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# Magnetic solid phase extraction based on phenyl silica adsorbent for the determination of tetracyclines in milk samples by capillary electrophoresis

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ABSTRACT

A magnetic solid phase extraction method coupled to capillary electrophoresis is proposed for the determination of tetracycline, oxytetracycline, chlortetracycline and doxycycline in milk samples. Five different magnetic phenyl silica adsorbents covered with magnetite were synthesized by varying the molar ratio of phenyltrimethylsilane and tetramethylorthosilicate; these adsorbents were evaluated in terms of their pH and degree of hydrophobicity for tetracycline retention. The optimal, selected combination of conditions was a pH of 10.0 and a magnetic sorbent ratio of 4:1; under these conditions, the retention capacity ranged from 99.7% to 101.2% for the four tetracyclines analyzed. The elution conditions and initial sample volume of the proposed extraction method were also optimized, and the best results were obtained with $1 \times 10^{-3}$ M acetic acid in methanol as eluent and a 200 ml of sample volume. Under optimal conditions, average recoveries ranged from 94.2% to 99.8% and the limits of detection ranged from 2 to 9 $\mu g\,l^{-1}$ for the four tetracyclines. After the proposed method was optimized and validated, 25 milk samples of different brands were analyzed, oxytetracycline residues were detected in five samples, in concentrations ranging from 98 to 213 $\mu g\,l^{-1}$. Subsequent analysis of positive samples by SPE–CE and magnetic solid phase extraction–HPLC revealed than no significant differences were found from results obtained by the proposed methodology. Thus, the developed magnetic extraction is a robust pre-concentration technique that can be coupled to other analytical methods for the quantitative determination of tetracyclines.



## 1. Introduction

Antimicrobials are used therapeutically in animals and humans to control bacterial infections. Tetracyclines (TCs, Fig. 1) are a family of compounds frequently employed due to their broad spectrum of activity as well as their low cost, compared with other available antibiotics. Currently, there are over 20 tetracyclines available; however, tetracycline (TC), chlortetracycline (CT), oxytetracycline (OT) and doxycycline (DT) are the most commonly used TCs in veterinary medicine [1,2]. In addition to therapeutic purposes, in Mexico and many other countries, TCs are often incorporated into livestock feed at sub-therapeutic doses as growth promoters. This practice is believed to enhance bacterial resistance, allergic reactions, liver damage and gastrointestinal disturbance [3]. In dairy cows, significant percentages of the administered TCs are excreted by milk. To protect human health from exposure to these drug residues in milk, the EU has established a maximum residue limit (MRL) of 100 $\mu g\,kg^{-1}$ for TC, OT and CT [4], while in the USA, the food and drug administration (FDA) has established a MRL of 300 $\mu g\,kg^{-1}$ for the combined residues of TC, OT and CT [5]. Doxycicline is not licensed in the aforementioned countries for use in animals that produce milk for human consumption, so no MRL has been set for this drug. In other countries whose regulatory agencies have not established MRLs, limits are usually adopted from the Codex Alimentarius [6], which has recommended a limit of 199 $\mu g\,kg^{-1}$ in milk for either the sum of the combined TC, OT and CT residues or each individual tetracycline.

During recent years, the problems caused by antimicrobial residues in food have stimulated the development of analytical methodologies for the determination of TC residues at $\mu g\,kg^{-1}$ or $\mu g\,l^{-1}$ levels, such as microbiological assays [7], spectrophotometry [8,9], chemiluminescence [10–12], high performance liquid chromatography (HPLC) [13–15] and capillary electrophoresis (CE) [16,17]. These techniques can be used individually or sequentially according to the complexity of the samples, the nature of the matrix and the target analytes. Sample preparation, including isolation, clean-up and preconcentration, is usually the bottle-neck for these analytical procedures.

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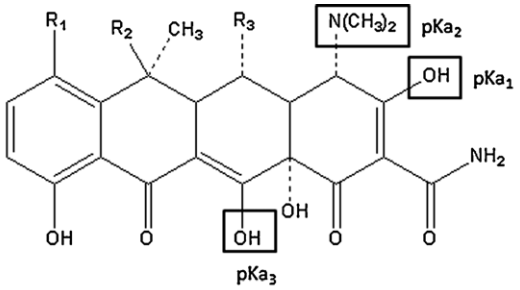


| Common denomination | Symbol | R Groups | | | pKa | | |
|---|---|---|---|---|---|---|---|
| | | R1 | R2 | R3 | $pKa_1$ | $pKa_2$ | $pKa_3$ |
| Tetracycline (TC) | TC | -H | -OH | -H | 3.2 | 7.5 | 8.9 |
| Oxytetracycline (OT) | OT | -H | -OH | -OH | 3.3 | 7.8 | 9.6 |
| Doxycycline (DT) | DT | -H | -H | -OH | 3.0 | 8.0 | 9.2 |
| Chlortetracycline (CT) | CT | -Cl | -OH | -H | 3.3 | 7.6 | 9.3 |

