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Chapter 3

Importance of Treatment Process on the Analysis of Penicillins in Milk Samples by Capillary Electrophoresis

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Additional information is available at the end of the chapter

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1. Introduction

Some veterinary drugs (such as antibiotics) are required in rational use at some stage of animal production in order to guarantee the hygienic management of farms. Antibiotics can be used at low levels for growth promotion (2.5 to 125 $mg_{antib}\ kg^{-1}{}_{feed}$, depending on the species treated), at intermediate levels for disease prevention (<200 $mg_{antib}\ kg^{-1}{}_{feed}$), and at high levels to treat infected animals. Prophylactic antimicrobial use is applied in intense livestock production to protect animal welfare by treating uninfected animals, thereby preventing epidemic spread of infectious animal diseases. This provides for a high efficiency of animal production. Antibiotic use is a potential risk for the development of antimicrobial resistant bacteria since these drugs can be found as residues in animal-derived food products [1-4].

Antibiotics used in veterinary and human medicine include mainly: penicillins, cephalosporins, tetracyclines, fluoroquinolones, sulfonamides and macrolides. Penicillins (PENs) have been widely used for more than 30 years and today are one of the most important groups of antibiotics [5].

Residue analysis is closely related to food safety. It establishes whether food is or is not safe for human consumption. Residue analysis is part of the monitoring programs of regulatory agencies. Its aim is to guarantee that concentration levels are below the established maximum residue levels (MRLs). Regulatory demands for the control of chemical contaminants in food have led to an increase in the demand for analytical methods for detecting concentrations below MRLs in food stuffs.

Advances in analytical instrumentation have been invaluable in assisting in each step of the analytical cycle. Different strategies have been developed for controlling the presence of PENs

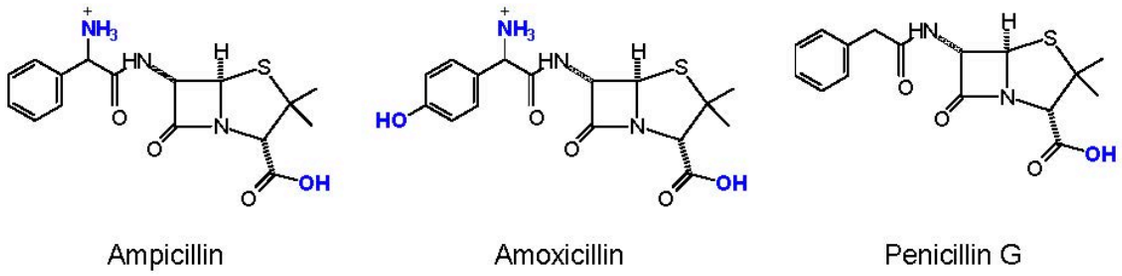

Figure 1. Chemical structures of the main PENs

residue in food samples. These methods vary in reliability, the speed of obtaining results, and cost of the analysis. These methods can be grouped into the following categories [6]:

1. Microbiological approaches based on bacterial growth inhibition
2. Electroanalytical methods
3. Immunochemical techniques
4. Separation techniques

Separation methods, such as gas chromatography and high performance liquid chromatography, are the techniques commonly used to determine the low concentration levels of this antibiotic group. Capillary electrophoresis (CE) is increasingly being used for confirmation purposes. The main drawback of CE is the low sensitivity when UV detection is used, as a consequence of the short optical path length and the extraction of PENs from complex matrices. Several sample treatments are required in most cases to extract and pre-concentrate the analytes. Additionally, during CE analysis some inorganic ions, proteins, and other major compounds can affect the electrophoretic mobility [7].

Recently, a review article focused on PENs analysis by CE can be found in the literature [8]. It describes the importance of high sample volumes in the analysis as an alternative for obtaining lower limits of detection. In this study, we propose the combination of magnetic solid phase extraction and capillary electrophoresis in the analysis of PENs in milk samples.

1.1. Penicillins

Penicillin was discovered by Alexander Fleming in 1928 and was purified and then synthesized by Florey and Chain in 1940. PENs belong to the β-lactam antibiotics. Their structure consist of a thiazolidine ring attached to a β-lactam ring, forming 6 aminopenicillanic acids and a side chain in the 6-position, which determines the stability and the antimicrobial activity of the different derivatives. The mechanism of action of PENs is via the inhibition of bacterial cell death of the offending bacteria due to the faulty production of the vital cell wall components [9].

The most common PENs are ampicillin (AMP), amoxicillin (AMO) and penicillin G (PEN G). Figure 1 shows the acid structure of the main PENs.

The existence of PENs charge molecules suitable for electrophoretic separation is a result of the presence of acid-base functional groups in their structure such as amino, carboxylic acid, and phenol. They can exhibit cationic and zwitterionic forms when an amino group is presented, while the carboxylic and phenol groups contribute to generate anions. The pKa values are: ampicillin (pKa_1=2.6, pKa_2=7.4), amoxicillin (pKa_1=2.6, pKa_2=7.2, pKa_3=9.6), and penicillin (pka_1=2.6). [10]

β-lactams are usually employed in treating mastitis in cows, therefore milk is the most frequently analyzed sample. As a result, the international organizations have defined MRLs for PENs. The European Union directive establishes MRLs of 4 μg kg^{-1} for the three main PENs [11]. On the other hand, the U.S. Food and Drug Administration established the MRLs for these drugs as follows: amoxicillin and ampicillin, 10 μg kg^{-1}; and penicillin G, 5 μg kg^{-1} [12]. In order to achieve the MRLs, sample preparation processes including both clean-up and pre-concentration are indispensable during determination of PENs residue in milk samples.

