involves initial SPE of the sample with acetonitrile (ACN) followed by liquid–liquid partitioning by the addition of anhydrous magnesium sulphate ($MgSO_4$) and sodium chloride. Removal of water and clean up are performed simultaneously on an aliquot of the ACN extract with dispersive SPE using $MgSO_4$ and primary secondary amine sorbent. The QuEChERS procedure has some advantages because it simplifies and reduces the time taken for the extraction and clean up processes. However, so far no studies were found where QuEChERS methodology was used as sample treatment in the determination of PENs in milk by CE.

Although a number of interesting methods testifying to the analytical usefulness of CE for determining PENs [5, 23, 25, 28, 31], no critical assessment of their effectiveness has so far been published to help others select the most suitable choice for specific extraction of PENs. It can be confirmed that there is a lack of sample treatment protocol that generate extracts compatible with CE analysis. In this work, a systematic study on the influence of different BGE composition and pH found in the literature, for the determination of PENs by CE

was presented. Also different procedures to extract and pre-concentrate AMX, AMP, CLX, OXA and PEN G present in bovine milk have been evaluated. Off-line preconcentration strategies (classical SPE and QuEChERS) and in-line, as large volume sample stacking (LVSS) were applied. In order to provide useful information enabling the determination of these analytes in routine laboratories, the advantages and disadvantages of each of the sample treatments tested are here discussed.

MATERIALS AND METHODS

Reagent and materials

All chemicals and solvents were of analytical grade. Sodium tetraborate ($Na_2B_4O_7$), sodium dihydrogenphosphate (NaH_2PO_4), hydrochloric acid (HCl), and ACN were purchased from Merck (Darmstad, Germany); sodium hydroxide (NaOH), trichloroacetic acid (TCA) and methanol (MeOH) were obtained from Panreac (Barcelona, Spain); and sodium docedyl sulphate (SDS) was purchased from Sigma. AMX, AMP, CLX, OXA, PEN G, and Naproxen (I.S) were obtained from Sigma (St. Louis, MO, USA).

Individual stock solutions containing a 100 μg mL^{-1} concentration of each penicillin were prepared in water and stored at 4°C (freezer Ignis, Comerio, Italy) prior to use. Under such conditions, they were stable for at least two months. Working solutions (containing all PENs) were prepared daily by diluting the stock solutions in water. All water used was purified by passage through a Milli-Q system from Millipore (Bedford, MA, USA).

The SPE cartridges used in this study were: Oasis hydrophilic-lipophilic balance (HLB) cartridge (500 mg, 12 cm^3; Waters, Milford, MA, USA), Bond Elut C18 (500 mg; Varian, Harbor City, CA, USA), Strata X-Phenomenex (Torrance, CA, USA). Kits SampliQ QuEChERS (kindly supplied by Agilent Technologies Inc., Wilmington, DE, USA) consisted on extraction tubes (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) and dispersive tubes (150 mg C18, 150 mg primary secondary amine (PSA) and 900 mg $MgSO_4$).