**Fig. 1.** Chemical structures for the tetracyclines evaluated in this study.

Protein precipitation followed by solid phase extraction (SPE) with polymer or silica sorbents is the strategy most commonly applied for milk analysis [13]. However, SPE procedure is expensive and time-consuming, and some factors, such as the vacuum and the cartridges dry out, are difficult to address [13,18,19]. A major concern in the analysis of TCs is the tendency of the compounds to bind irreversibly to silanol groups contained on silica-based materials ($C_8$, $C_{18}$) by interacting with the TC ketone groups or forming chelate complexes with metal ions, which results in low recovery yields during sample pre-treatment [19].

In contrast, magnetic solid phase extraction (MSPE) has received considerable attention in recent years due to its potential applications in cell isolation, enzyme immobilization, protein separation and pre-concentration of organic compounds from large volume samples [20]. A unique and attractive property of this technology is that magnetic adsorbents can be isolated from sample solutions by the application of an external magnetic field. Thus, suspended magnetic particles tagged with the analytes can be removed from large volume samples by a magnet [21].

In this work, a new method for the analysis of TCs in milk samples at EU and USA MRLs is proposed. This method involves sample pre-treatment by magnetic solid phase extraction using a paramagnetic, phenyl-functionalized silica adsorbent and analytical determination by capillary zone electrophoresis (CE) with photo diode array detection. The advantages of the developed method and its application for the determination of TCs residues in milk samples are also discussed.

## 2. Experimental

### 2.1. Reagents and chemicals

All solutions were prepared by dissolving the respective analytical grade reagent in deionized water with a resistivity not less than 18.0 MΩ cm, which was provided by a Milli-Q system (Millipore, Bedford, MA, USA). Sodium phosphate was obtained from Sigma (St. Louis, MO, USA). EDTA sodium salt, sodium hydroxide and hydrochloric acid were obtained from J.T. Baker (Phillipsburg, NJ, USA). Methanol was obtained from Mallinckrodt Baker (Xalostoc, Mexico), and 2-propanol was obtained from Fluka (St. Gallen, Switzerland). The electrolyte solution consisted of 30 mM sodium phosphate, 2 mM EDTA disodium salt and 2% 2-