1.2. Sample preparation

PENs exhibit significant binding to proteins contained in milk samples, thus the first step during milk analysis by CE is to deproteinize the sample. Additionally, this procedure helps to prevent emulsions during partition equilibriums. The main strategies used are acidic precipitation or addition of acetonitrile. Methanol should be avoided as a precipitation solvent because it degrades PENs to the corresponding alkyl-*α*-D-penicilloic acids. [13]. Strong acidic deproteinizong agents are not recommended because of the degradation of PENs. However, good recoveries have been described when trichloroacetic acid was used for the precipitation of milk samples containing PENs concentrations higher than the MRLs [14].

Acetonitrile provided satisfactory results for precipitating proteins and, since the solubility of lipids in the organic solvent is low, most of the lipid fraction was co-precipitated. For the recovery of PENs from milk, a volume ratio of acetonitrile:milk higher than 2:1 was optimal. The use of lower ratios has been described for the analysis of PENs during the first step using the QuEChERS technique [15].

Once the sample is deproteinize, the acetonitrile phase is separated and evaporated. The sample is reconstituted in aqueous phase to be suitable for solid phase extraction. Oasis HLB and C18 cartridges have been applied during sample pre-concentration and clean-up. Acetonitrile was commonly used for the elution of the sample. When an on-line pre-concentration system such as large volume sample stacking is employed, a low conductivity sample is required. The acetonitrile extract is then passed through a polar sorbent (alumina N) and the PENs are eluted with deionized water.

Recently discovered, dispersive techniques (liquid and solid) have shown to improve the contact between the analyte and the extracting phase [16-17]. A clear example is the so-called QuEChERS process, which involves a second clean up step based on dispersive solid phase extraction. During PENs analysis, a mixture of silica C18, primary and secondary amine and acetonitrile were added to an aliquot of the organic extract in order to remove sugars and fatty

acids. The extract was centrifuged, evaporated, and reconstituted in a phosphate buffer before its analysis.

There are a few reports related to the analysis of PENs in milk samples by CE. Table 1 shows the conditions used for the preparation of a milk sample for analysis of AMP, AMO and PEN G.

Recovery of the solid phase in dispersive solid phase extraction requires the use of additional procedures such as filtration or centrifugation. The loss of solid during extraction might affect precision and accuracy. In order to minimize the steps involved, magnetic compounds have been incorporated (mainly, Fe_3O_4 or γ-Fe_2O_3) in the solid phase. These magnetic supports can be dispersed and isolated from the sample by the use of an external magnetic field [18-19].

Sample pre-treatment	Separation mode	Limit of detection	Reference
a) 50 ml milk+20 ml trichloroacetic acid (20%) b) SPE (Oasis-HLB), elution with ACN c) Evaporation and reconstitute in 5 ml of H_2O:ACN (1:1)	CZE-UV	0.48-1.09 μg ml^{-1}	[14]
a) 5 ml milk+15 ml ACN b) SPE (Oasis-HLB), elution with ACN c) SPE (Alumina N), elution with H_2O d) Evaporation and reconstitute in 1 ml of H_2O e) On-line concentration	CZE-DAD	4 μg kg^{-1}	[17]
a) 2 mL milk + 4 mL ACN b) The liquid phase was dried and reconstituted in H_2O (1 ml) d) SPE (C18 cartridge), elution with ACN e) Evaporation and reconstitute in H_2O (1 ml) f) On-line sample concentration	CEC-MS	0.05-0.11 μg l^{-1}	[10]
a) 50 ml milk+HCl to reach a pH 3.4-3.8 b) SPE (Oasis HLB), elution with ACN:MeOH (1:1 v:v) c) Evaporation and reconstitute in H_2O (0.25 ml)	MEKC-UV	2.4-3.2 μg kg^{-1} (in water)	[15]
a) 10 g milk+ 8 ml (PBS) + 10 ml (ACN) + QuEChERS mixture b) 4 ml of liquid phase + dispersive SPE (C18, PSA, $MgSO_4$) c) Evaporation and reconstitute in H_2O (0.25 ml)	MEKC-UV	N.R.	[15]

SPE: solid phase extraction, **ACN**: acetonitrile, **MeOH**: methanol, **CZE**; capillary zone electrophoresis, **CEC**: capillary electrophoresis, **MEKC**, micellar electrokinetic chromatography, **UV**: ultraviolet, **DAD**: diode array detection, **MS**: mass spectrometry.