Electrophoretic conditions

P/ACE MDQ CE System from Beckman (Palo Alto, CA, USA) equipped with a diode array detector (DAD) were used for the separation and quantification of PENs. Electrophoresis experiments were performed in a 60.2 cm x 75 μm id, uncoated fused-silica capillary (Beckman Coulter) with an optical path length of 220 mm and an effective length of 50 cm.
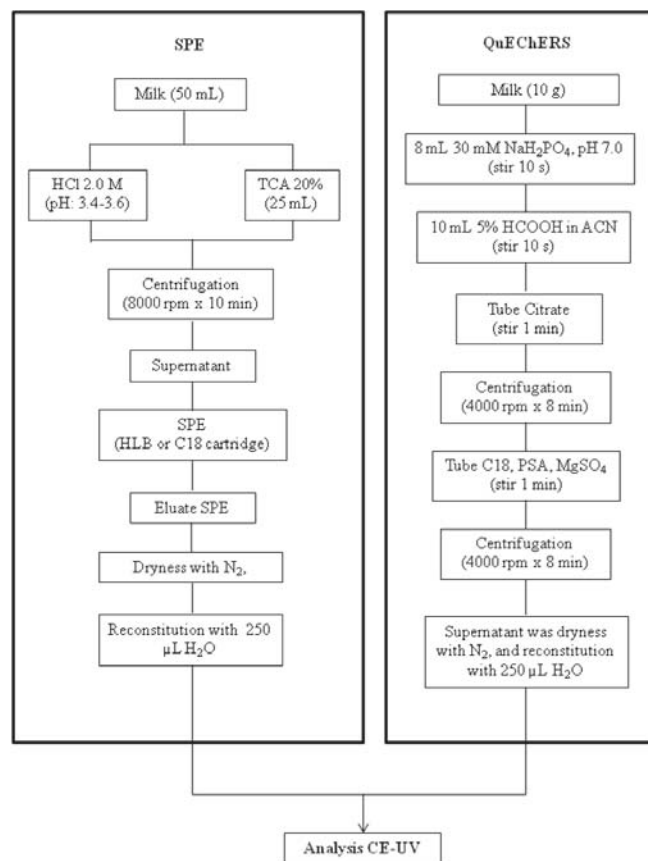
The BGE used was 35 mM of sodium tetraborate and 75 mM of SDS adjusted at pH 8.5. Prior to first use, the capillary was conditioned by rinsing with 1 M HCl for 5 minutes (min), 0.1 M NaOH for 10 min, water for 5 min and separation buffer for 15 min. The capillary was prepared for daily use by rinsing

with 0.1 M NaOH for 5 min, water for 5 min and separation buffer for 15 min; between runs, the capillary was rinsed with water for 1 min, 0.1 M NaOH for 2 min, water for 1 min and separation buffer for 5 min. These solutions were filtered through a 0.45 μm nylon membrane (Millipore, Bedford, MA, USA) before analysis.

Extraction procedure

Before the extraction procedure, milk samples were spiked with different aliquots of stock standard solution of the PENs studied: AMX, AMP, CLX, OXA and PEN G. Samples were shaken on a vortex mixer (Fisher Scientific, Madrid, Spain) for 30 s and then allowed to stand for at least 20 min, to enable sufficient equilibrium with the milk matrix. In total, six milk samples (at different concentration levels) were analyzed and three replicates were prepared for each concentration level.

Two different methodologies for extraction and off-line preconcentration of analytes presents in milk samples were evaluated: classical SPE format and QuEChERS (FIG. 1).

FIGURE 1. DIFFERENT SAMPLE TREATMENTS (SPE AND QuEChERS METHODOLOGY) FOR THE DETERMINATION OF PENs APPLIED IN THIS STUDY.

SPE

A study related to the recoveries of the PENs using standard solutions was carried out in order to get an idea of the most advantageous sorbents for these analytes, testing three types of sorbents: HLB, C18 and Strata-X. All the SPE sorbent material was preconditioned by flushing 2 mL of MeOH and 2 mL of water. After rinsing with 2 mL water, the PENs were eluted with 1 mL MeOH and 1 mL ACN, successively. The collected eluate was evaporated to dryness using a stream of nitrogen at 40°C. The residue was resuspended in 250 µL of water and analyzed by CE system. All the SPE experiments were performed at room temperature and the optimum sorbent was used to extract PENs in milk samples.

Different deproteination procedures used HCl 2 M or TCA 20% were tested prior to SPE. A volume of 50 mL of milk was spiked with known variable amounts of the analytes. The milk sample was deproteinated by adding of 2.0 M HCl to decrease the pH to 3.4–3.6 using a pH meter (Crison model pH 2000, Barcelona, Spain) or by adding 25 mL of 20% aqueous TCA. Thus the extract obtained was defatted by centrifugation (J. P. Selecta, Barcelona, Spain) at 9500 *g* for 10 min. Finally, the extract was passed through a SPE cartridge.