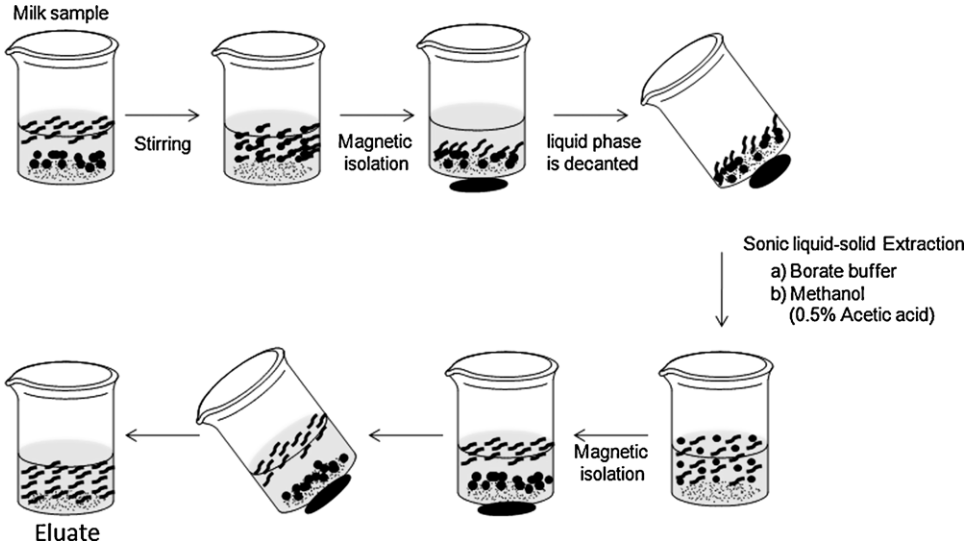


**Fig. 2.** Schematic procedure for the isolation of TCs from milk samples using MSPE.

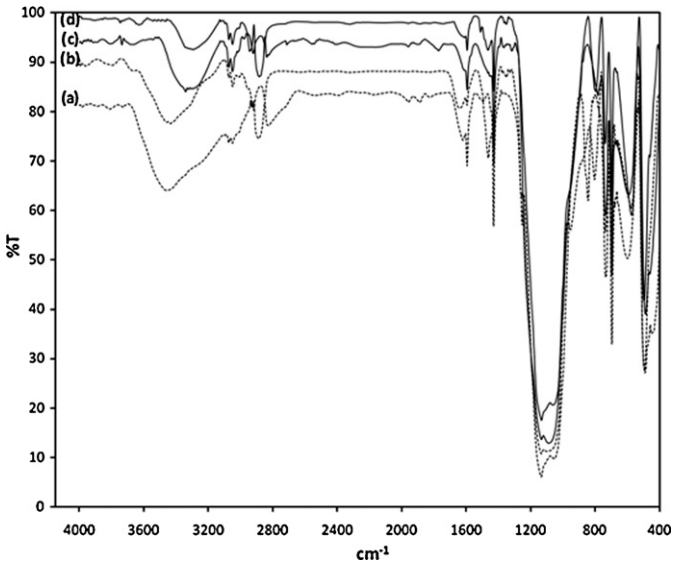


**Fig. 3.** FTIR spectra for magnetic adsorbents at different PTMS:TMOS molar ratios: (a) 0.5:1.0, (b) 1.0:1.0, (c) 2.0:1.0, and (d) 4.0:1.0.

propanol; the solution pH was adjusted to 12.0 with 0.1 M sodium hydroxide.

Tetracycline hydrochloride (95%), chlortetracycline hydrochloride (95%), oxytetracycline dihydrate (95%), doxycyline hyclate (98%) and picric acid (98%) were obtained from Sigma. The different standard solutions were prepared daily by dilution of a stock solution of each TC at a concentration of 1.0 g $l^{-1}$, which were prepared by dissolving the pure substances in 0.01 M hydrochloric acid. These solutions were stored in the dark and refrigerated at 4 °C. Picric acid at a concentration of 50 mg $l^{-1}$ was used as internal standard. Solid phase extraction was applied using Sep-Pack Vac C18 cartridges (Waters, Milford, MA, USA). Magnetite embedded in phenyl silica adsorbents was synthesized by the sol–gel method using magnetite, phenyltrimethoxysilane (97%, PTMS) and tetramethylorthosilicate (98%, TMOS) from Sigma. Emulsion polymerization of the monomers was performed using Triton X-100 and cetyltrimethyl ammonium bromide (CTAB) as surfactants.

### 2.2. Synthesis of the adsorbent

The synthesis of the adsorbents was carried out in two steps. First, magnetite was obtained through partial oxidation and precipitation of Fe(II) in the presence of oxygen in basic media [22]. The magnetic adsorbents were obtained by emulsion polymerization by mixing the previously synthesized magnetite, PTMS and TMOS at different molar ratios. The silica precursors were previously solubilized in 24 ml of a solution containing 2.0% (w/v) Triton X-100, 0.02% (w/v) CTAB, 12.5% (v/v) methanol and 200 μl, 28% (w/v) $NH_3$ as the catalyst. The mixture was heated and refluxed at 120 °C for 16 h with stirring [21].

Once the magnetic adsorbents were obtained, derivatization was conducted to block superficial silanol groups (–Si–OH). A mixture of 0.9 g of chlorotrimethylsilane (CTMS) and 1.0 ml of pyridine per gram of magnetic adsorbent in 25 ml of toluene was used. Pyridine was added to neutralize the HCl produced during the derivatization. The magnetic adsorbents were sequentially washed with 20.0 ml portions toluene, ethanol and deionized water, until the washing solvent was colorless. The magnetic adsorbent was dried at 60 °C for 24 h [21].

### 2.3. Apparatus

Infrared characterization of the magnetic adsorbents was performed in a PerkinElmer Fourier Transform infrared (FTIR) spectrophotometer model IRDM. The samples were analyzed as KBr sample pellets. The particle size distribution was determined using a Beckman Coulter LS 13320 light scattering (LS) particle size distribution analyzer with a diode laser at a wavelength of 750 nm. A mass of 0.1 g of the adsorbent was initially dispersed in 5 ml of methanol; the organic suspension was diluted to 500.0 ml with water to disperse the solid particles in the aqueous matrix. The morphological analysis of the magnetic adsorbents was performed using a JEOL JSM-820 scanning electron microscope (SEM).