Table 1. Summary of the main PENs analysis in milk samples by CE

Core-shell particles with paramagnetic core and silica shell are the most common particles synthesized. The magnetic solids offer adequate surface area and the possibility of functionalization and paramagnetic properties. Their application as dispersed sorbents in liquid

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samples is called magnetic solid phase extraction (MSPE) [20]. This technique has demonstrated several advantages such as the decrease in sample treatment time, the decrease in solvent use, and the easy treatment of high volume samples [21-22]. The basic procedure involved in MSPE is represented in Figure 2. Initially, the pre-activated magnetic solid is added to the sample (Fig. 2.a.), the solid is dispersed in the sample and the analytes are retained on the support (Fig. 2.b). Once the extraction is complete, a magnet is placed on the external wall of the sample container to isolate of the solid containing the analytes (Fig. 2.c). Finally, the analytes are eluted from the solid using an appropriate organic solvent (Fig. 2.d).

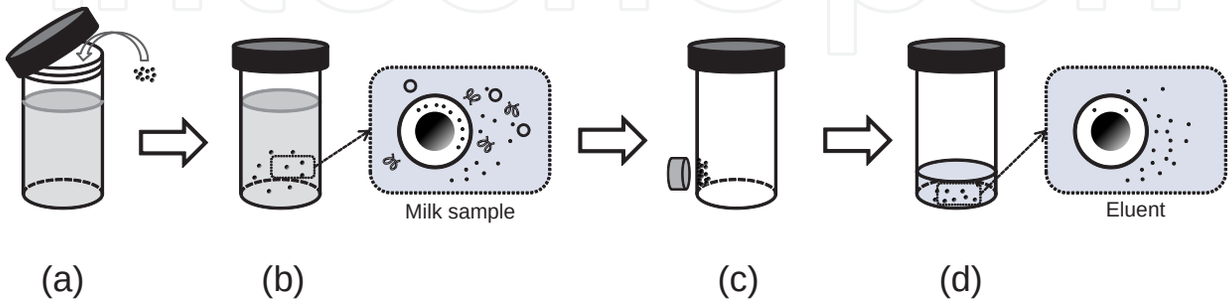

Figure 2. Representation of the magnetic solid phase extraction methodology

MSPE can be coupled with different analytical techniques including gas and liquid chromatography and CE. MSPE-CE has been applied for the selective separation of tetracyclines and fluoroquinolones from milk samples. The possibility of modification has allowed the synthesis of magnetic solid modified with phenyl and phenyl-octyl in order to improve the selectivity during the sample treatment through mixed interaction modes [22-23].

One of the limits of detection obtained with CE-UV is that it requires the use of pre-concentration strategies. On-line concentration by large volume sample stacking has been successfully applied. Taking into account that MSPE allows the use of high volume samples (until 1 liter) during the extraction process, simultaneously with the use of dispersive extraction mode, we propose the analysis of PENs by MSPE-CE-UV.

2. Experimental conditions

2.1. Reagents and dissolutions

All solutions were prepared in deionized water with specific resistivity greater than 18 MΩ cm. PENs solutions were prepared daily by dilution of a 1.0 g l^{-1} standard solution of AMO, AMP, and Pen G (all reagents are analytical grade, Sigma, St. Louis, MO, USA). The background electrolyte or buffer solution used for analysis by MEKC consisted of a mixture of: 20 mM sodium tetraborate and 50 mM sodium dodecyl sulfate (SDS) at pH 8.5. The internal standard used was acid (S)-6-methoxy-α-methyl-2-naphthaleneacetic, which was dissolved in

the background electrolyte at a concentration of 5 mg l^{-1}. All the diluted solutions were stored at 4°C for a period no more than seven days.

Milk samples analyzed included 5 of pasteurized milk, 5 of ultra-pasteurized milk and 3 without heat treatment. All the samples were acquired in markets in Pachuca, Hgo, Mexico.

2.2. Synthesis of magnetic support

Magnetite particles (Fe_3O_4) were synthesized as follows: 12 g of $FeSO_4.7H_2O$ was dissolved in 100 ml of water. The mixture was heated with stirring until it reached 60°C. The pH value was adjusted to 10 while airflow was bubbled during the reaction. After 1 hour, the magnetic precipitate was isolated from the solution with a magnet and it was washed with 50 ml portions of deionized water several times. The magnetite obtained previously was added to a flask containing 5.910 g of octyltriethoxysilane (C8-TEOS) and 1.182 g of tetramethoxysilane (TMOS), dissolved in 24.0 mL of a solution which contained: Triton X-100 (2.0%, w/v), cetyltrimethylammonium, methanol (12.5%, v/v), and 200.0 μl of catalyst (NH_3 28 %, v/v). The mixture was heated and refluxed at 120°C for 12 hours with stirring. The obtained magnetic particle was dried at 60°C for 120 hours [21].

2.3. Characterization of the magnetic solid

The structures of the magnetic solid were examined by X-ray diffraction (XRD) patterns using a PHILIPS PW1710 instrument equipped with a Cu anode, automatic divergence slit, and a graphite monochrometer under the following experimental conditions: CuKα radiation, 1.54 Å; generator tension, 40 kV; generator current, 30 mA; intensity ratio (α2/α1), 0.500; divergence slit, 1°; receiving slit, 0.1; start angle (2θ), 5; end (°2θ), 70. The morphological analysis of the products was performed using a JEOL JSM-820 scanning electron microscope (SEM).