QuEChERS

The QuEChERS procedure was adapted from that described by Agilent Technologies for the determination of quinolones in bovine liver [29]. Samples of 10 g of milk were spiked at different concentration levels of PENs using the working standard solutions. They were placed into 50 mL centrifuge tubes and homogenized in vortex. Then 8 mL of 30 mM NaH_2PO_4 buffer pH 7.0 was added, shaking by hand for 10 s. Subsequently, 10 mL of 5% formic acid in ACN was added to the tube, shaking by hand for 10 s. Agilent SampliQ QuEChERS extraction tubes ($MgSO_4$, NaCl, sodium citrate, and disodium citrate sesquihydrate) was added and the tube was shaken vigorously for 1 min. After that, the sample was centrifuged at 4700 *g* for 8 min and 4 mL of the upper ACN layer was transferred to another tube containing the dispersive SPE (C18, PSA and $MgSO_4$) and stirred in vortex for 1 min. The tube was centrifuged at 4700 *g* for 8 min. Then, all supernatant was transferred to a vial, dried at 40°C under a stream of nitrogen and reconstituted with 250 µL of water and analyzed by CE system.

LVSS procedure

Standard solutions containing the PENs were loaded for 270 s into the electrophoretic system so that the whole capillary was filled with the sample solution. Water was used as the sample solvent to produce a low-conductivity analyte matrix. A negative voltage (-20 kV) was then applied and the sample stacking started. Reverse polarity was applied for a time of 2.1 min. A positive voltage (20 kV) was then applied to separate the compounds.

RESULTS AND DISCUSSION

Selection of the appropriate instrumental CE variables

All the preliminary studies were focused on the optimization of the experimental parameters affecting the CE separation of the target compounds by using UV-vis detection. The UV-vis spectra of the analysis was registered choosing a wavelength of 210 nm with a bandwidth of 8 nm for monitoring the selected PENs. To optimize the separation, the influence of the running buffer nature, its concentration and the pH were studied. Twenty different running buffers found in the literature for the determination of PENs were initially tested (TABLE I). The different works have been ordered in the table according to the pH of the running buffer. CZE and MEKC modality were mainly used and basic pH was dominant over acid pH. The best results were obtained by using a mixture of sodium tetraborate and SDS as surfactant (MEKC mode). In general, this modality allows the separation of neutral and/or ionic antibiotics.

The influence of the concentration of sodium tetraborate (20–40 mM) and the concentration of SDS (60–100 mM) were investigated. The concentrations of sodium tetraborate and SDS are parameters with a more significant influence on sensitivity. A concentration of 35 mM of sodium tetraborate and 75 mM of SDS increased the area of all peaks, obtaining also an adequate electric current (below 110 µA). Due to this fact, both concentrations were selected for the separation of the PENs. The influence of buffer pH was also studied. The pH of the running electrolyte is one of the critical factors in resolution due to its impact on EOF in a fused-silica capillary, and the possible effect on solute charge altering relative migrations. The effect of pH value was investigated over the range of 7.5-8.5. From the experimental results can conclude that the pH of the buffer solution affects the resolution of the PENs studied. The best results were achieved at a pH of 8.5. With this buffer it was carried out the separation of the PENs tested (AMX, AMP, CLX, OXA, and PEN G), in less than 11 min, as it is shown in FIG. 2. These results are consistent with several studies found in the literature that employ a similar BGE composition and pH, as it can be seen in the works highlighted in the TABLE I.
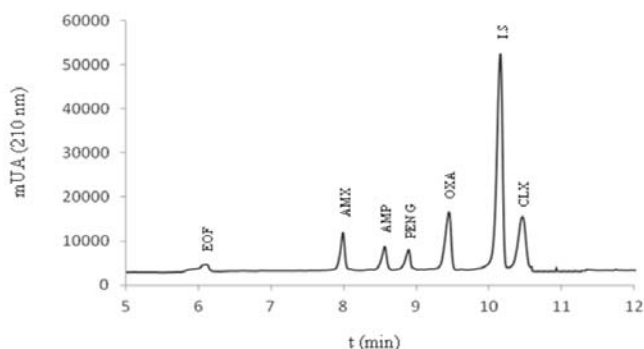
A voltage of 15 kV was applied as optimum so as to achieve a good compromise between the running time, the resolution and the electric current. The effect of the temperature on the separation was investigated in the range of 20-30°C, lower values did not provide an adequate resolution for all the analytes. A capillary temperature of 25°C was selected as optimum. The figures of merit corresponding to PENs studied are shown in TABLE II.