Electrophoresis was performed using a Beckman Coulter P/ACE 5500 (Fullerton, CA, USA) with a photo diode array detector. Data were collected and analyzed with Beckman P/ACE system MDQ version 2.3 software. Separations of the TCs were performed in a fused-silica capillary (41.7 cm × 75 μm I.D.). A pH/ion analyzer model 450 from Corning (Corning Science Products, NY, USA) was used to accurately adjust the pH of the electrolyte solution to 0.01 pH unit. A Branson Ultrasonic system (Danbury, CT, USA) model 3510 was used in the dispersion of magnetic adsorbent and elution of the analytes. Finally, a Maxi-Mix I (Barnstead/Thermolyne, IA, USA) was used as a vortex mixer. Chromatography determinations were performed using a PerkinElmer Series 200 liquid chromatography system (PerkinElemer MA, USA) with a UV–vis detector at 360 nm and a manual injector equipped with a 20 μl loop, with a Spheri-5 ODS column (5 μm; 240 × 4.6 mm) from PerkinElmer. The mobile phase employed was 0.01 mol $l^{-1}$ aqueous oxalic acid: methanol: acetonitrile (64:18:18, v/v) at a flow rate of 1.0 ml $min^{-1}$ [1].

### 2.4. Sample treatment

MSPE was performed as follows [23]: Initially, 0.1 g of the magnetic adsorbent material was added into a beaker. The particles were conditioned with 5.0 ml of methanol in an ultrasonic bath for 5 min. Then, the adsorbent was magnetically isolated and washed twice with 10.0 ml of deionized water for 3 min, and the supernatant was discarded. Subsequently, an adequate volume (50–200.0 ml) of milk sample and 12.0 ml of borate buffer (0.1 M, pH 10.0) were mixed with the pre-activated magnetic adsorbents. After sonication for 15 min, an external magnetic field was applied to isolate the adsorbent with the adsorbed analytes. The liquid phase was decanted, while the solid phase was washed three times with 5.0 ml of borate buffer. The TCs were eluted from the magnetic adsorbent by dispersion of the solid in 3.0 ml of methanol solution containing 0.5% (v/v) acetic acid for 5 min. The resulting solution was evaporated to dryness, and the residue was reconstituted with 1.0 ml of 0.01 M HCl/50 mg $l^{-1}$ picric acid. Finally, the solution was filtered through a 0.2 μm nylon filter and analyzed by CE. The procedure is illustrated in Fig. 2.

Solid phase extraction conditions with C18 cartridges were carried out as follows: 10 ml of milk sample were mixed with 30 ml of the McIlvine/EDTA solution; the mixture was stirred for 1 min and centrifuged for 15 min (4000 rpm). The precipitate was disposed of and the solution was passed through a C18 SPE cartridge previously activated with 2.0 ml of methanol followed by 2.0 ml of the McIlvane/EDTA solution at a maximum flow rate of 5.0 ml/min. After preconcentration of the analytes the cartridge was washed with 2.0 ml of 5.0% methanol. The retained TCs were eluted with 3.0 ml of methanol. The eluted solution was evaporated to dryness and redissolved in 1 ml of 0.01 M HCl/50 mg $l^{-1}$ picric acid [1].

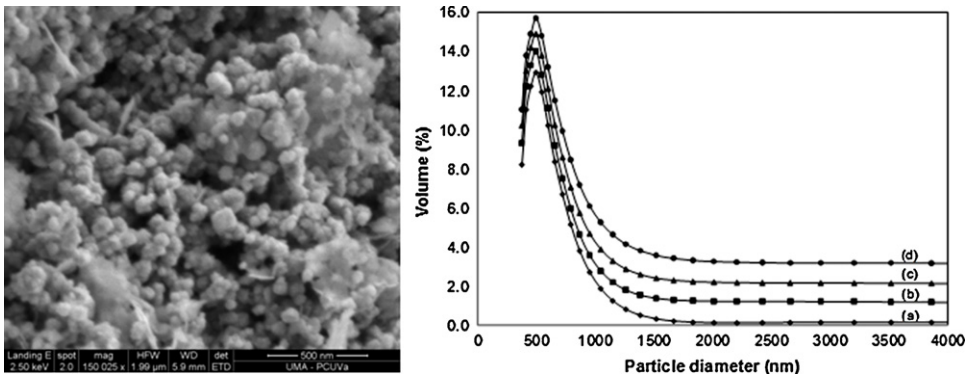


**Fig. 4.** (A) Scanning electron microscopy image and (B) particle size distribution of phenyl magnetic adsorbents at different PTMS:TMOS molar ratios: (a) 0.5:1.0, (b) 1.0:1.0, (c) 2.0:1.0, and (d) 4.0:1.0.