2.4. Sample treatment

The MSPE consisted in the activation of 0.1 g of the magnetic support with 4.0 ml of methanol for 5.0 min in an ultrasonic bath; the solid was isolated magnetically and washed with 5.0 ml of deionized water for 4 min, then the milk sample was added (50,100 and 200 ml), dispersing the magnetic solid for 8.0 min. Once the dispersion was concluded, the magnetic support was magnetically isolated and the milk was decanted and the solid was washed with 20 ml of phosphate buffer solution (pH 7.0, 0.1 M). Finally, the adsorbed analytes were eluted with 5.0 ml of acetonitrile for 4 min and the organic phase was evaporated under an air steam until dried. The residue was reconstituted in the background electrolyte containing the internal standard at 5 mg l^{-1} concentration.

2.5. Procedure of separation by MEKC

Capillary electrophoresis experiments were performed on a Beckman Coulter P/ACE 5500, with a diode array detector. The data obtained was analyzed by the P/ACE MDQ software version 2.3. Separations of the PENs were performed in a fused-silica capillary (57 cm× 50 μm I.D.). The detection wavelength was set a 200 nm and the temperature capillary was thermo-

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stated at 30°C during separation. The separation voltage was set at 16 kV. The sample was introduced using a pressure of 0.5 psi.

Initially, the capillary was rinsed with 1.0 M NaOH (20 min), followed by 0.1 M NaOH (20 min), deionized water (15 min), and finally with the background electrolyte (20 min). Each separation in samples was preceded by a rinse with 1.0 M NaOH (2 min), 0.1 M NaOH (2 min), followed by H_2O (5 min), and background electrolyte (5 min).

3. Results and discussion

3.1. Optimization of background electrolyte composition

PENs can be separated in different modes including capillary zone electrophoresis, micellar electrokinetic chromatography, and capillary electrochromatography. Different background electrolytes have been described for PENs analysis in different matrices. The pH value used is in the interval from 3.6 to 9.2 using phosphate or borate and sodium dodecyl sulphate salts [24-28].

Figure 3 shows the predominance zone diagrams for the studied PENs using the pKa values reported for the analytes. The zone marked in the Figure denotes the pH values used for separation of PENs. It can be seen that neutral and anionic forms are presented, then MEKC (using SDS) is the most suitable mode for the separation of PENs.

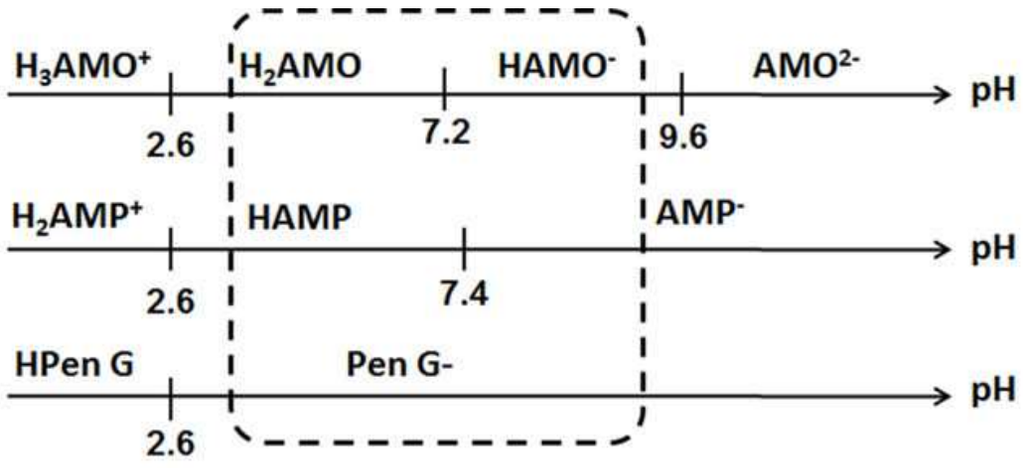

Figure 3. Predominance zone diagrams for PENs

Different factors affecting the separation with the background electrolyte required evaluation. A Taguchi parameter design (TPD) was selected as the optimal method since this provides the necessary information with the minimal experimentation. TPD discriminates between control factors, treating them separately by a means of special design matrices (orthogonal arrays) in which the columns (corresponding to factors) and rows (corresponding to trials) are arranged in a conveniently fixed manner. These matrices indicate the combination of factor settings in

each experiment and allows for the simultaneous evaluation of several variables with the minimum number of trials. The results obtained were analyzed statistically to adjust each variable to its optimum [29].

Optimization of the system with TPD involves 5 steps, I) identifying the output variable to optimize, II) identifying and selecting factors that affect the system, III) selecting the appropriate orthogonal array and assigning adequate settings to the chosen factors, IV) analyzing the data and determining the optimum settings, and V) conducting a confirmatory experiment under the optimal conditions obtained [30].

In CE techniques, the effect of the background composition on the separation can be evaluated using the sum of the electrophoretic mobilities (μ_{ion}) of the ions evaluated according the following relation:

$$\mu_{ion}=\frac{L_t L_d}{V}\left[\frac{1}{t_{ion}}-\frac{1}{t_{eof}}\right]$$

Where L_t is the total capillary length (0.57 m), Ld is the capillary length from the injection inlet to the detector (0.32 m), V is the applied voltage (16 kV), and t_{ion} and t_{eof} are the migration times (s) of the analyte and the electroosmotic flow, respectively [14].