The electrophoretic method was validated directly with standard solutions of PENs. In order to establish the standard calibration curve, solutions containing PENs were prepared at six concentration levels. TABLE II summarizes the LODs obtained with this methodology. LODs were determined by calculating three times the SD of the intercept divided by slope.

BGE COMPOSITION OF THE CE METHODS USED PREVIOUSLY FOR THE SEPARATION OF PENs
(AMX, AMP, CLX, OXA, AND PEN G)

BGE composition	pH	Ref.
50 mM Phosphoric acid and 5.2 mM 2-hydroxypropylbeta-cyclodextrin	3.6	[6]
20 mM Ammonium acetate	5.1	[23]
60 mM Ammonium acetate	6.0	[8]
70 mM Sodium dihydrogenphosphate, 125 mM SDS, 5% ACN	6.0	[18]
20 mM Ammonium acetate and 20 mM ammonium acetate in ACN/MeOH(60/40 v/v)	6.5	[35]
40 mM Sodium dihydrogenphosphate and 100 mM SDS	7.0	[20]
40 mM Phosphate-borate and 75 mM SDS	7.5	[15]
175 mM Tris buffer with 20% ethanol	8.0	[4]
2.7 x 10^{-2} M Potassium dihydrogenphosphate and 4.3 x10^{-2} M sodium tetraborate	8.0	[5]
60 mM Ammonium acetate with 10% of MeOH	8.0	[9]
20 mM Sodium tetraborate with 60 mM SDS	8.0	[14]
80 mM Sodium tetraborate decahydrate	8.0	[17]
26 mM Sodium tetraborate with 100 mM SDS	8.5	[11]
20 mM Sodium tetraborate and 75 mM SDS	8.5	[21]
40 mM Sodium tetraborate and 100 mM SDS	8.5	[22]
3.12 g/L Disodium hydrogenphosphate, 7.64 g/L sodium tetraborate and 14.4 g/L SDS	8.7	[16]
20 mM Sodium tetraborate	9.0	[25]
10 mM Sodium dihydrogenphosphate, 6 mM sodium tetraborate	9.0	[26]
30 mM Tetraborate	9.2	[19]
30 mM Sodium tetraborate	9.2	[24]

FIGURE 2. ELECTROPHEROGRAM CORRESPONDING TO STANDARD SOLUTION MIXTURE OF FIVE PENs (5 mg L^{-1} IN ULTRA-PURE WATER). AMX: AMOXICILLIN; AMP: AMPICILLIN; PEN G: PENICILLIN G; OXA: OXACILLIN; CLX: CLOXACILLIN; I.S: NAPROXEN (2.5 mg L^{-1}). EXPERIMENTAL CE CONDITIONS WERE: 35 mM SODIUM TETRABORATE AND 75 mM SDS, pH 8.5, SEPARATION VOLTAGE 20 kV, TEMPERATURE 25°C, HYDRODYNAMIC INJECTION (APPLYING 0.5 psi 10 s) AND DETECTION AT 210 nm.

With the CE using UV-vis detection was not possible to obtain LODs below MRLs established by legislation.

Optimization of LVSS

LVSS was applied to increase the analyte concentration prior to their separation. Significant parameters that influence the LVSS, such as injection time, voltage and time reverse polarity, and voltage normal polarity were studied. To optimize the injection time, different values (90, 180, 210, 270 and 360 s) were used in each experience. These values were estimated considered the diameter and length of the capillary and the applied pressure. Injection time of 270 s was selected, since no gain in preconcentration factor by using longer times. The negative voltage was studied in the interval of -15 kV to -25 kV. The stacking voltage was kept at –20 kV in this step for 2.1 min, and a voltage of 20 kV applied for MEKC analysis. Electropherogram corresponding to a mixture of the selected PENs, after the application of the LVSS procedure, is shown in FIG. 3, where a significant increase in the signal scale is observed. The statistic parameters calculated and the performance characteristics of the LVSS-CE method are presented in TABLE III. The LOD obtained for the analytes in aqueous solution was up to 29 times lower when LVSS was used compared with CE method using normal injection mode.