### 2.5. Electrophoretic procedure

At the beginning of each working day, the capillary was activated with 1.0 M NaOH at 35 °C for 15 min, followed by 0.1 M NaOH for 10 min, deionized water at 25 °C for 10 min, and then electrolyte solution at 25 °C for 10 min. The capillary was washed out between successive analyses using: 1.0 M NaOH for 1 min, 0.1 M NaOH for 2 min, deionized water for 2 min and electrolyte solution for 2 min. All flushing procedures were performed at a pressure of 20 psi.

The wavelength detector (λ) was set at 360 nm to monitor TCs separations. Samples were injected in hydrodynamic mode under a pressure of 0.5 psi for 5 s. The capillary was kept at 25 °C, and a voltage of 14 kV was applied to separate the analytes. The different peaks were identified by migration times and co-injection of standard solutions [24].

## 3. Results and discussion

### 3.1. Characterization of magnetic adsorbents

To evaluate the effect of hydrophobicity on the retention of TCs, five different magnetic adsorbents were synthesized and characterized. The magnetic adsorbents were obtained by varying the molar ratio of functionalized monomer (PTMS) and crosslinking monomer (TMOS) in the following ratios: 0.5:1.0, 1.0:1.0, 2.0:1.0, 4.0:1.0 and 5.0:1.0 (PTMS:TMOS). Solids with high surface area were obtained in the four former experiments while in the last ratio (5.0:1.0) a viscous gel phase with small surface area was obtained. Therefore, magnetic adsorbents with ratios below 4.0:1.0 were evaluated for TCs retention by MSPE. Fig. 3 shows the FTIR spectra for the four synthesized magnetic adsorbents. The spectra show an intense stretching band at 3600–3250 $cm^{-1}$ attributed to the vibration of the silanol group (Si–OH). Two bands above 3100 $cm^{-1}$ correspond to the =C–H vibration of the phenyl group, and two bands around 2900 $cm^{-1}$ are assigned to the vibration of the C–H bond. A series of weak bands from 1900 to 1600 $cm^{-1}$ are also characteristic of C–H vibrations from aromatic groups. A bending band at 1450 $cm^{-1}$ is attributed to the $H_2O$ contained in the magnetic adsorbent. A stretching band at 1250–950 $cm^{-1}$ is assigned to the siloxane group (Si–O–Si), and the deformation band around 850–750 $cm^{-1}$ corresponds to the Si–OH group.

The FTIR spectra (Fig. 3) show that the stretching band at 3600–3250 $cm^{-1}$ corresponding to the Si–OH bond decreases as the PTMS:TMOS ratio increases, suggesting that the hydrophobicity of the magnetic adsorbent increases in the same way. This conclusion is further supported by the corresponding decrease in the intensity of the band attributed to $H_2O$ at 1450 $cm^{-1}$.

The overall morphology of the magnetic adsorbents was studied by SEM. As shown in Fig. 4A, the adsorbents under study show