The composition (concentration of borate and SDS) and pH value are the variables involved. The selected orthogonal array must have a number of columns equal to or higher than the number of degrees of freedom of the system; thus an $L_9(3^3)$ array was used. The three settings selected for each factor were chosen using MEKC electrolytes reported during PENs analyses. The concentrations of borate and SDS affect the conductivity and the micelle formation. With respect to the pH, the value influences the formation of anionic forms of the analytes. Table 2 shows the settings for each control and noise factor used in the optimization experiments.

Factors	Level		
	1	**2**	**3**
$[BO_3^-]$ (mM)	10	20	30
[SDS] (mM)	50	60	70
pH	7.5	8.0	8.5

Table 2. Control factor settings for the optimization experiment

Table 3 shows the factorial design matrix and the results obtained for each trial. All experiments were performed in duplicate in order to calculate the residual error; the total number of experiments was therefore 9 experiments x 2 replicates. Measurements were performed with solutions containing 50.0 mg l^{-1} of each PEN.

Exp	Control factors and levels			$\lvert \Sigma\mu_{ion} \rvert$ *
	$[BO_3^-]$	[SDS]	pH	(10^{-8} $m^2V^{-1}s^{-1}$)
1	1	1	1	3.21
2	1	2	2	3.31
3	1	3	3	3.48
4	2	1	2	4.65
5	2	2	3	3.88
6	2	3	1	4.41
7	3	1	3	4.71
8	3	2	1	4.08
9	3	3	2	2.58

* mean values (n=2)

Table 3. $L_9(3^3)$ orthogonal array and sum of ion mobilities (each value is the mean of two readings).

The results were analyzed statistically to adjust each variable to its optimum with the least variability possible. All calculations were made using ANOVA-TM v2.5 software. Table 4 shows the results for these analyses. The values of the variance ratio (F) and the critical variance ratio (3.98 for a 95% confidence level) show that all the factors taken into account were critical ($F_{calculated} > F_{critical}$). The factor with the greatest influence on the response was the borate concentration, which accounted for 55.0% of the total variance of the results, followed by SDS concentration (28.8%). The contribution of the residual error was 0.1%; this indicates the correct selection of experimental parameters.

Figure 4 shows the effects of the control factors on the output variable (sum of ion mobilities), among which the sample volume is the most important. The combination of settings that allowed the highest output peak was $[BO_3^-]$ 20 mM, [SDS] 50 mM and pH 8.5.

Variance source	Degree of freedom	Sum of squares	Variance	Variance ratio (F)[a]	Influence (%)[b]
$[BO_3^-]$	2	102.77	51.39	2569.50	55.0
[SDS]	2	53.68	26.84	1344.50	28.8
pH	2	30.22	15.11	755.50	16.1
Residual	11	0.22	0.02	1.00	0.1
Total	17	186.69			100.0

[a] The critical variance ratio for a 95% confidence level is 3.98 (2,11 d.f.)

[b] Contribution is defined as 100 x (sum of squares/total sum of squares)

Table 4. ANOVA for sum of ion mobilities in Table 3.

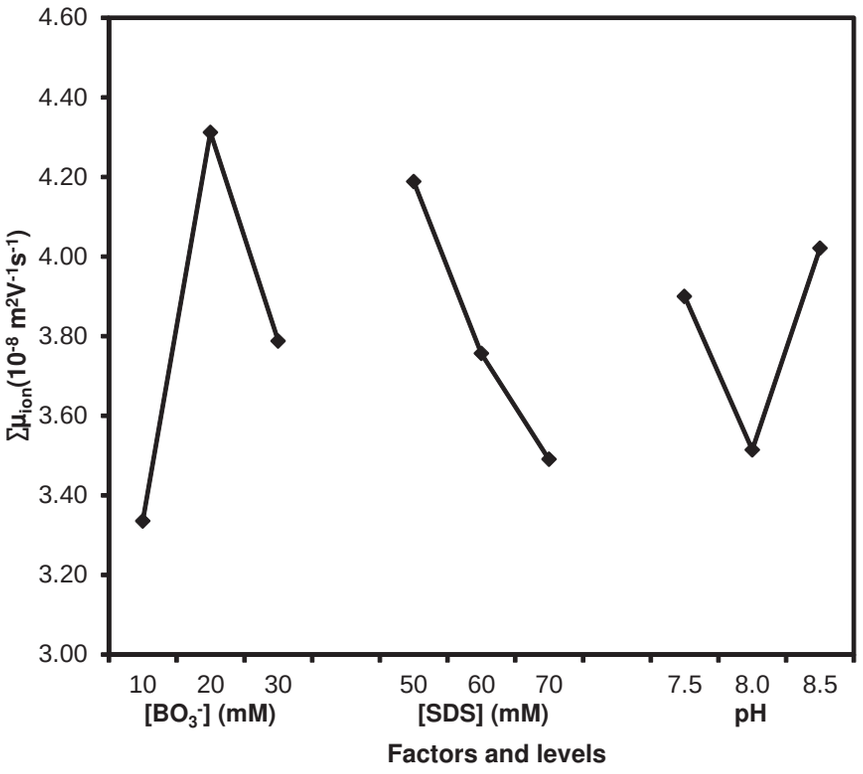

Figure 4. Effect of control factors on the sum of ion mobilities.