Off-line preconcentration SPE

Three SPE sorbents like (Oasis HLB, C18 and Strata-X) were tested. Oasis HLB provided higher recoveries for AMX, AMP and PEN G and C18 sorbent for CLX and OXA. The statistic parameters calculated and the performance characteristics of the SPE-CE method are presented using HLB (TABLE IV). With this preconcentration method was reached MRLs established for PENs studied. Electropherogram corre-

CALIBRATION CURVES WITH CE METHOD FOR THE DETERMINATION OF PENs

Analyte	$y = ax + b$	R^2	$S_{y/x}$	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)
AMX*	$a = 7.4 \times 10^{-5} \pm 1.7 \times 10^{-6}$ $b = -0.06271 \pm 0.00559$	0.992	0.010	226.6	755.4
AMP*	$a = 6.4 \times 10^{-5} \pm 1.6 \times 10^{-6}$ $b = -0.04563 \pm 0.00518$	0.991	0.009	242.8	809.4
CLX*	$a = 1.9 \times 10^{-4} \pm 7.2 \times 10^{-6}$ $b = -0.19161 \pm 0.02322$	0.981	0.041	366.6	1222.1
OXA*	$a = 1.3 \times 10^{-4} \pm 3.5 \times 10^{-6}$ $b = -0.09466 \pm 0.01134$	0.990	0.020	261.7	872.3
PEN G*	$a = 5.7 \times 10^{-5} \pm 1.8 \times 10^{-6}$ $b = -0.02521 \pm 0.00604$	0.985	0.011	317.9	1059.6

* The values were obtained with respect to the internal standard (Naproxen). Concentration of penicillins in $\mu g\ kg^{-1}$; y: absorbance; *a:* slope; *b:* intercept; R^2: correlation coefficient; AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; OXA: oxacillin; PEN G: penicillin G.
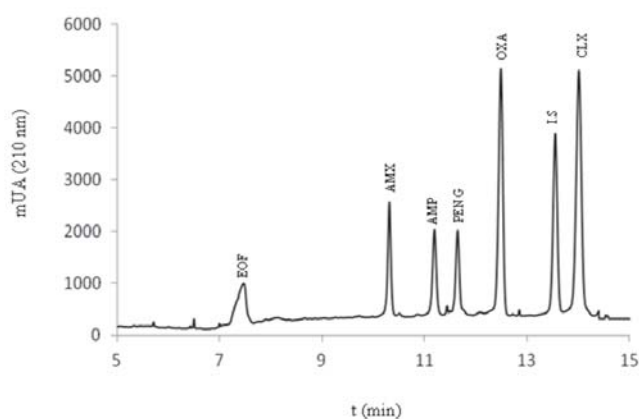

FIGURE 3. ELECTROPHEROGRAM CORRESPONDING TO STANDARD SOLUTION MIXTURE OF FIVE PENs (1 mg L^{-1} IN ULTRA-PURE WATER). AMX: AMOXICILLIN; AMP: AMPICILLIN; PEN G: PENICILLIN G; OXA: OXACILLIN; CLX: CLOXACILLIN; I.S: NAPROXEN (0.1 mg L^{-1}). EXPERIMENTAL LVSS-CE CONDITIONS WERE: 35 mM SODIUM TETRABORATE AND 75 mM SDS, pH 8.5, HYDRODYNAMIC INJECTION (APPLYING 0.5 psi 270 s), SEPARATION VOLTAGE (REVERSE POLARITY) -20 kV, TIME VOLTAGE IN REVERSE POLARITY 2.1 min, SEPARATION VOLTAGE (NORMAL POLARITY) 20 kV, TEMPERATURE 25°C AND DETECTION AT 210 nm.

sponding to a mixture of the selected PENs, after the application of the SPE procedure with HLB is shown in FIG. 4. SPE allowed better LOD for different analytes studied in aqueous matrix compared with CE and LVSS-CE.

Determination of PENs in real samples

Before conducting the SPE procedures to milk samples, a protein precipitation step was required. Different organic solvents, inorganic salts or strong acids have been currently used to precipitate proteins. The precipitating agent most appropriate to avoid compatibility problems between the extract and the electrophoretic system were the acids. In this study, HCl removed more interference and has a low dilution effect compared with TCA. HCl was added to reduce the pH in the range 3.4–3.6 and to remove the proteins present in milk sample (casein, mainly). Subsequently, the sample was centrifuged to remove precipitated proteins and the fatty material in the sample. HCl has been used in a previous study as deproteinization agent on determination of organic acids by CE in milk samples obtained from goats *(Capra hircus)* of the *Payoya* breed [7]. This sample treatment allows partial removal of matrix interferences, making possible only the determination of AMP and PEN G in milk samples, as shown in FIG. 5.