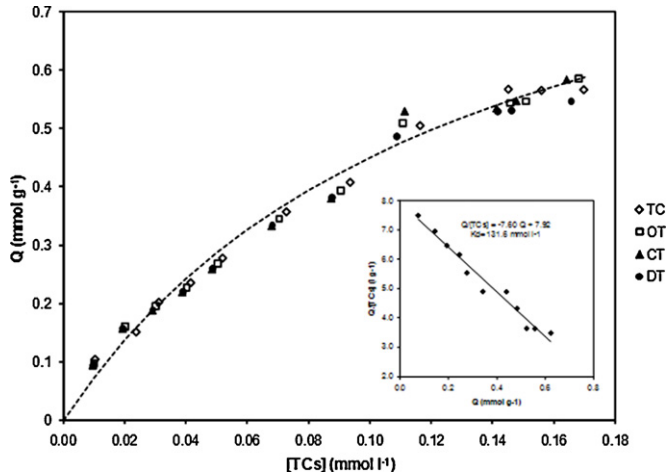


**Fig. 5.** Adsorption isotherm and Scathchard analysis of the binding of TCs onto the magnetic adsorbent.

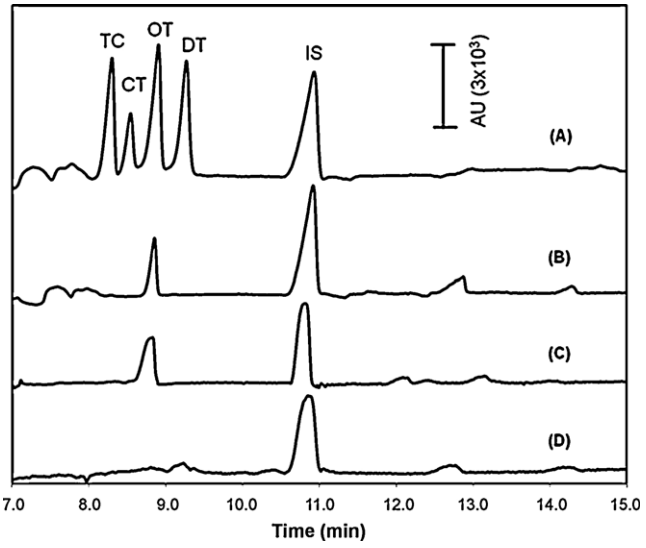


**Fig. 6.** Electropherograms obtained from the analysis of tetracyclines by MSPE: (A) spiked milk sample (250 μg $l^{-1}$), (B) real milk sample, (C) real milk sample by SPE–CE and (D) reference sample. CT, chlortetracycline; OT, oxytetracycline; TC, tetracycline; DT, doxycycline; IS, internal standard. CE conditions: a fused-silica capillary (41.7 cm × 75 μm I.D.); running buffer composition, 30 mM sodium phosphate, 2 mM EDTA disodium salt and 2% 2-propanol at pH 12.0; running voltage, 14 kV; injection at 0.5 psi during 5 s.

**Table 1**
% Retention (mean and %RSD, $n = 3$) at different pH values and magnetic adsorbent proportion (PTMS:TMOS).

| PTMS:TMOS | | pH | | | | |
|---|---|---|---|---|---|---|
| | | 4.0 (RSD) | 6.0 (RSD) | 8.0 (RSD) | 10.0 (RSD) | 12.0 (RSD) |
| 0.5:1.0 | TC | 17.7 (0.8) | 23.4 (0.7) | 35.8 (0.6) | 11.2 (0.5) | 3.2 (1.8) |
| | CT | 18.0 (0.2) | 25.9 (0.6) | 39.9 (0.9) | 10.2 (0.1) | 2.9 (1.0) |
| | OX | 12.1 (0.3) | 23.5 (0.5) | 35.4 (1.1) | 10.1 (0.5) | 5.5 (1.3) |
| | DX | 17.4 (0.7) | 24.8 (0.3) | 37.1 (0.5) | 11.2 (0.7) | 4.3 (2.2) |
| 1.0:1.0 | TC | 30.7 (1.1) | 30.6 (0.7) | 43.4 (0.8) | 43.2 (0.8) | 5.8 (1.9) |
| | CT | 31.3 (0.9) | 29.9 (0.9) | 41.9 (0.1) | 44.2 (1.3) | 6.9 (1.5) |
| | OX | 32.3 (1.5) | 28.4 (0.5) | 43.1 (0.1) | 48.4 (0.4) | 4.4 (0.8) |
| | DX | 31.2 (0.7) | 28.0 (0.4) | 42.9 (0.1) | 43.5 (0.7) | 7.9 (2.7) |
| 2.0:1.0 | TC | 50.8 (0.4) | 41.2 (0.2) | 55.3 (0.6) | 44.6 (0.8) | 6.7 (2.3) |
| | CT | 47.1 (0.3) | 40.4 (0.6) | 56.9 (1.3) | 47.1 (1.3) | 5.9 (1.8) |
| | OX | 50.1 (0.9) | 40.9 (0.6) | 57.4 (1.1) | 40.2 (0.6) | 4.7 (2.2) |
| | DX | 53.5 (0.3) | 47.6 (0.8) | 53.5 (0.1) | 50.7 (0.9) | 7.8 (2.1) |
| 4.0:1.0 | TC | 82.6 (0.4) | 80.4 (1.0) | 90.7 (0.4) | 99.6 (0.5) | 6.9 (2.1) |
| | CT | 80.8 (0.5) | 81.8 (1.4) | 91.9 (1.2) | 100.5 (1.3) | 3.4 (1.4) |
| | OX | 83.3 (1.1) | 82.9 (1.1) | 91.8 (0.3) | 100.1 (1.2) | 5.9 (1.0) |
| | DX | 80.8 (0.3) | 79.9 (1.4) | 87.9 (0.1) | 101.2 (1.5) | 2.4 (0.6) |

**Table 2**
% Recovery (mean and %RSD, $n = 3$) in the re-extraction of TCs.

| Eluate solutions | % Recovery | | | |
|---|---|---|---|---|
| | CT (RSD) | TC (RSD) | OT (RSD) | DX (RSD) |
| MeOH–$CH_3COOH$ ($1 \times 10^{-3}$ M) | 94.2 (3.0) | 99.4 (1.8) | 99.8 (4.3) | 97.1 (3.2) |
| MeOH–Ethyl acetate (1:1, v/v) | 29.5 (1.4) | 34.9 (3.5) | 34.5 (3.2) | 32.7 (4.3) |
| MeOH–HCl ($1 \times 10^{-3}$ M) | 82.6 (3.4) | 87.1 (2.1) | 87.2 (4.9) | 85.3 (3.7) |

a spherical morphology. The size distribution of the particles (previously dried at 100 °C, 24 h) was determined to be in the range of 63–100 nm based on measurements of the sizes of particles belonging to a homogeneous sample of the magnetic adsorbent.