3.2. Characterization of magnetic solid

The results of analysis by the XRD are displayed in Fig. 5.a; the diffractogram obtained shows diffraction lines characteristic of magnetite (2θ=30.1°, 35.5°, 43.1°, 53.4°, 57.0° and 62.6°) expressed in the Figure as "m". The wide band observed at 2θ angle from 10° to 25° is consistent with the amorphous phase of silica gel [31]. The micrograph obtained for the magnetic support (Fig. 5.b) shows a spherical morphology for the solid with a particle size around to 1-2 μm.

Figure 6 shows FTIR spectra for the magnetic supports. Magnetite has an inverse spinal type structure with a characteristic vibration M_T-O-M_O at ≈ 600-550 cm^{-1}, where M_T and M_O correspond to the metal occupying tetrahedral and octahedral positions, respectively. On the other hand, the spectra showed a band of intense stretching at ≈3450 cm^{-1}, attributable to the vibration of the silanol group (SiOH). The peaks below 3000 cm^{-1} corresponds to the vibration of-CH_2-. The bending band at ~1630 cm^{-1} corresponds to H_2O occluded in the support. The flexion vibration band at ~1400 cm^{-1} represents the C-H bond of the alkyl groups joined to the silicon. The stretching band at 1100-1000 cm^{-1} is assigned to the Si-O-Si bond and the deformation band at ~888 cm^{-1} belongs to Si-OH bonds [32].

The synthesis protocol used to obtain the magnetic solid is based on the hydrolysis of alkoxysilanes. This process produces solids with silanol (Si-OH) and octyl groups (Si-C_8H_{17}), modifying the retention mechanism [23,33].

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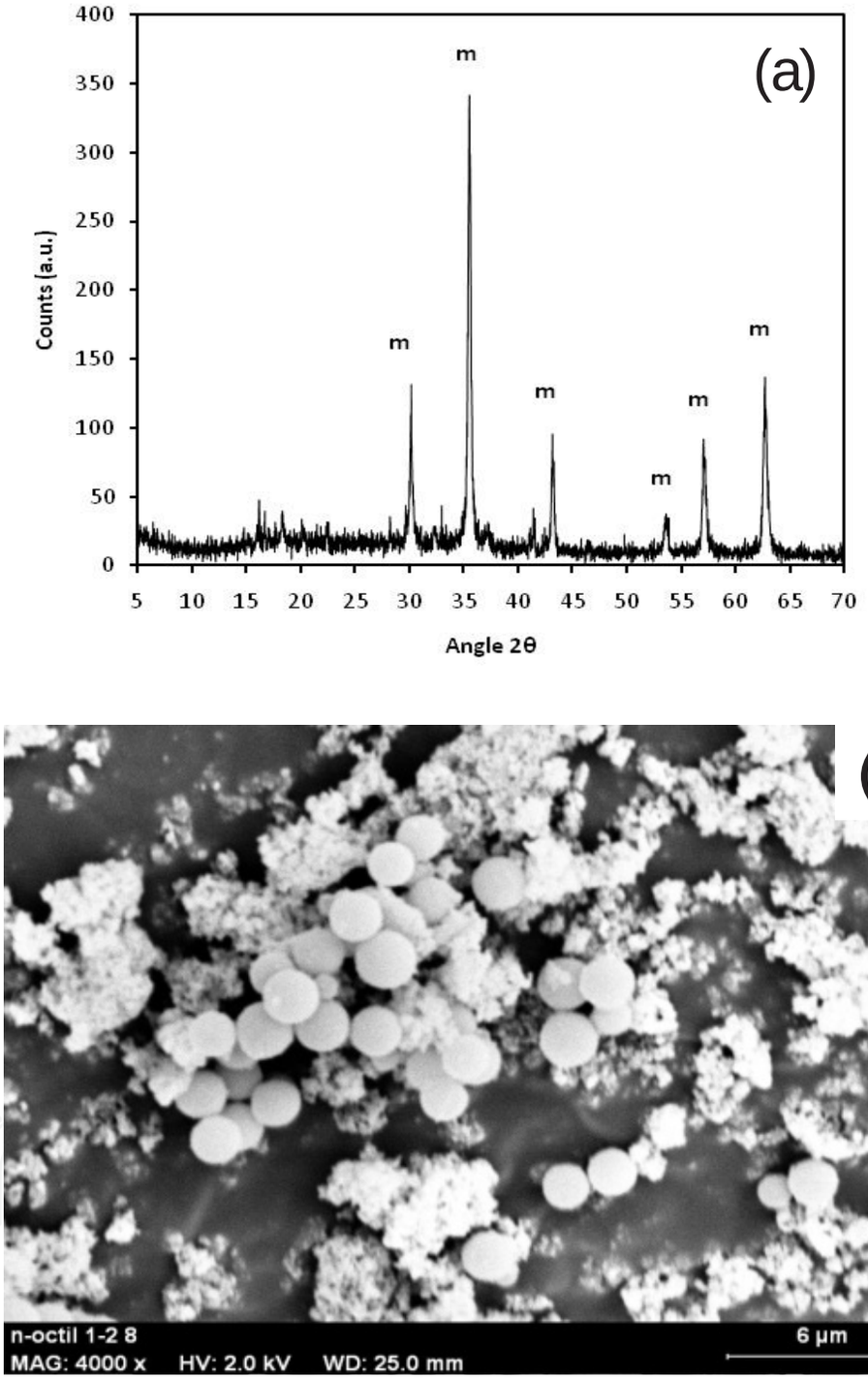