Also, the QuEChERS procedure described for the determination of quinolones in bovine liver [29] has been adapted in this work for bovine milk samples. The FIG. 6 shows an electropherogram of bovine milk sample (solid line) and spiked bovine milk sample (dashed line) treated following the QuEChERS procedure using UV-vis detection at optimum method conditions. As can be observed, all examined analytes were resolved from matrix, except for AMP. An interference peak was found co-migrating with CLX. With this sample treatment, AMX, PEN G and OXA can be detected in milk samples.

Finally LVSS was used to preconcentrate the extract obtained from real samples (bovine milk) after the different sample treatments were applied. Notice that LVSS requires extracts with very low conductivity to obtain the best focusing of the analytes. For this reason was impossible to implement this strategy in the milk matrix. In others samples such as farm waste water, LVSS could be an effective preconcentration strategy. To increase the sensitivity of the CE method optimized other detectors, such as mass spectrometry (MS) or fluorescence (FL) should be used.

TABLE III
CALIBRATION CURVES WITH LVSS-CE METHOD FOR THE DETERMINATION OF PENs

Analyte	y = ax + b	R^2	$S_{y/x}$	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
AMX*	a = 0.00082 ± 0.00002 b = 0.02662 ± 0.01159	0.990	0.029	42.4	141.3
AMP*	a = 0.00094 ± 0.00001 b = -0.01796 ± 0.00483	0.997	0.013	15.4	51.3
CLX*	a = 0.00283 ± 0.00006 b = 0.04472 ± 0.03986	0.992	0.092	42.2	140.8
OXA*	a = 0.00228 ± 0.00001 b = -0.02367 ± 0.00694	0.998	0.022	9.1	30.4
PEN G*	a = 0.00101 ± 0.00001 b = -0.01677 ± 0.00545	0.996	0.015	16.1	53.9

*The values were obtained with respect to the internal standard (Naproxen). Concentration of penicillins in µg kg^{-1}; y: absorbance; *a:* slope; *b:* intercept; R^2: correlation coefficient; AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; OXA: oxacillin; PEN G: penicillin G.
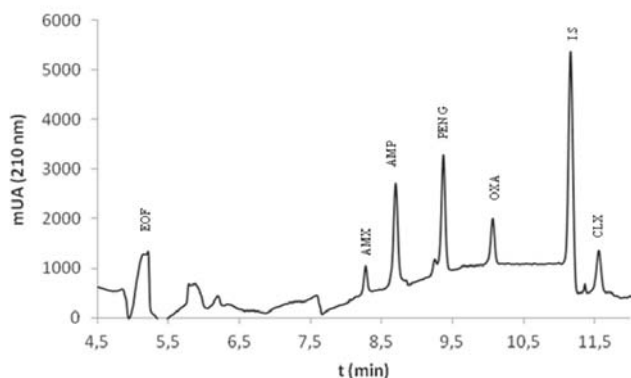