The particle size of the magnetic adsorbents in aqueous solution was analyzed by LS. Fig. 4B shows the results obtained for all of the magnetic adsorbents. The particle size is uniform in all cases, with a mean size of around 500 nm. The increase in particle size is attributed to hydration and swelling that are characteristic of the hydrophilic silica agglomerates, which are obtained using TMOS monomer during the synthesis protocol.

### 3.2. Retention and elution of TCs in milk samples

The pH dependence of sample extraction and isolation is commonly reported during isolation of TCs from complex matrices. The existence of various ionization degrees on the TCs structure is related to their acidic dissociation constants ($pK_a$). The pKa values of TCs in aqueous solution are around 3.3, 7.5 and 9.0 [25]. To evaluate the effect of pH and hydrophobicity, retention experiments were carried out in pH interval 4.0 to 12.0. All the experiments were performed using 25 ml of a 10 mg $l^{-1}$ standard solution of TCs and 0.1 g of magnetic adsorbent. Once completed the extraction, the TCs remaining in the solution were determined by SPE–CE [1]. The % retention and % recovery were calculated as a function of the concentration added and concentration found after extraction. Table 1 shows the % retention obtained in each experiment.

**Table 3**
Limits of detection for different volumes of milk using MSPE.

| Volumes (ml) | Limits of detection (LODs) [μg $l^{-1}$] | | | |
|---|---|---|---|---|
| | CT | TC | OX | DX |
| 200 | 6 | 9 | 2 | 5 |
| 100 | 63 | 91 | 64 | 77 |
| 50 | 187 | 210 | 165 | 158 |

The % retention increases with the hydrophobicity of the TCs. For the whole pH range investigated, maximum retention is achieved using the magnetic adsorbent with a 4:1 PTMS:TMOS ratio. The pH effect on the TCs retention demonstrates a higher affinity of the adsorbent for their deprotonated forms than for their protonated forms. Therefore, the optimal combination of conditions selected for TCs retention was a pH of 10.0 and a magnetic sorbent ratio of 4:1 because under these conditions, the % retention ranges from 99.6% to 101.2% for all TCs determined. At pH values above 10 a decrease of adsorption is observed because the magnetite particles acquire a negative charge due to the binding of hydroxide groups, thus causing electrostatic repulsion between the adsorbent and the anionic TCs [26].

Once the retention conditions were selected, the elution step was evaluated. Three different eluent solutions were used [13]: 5 ml methanol–acetic acid ($1 \times 10^{-3}$ M), 5 ml methanol–ethyl acetate (1:1, v/v) and 5 ml methanol–hydrochloric acid ($1 \times 10^{-3}$ M). The organic eluate was evaporated, and the residue was re-dissolved and filtered prior to CE analysis. Table 2 shows the results obtained using the different elution conditions. The acidified methanolic solutions provide better recoveries (82.6–99.8%) than the methanol:ethyl acetate solution. Because the eluent solution containing acetic acid is less reactive with the magnetite contained in the adsorbent, chelation between the Fe(III) and the TCs is inhibited, which results in increased recoveries [13,24]. Methanol–acetic acid ($1 \times 10^{-3}$ M) was therefore selected for subsequent analysis.

To determine the adsorption capacity and affinity of the magnetic adsorbent for the analytes of interest, the adsorption isotherm and the corresponding Scatchard analysis of the isotherm were acquired. To construct the adsorption isotherms, a set of samples consisting of 25 ml of different amounts of TCs at concentrations ranging from 0.01 to 0.17 mM were subjected to the proposed methodology and analyzed by CE. Fig. 5 shows that the amount of TCs bound to the magnetic adsorbent at the binding equilibrium increased when the initial concentration of the TC increased.