Figure 5. a) Diffractogram of the diffraction lines of magnetite of C_8-TEOS; b) Scanning electron micrograph of the magnetic C_8-TEOS

Because they have a higher affinity for the polar part of the solid, proteins interact with the silanol groups, releasing penicillins, which then are partially retained by the MSPE octyl group.

Thus, the presence of both groups promotes a mix mode retention mechanism. In consequence a single MSPE process can be performed without a protein precipitation and multiple SPE procedures [34].

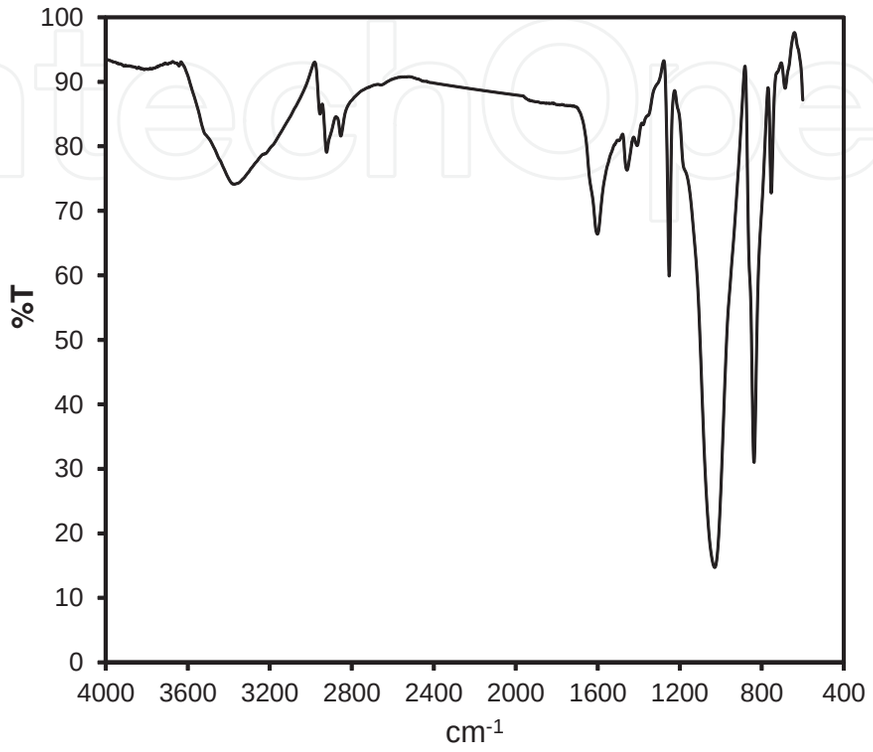

Figure 6. FTIR spectra for magnetic solid (C8) used for MSPE experiments

3.3. Analysis of PENs by MEKC-CE

Quantification of PENs at maximum residual limits involves clean-up and pre-concentration steps. In order to achieve the limits of detection (LOD) required by the international norms, the effect of initial sample volume used on the MSPE methodology was evaluated in the range from 25 to 200 ml. Table 5 shows the LOD obtained; the values decrease with increasing volume. Since a volume of 200 mL yields adequate LODs, it was chosen as the optimal original sample volume.

Sample volume initial (ml)	Limits of detection obtained (μg l^{-1})
25	100.0-120.0
100	24.0-30.0
200	0.20-3.0

Table 5. Limits of detection (μg l^{-1}) reached at different initial sample volumes.

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Once the appropriate initial sample volume for analysis is established, a series of penicillin standard solutions with concentrations ranging from 10-70 μg l^{-1} were analyzed. Table 6 shows the evaluation of analytical performance of the MEKC methodology. Precision of the method (reproducibility and repeatability) was expressed as relative standard deviation (%RSD) calculated from three assays using a concentration of 10 μg l^{-1} penicillin. A maximum %RSD value of 20% is required during ultratrace analysis, thus the PENs determination with the proposed method is precise [1,35].

Parameter	AMO	AMP	PEN G
Slope confidence interval $b_1 \pm t\ s(b_1)$	0.267±0.012	0.342±0.007	0.709±0.001
Intercept confidence interval $b_0 \pm t\ s(b_0)$	0.027±0.002	0.023±0.001	0.032±0.002
Correlation coefficient, r^2	0.999	0.999	0.999
Limit of detection (μg l^{-1})	2.7	1.7	0.2
Limit of quantification (μg l^{-1})	8.1	5.1	0.6
Linear range (μg l^{-1})	2.7-60	1.7-60	0.2-60
Repeatability (%RSD, 10 μg l^{-1}, n=3)	6.8	1.2	5.1
Reproducibility (%RSD, 10 μg l^{-1},n=3)	13.0	9.9	11.6

Table 6. Analytical parameters obtained of analysis of penicillins in milk.