FIGURE 4. ELECTROPHEROGRAM CORRESPONDING TO STANDARD SOLUTION OF FIVE PENs (25 µg L^{-1} IN ULTRA-PURE WATER) USING SPE-CE METHOD WITH HLB CARTRIDGE. AMX: AMOXICILLIN; AMP: AMPICILLIN; PEN G: PENICILLIN G; OXA: OXACILLIN; CLX: CLOXACILLIN; I.S: NAPROXEN (25 µg L^{-1}). EXPERIMENTAL CONDITIONS WERE THE SAME AS IN FIG. 2.
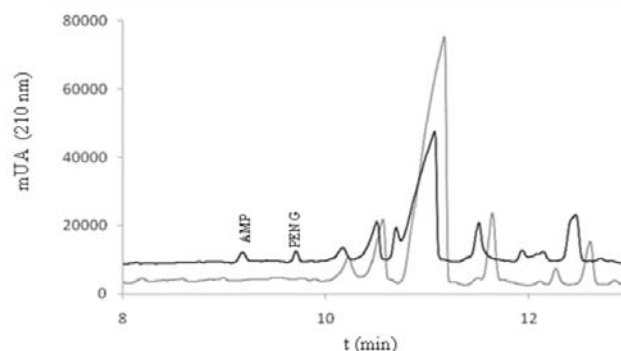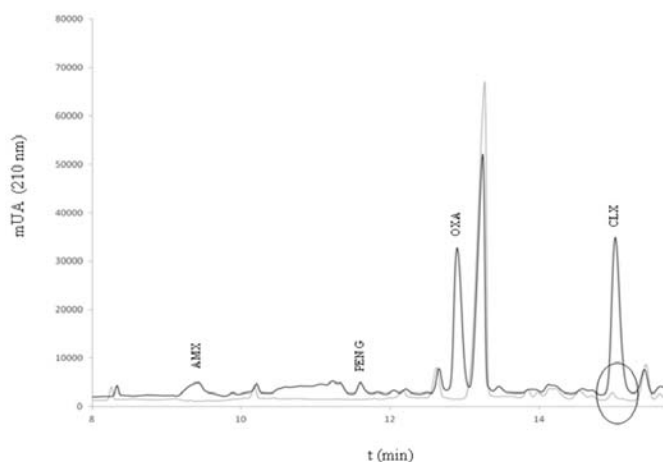
FIGURE 5. ELECTROPHEROGRAM CORRESPONDING TO BLANK AND SPIKED MILK SAMPLE WITH PENs AT 2 mg L^{-1} USING SPE-CE METHOD WITH HLB CARTRIDGE. AMP: AMPICILLIN; PEN G: PENICILLIN G. EXPERIMENTAL CONDITIONS WERE THE SAME AS IN FIG. 2.

TABLE IV
CALIBRATION CURVES WITH SPE-CE METHOD USING HLB FOR THE DETERMINATION OF PENs

Analyte	y = ax + b	R^2	$S_{y/x}$	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
AMX*	a = 0.00148 ± 0.00004 b = 0.00217 ± 0.00158	0.991	0.003	3.2	10.7
AMP *	a = 0.00763 ± 0.00015 b = 0.00631 ± 0.00617	0.995	0.014	2.4	8.1
CLX*	a = 0.00132 ± 0.00002 b = 0.00743 ± 0.00148	0.994	0.003	3.3	11.2
OXA*	a = 0.00115 ± 0.00002 b = 0.00812 ± 0.00162	0.990	0.041	4.2	14.1
PEN G*	a = 0.01126 ± 0.00025 b = -0.00317 ± 0.01010	0.994	0.023	2.7	9.0

*The values were obtained with respect to the internal standard (Naproxen). Concentration of penicillins in µg kg^{-1}; y: absorbance; *a:* slope; *b:* intercept; R^2: correlation coefficient; AMX: amoxicillin; AMP: ampicillin; CLX: cloxacillin; OXA: oxacillin; PEN G: penicillin G.

FIGURE 6. ELECTROPHEROGRAM CORRESPONDING TO BLANK AND SPIKED MILK SAMPLE WITH PENs AT 10 mg L^{-1} EXTRACTED WITH QuEChERS AND ANALYZED BY CE METHOD: AMX: AMOXICILLIN; PEN G: PENICILLIN G; OXA: OXACILLIN; CLX: CLOXACILLIN. EXPERIMENTAL CONDITIONS WERE THE SAME AS IN FIG. 2.

CONCLUSIONS

Two different analytical strategies that combine MEKC-UV analysis with SPE or QuEChERS procedure, for the determination of PENs in milk samples were proposed. Deproteination with HCl proved to be efficient for removing matrix interferences, showing higher selectivity than other procedures evaluated. The sample treatment with SPE allowed the determination of AMP and PEN G, while QuEChERS procedure can be used for the determination of AMX, PEN G and OXA. The sample treatments proposed are useful to reduce the number of interferences present in the milk samples but not to achieve the LODs required by the actual legislation. The sensitivity of the method can be increased by using MS and FL detector.

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