The accuracy was evaluated by recovery assays through spiked milk samples with the penicillins studied in a range of 10-30 μg l^{-1}. Table 7 shows the recovery percentages obtained from the assay. The results generated by the proposed methodology are accurate according to the determined recovery percentages. The recovery must be in the range of 80-120% during ultratrace analysis to be considered an acceptable parameter [1].

Concentration (μg kg^{-1})	% Recovery		
	AMO	AMP	PEN G
10	87	97	106
20	97	80	83

Table 7. Recovery values obtained of analysis of spiked milk samples.

The developed method was applied to the determination of penicillins in 15 commercial milk samples. Three replicate determinations were carried out on each sample. Overall, two samples of ultrapasteurized milk were found positive for the presence of residues of penicillins, one with AMP and other with AMO. The concentrations found were 35±1 and 34±1 μg l^{-1} for AMP

and AMO, respectively. The concentration found is higher than the MRLs. Figure 7 shows the electropherograms obtained during the analysis of the spiked blank sample (Fig. 7a), commercial milk sample positive to ampicillin (Fig. 7b), and the blank sample (Fig. 7c). These results are congruent with the antibiotic resistance found for *Escherichia coli* isolated from Food Stuffs in Hidalgo [36].

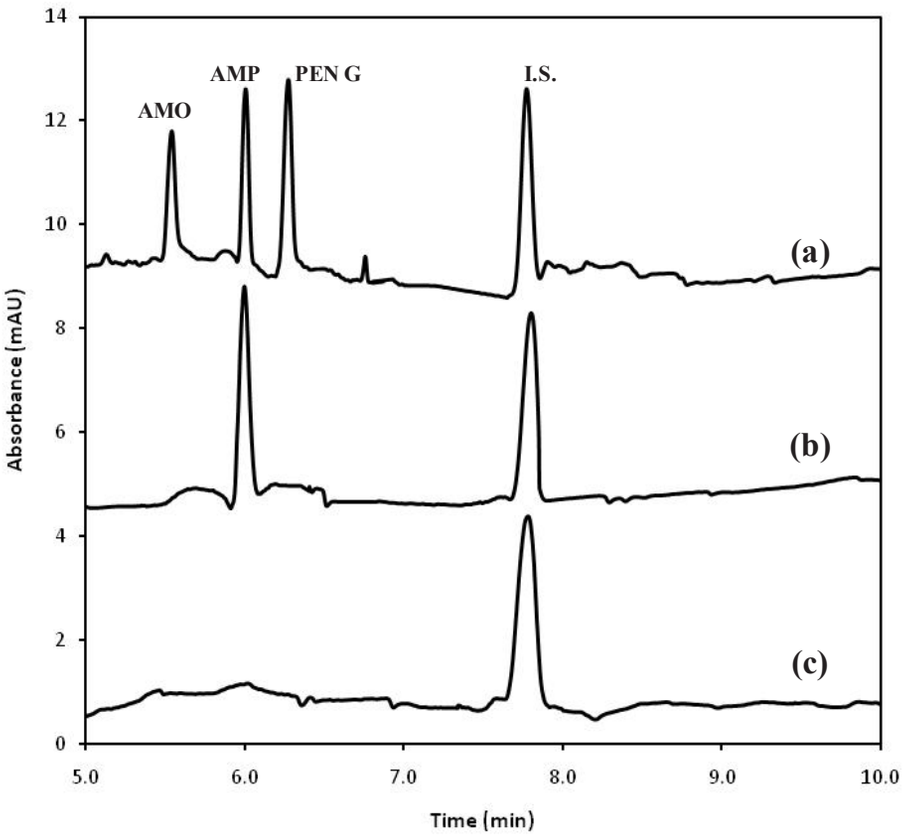

Figure 7. Electropherograms obtained at the optimized conditions in the analysis of: a) PENs spiked blank sample, b) commercial milk sample analyzed by MSPE and c) milk blank sample

The mixed mechanism offered by the solid supports contributes to a better clean-up of the sample, as can be seen in the electropherogram. Although the re-usability of the solid extracting phase is not possible due to the adsorption of other components of the sample, the proposed sample treatment has advantages such the use of fewer organic solvents and the time required for sample treatment is faster than SPE methodologies described.

4. Conclusions

In this study, an octyl-silica based magnetic support was synthesized and successfully applied for the separation of penicillin antibiotics from milk samples. The penicillins were isolated and the matrix interferences were eliminated using this support. The extraction technique is a

robust preconcentration technique that can be coupled successfully with MEKC. The methodology described (MSPE-MEKC) is faster than classical sample preparation procedures such as solid phase extraction, with a minimum sample handling, less solvent consumption, and is a promising methodology for routine milk sample analysis. Moreover, the linear range covers the MRL for PEN and the recovery studies are acceptable regarding to the range for ultratrace analysis.

In the analysis of commercial products, some samples contain residues of penicillins at higher levels than the MRLs. These results are consistent with the antibiotic resistance found for *Escherichia coli isolated* from Food Stuffs in Hidalgo, which shows the congruence between MSPE-MEKC and other microbiological studies.

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