**Table 4**
Regression parameters of calibration lines (mUA) vs. concentration of TC ($\mu g\,l^{-1}$) in 200 ml of milk.

| Parameters | | CT | TC | OX | DT |
|---|---|---|---|---|---|
| Correlation coefficient, $r^2$ | | 0.992 | 0.996 | 0.999 | 0.997 |
| Lack of fit test ($F_{calculated}$) | | 1.50 | 1.70 | 1.24 | 5.14 |
| Intercept, $b_0 \pm ts(b_0)$ | | $118 \pm 218$ | $124 \pm 256$ | $124 \pm 215$ | $101 \pm 217$ |
| Slope, $b_1 \pm ts(b_1)$ | | $85716 \pm 1467$ | $99690 \pm 2865$ | $81366 \pm 615$ | $79264 \pm 1182$ |
| Repeatability intra-day | $100\,\mu g\,l^{-1}$ | 2.3 | 1.7 | 2.2 | 2.7 |
| (%RSD, $n = 3$) | $150\,\mu g\,l^{-1}$ | 1.7 | 1.6 | 2.0 | 2.3 |
| Repeatability inter-day | $100\,\mu g\,l^{-1}$ | 1.2 | 3.2 | 1.9 | 1.9 |
| (%RSD, $n = 3$) | $150\,\mu g\,l^{-1}$ | 1.8 | 2.2 | 2.3 | 0.8 |
| Linearity range ($\mu g\,l^{-1}$) | | 18–250 | 27–250 | 4–250 | 25–250 |
| Limit of detection ($\mu g\,l^{-1}$) | | 6 | 9 | 2 | 5 |
| Limit of quantification ($\mu g\,l^{-1}$) | | 18 | 27 | 6 | 15 |

$F_{tabulated(0.05,4,6)} = 6.22$.

In the Scatchard analysis, the amount of TC bound on the magnetic adsorbents was obtained by subtracting the free concentration from the initial concentration of each TC. When the TC concentration was varied, the Scatchard plot was obtained according the following equation:

$$\frac{Q}{[TCs]} = \frac{(Q_{max} - Q)}{K_d}$$

where $Q$ is the amount of each TC bound on the magnetic adsorbent at equilibrium, [TCs] is the free TC concentration at equilibrium, $K_d$ is the dissociation constant (the affinity of the magnetic adsorbent for the TCs), and $Q_{max}$ is the apparent maximum binding amount. The $K_d$ and $Q_{max}$ are estimated from the slope and intercept of the linear plot of $Q$ vs. $Q$/[TCs]. The linear regression equation of this curve was $Q$/[TCs] = −7.60$Q$ + 7.92. The $K_d$ and $Q_{max}$ values were calculated to be 131.6 mmol $l^{-1}$ and 104 μmol $g^{-1}$, respectively. These values correspond to a moderate degree of interaction between the adsorbent and TCs, which is desired in retention–elution processes. The linearity observed in the Scatchard plot indicates the presence of homogeneous active sites on the magnetic adsorbent. The magnetic adsorbent of 4:1 PTMS:TMOS ratio shows an affinity and selectivity for TCs similar to those reported using a molecularly imprinted polymer [27].

Retention, elution and affinity experiments demonstrate the usefulness of the developed magnetic adsorbent, for tetracycline determination in milk samples by magnetic solid phase extraction.

### 3.3. Method validation

The limits of detection (LODs) of the MSPE–CE method were evaluated using different volumes (50, 100 and 200 ml) of milk samples spiked with TCs in the concentration range of 50–250 $\mu g\,l^{-1}$. The LODs were calculated for a signal-to-noise ratio equal to (S/N = 3.29). The results of these experiments are listed in Table 3. According to these results, the LOD is observed to decrease when higher sample volumes are used. The lower LODs estimated range from 2 to 9 $\mu g\,l^{-1}$ using 200 ml of initial sample. Based on the results and taking into account the most restrictive MRLs established by EU regulations (100 $\mu g\,l^{-1}$ [4]), a sample volume of 200 ml was selected as the most suitable for real sample analysis.

In the optimized conditions, the analytical parameters and precision data were determined using spiked milk samples in which TCs were initially undetectable. Aliquots of 200 ml of blank milk samples were spiked with the four TCs in the interval concentration of 25–250 $\mu g\,l^{-1}$.Each standard was prepared in triplicate. The resulting standards were thoroughly homogenized, and preconcentrated using the proposed methodology as described in the experimental section. The peak heights obtained (AU) were measured and the calibration line was constructed from the average peak heights. The calibration line showed a linear dependence between peak height and the concentration of TCs in the spiked milk sample. Regression parameters of the calibration lines are shown in Table 4 [28].

The proposed methodology was applied to the determination of TCs in 25 commercial milk samples from different brands. Three replicate determinations of the analytes were carried out on each sample. According to the results obtained by analysis of TCs in milk, 5 out of the 25 samples tested were positive for the presence of OT residues, which was identified from its migration time. Fig. 6 shows the electropherograms obtained from the analysis of a spiked sample (Fig. 6A), a real sample (Fig. 6B), a real sample by SPE–CE (Fig. 6C), and a reference sample (Fig. 6D)

The accuracy of the method was investigated by determining the recoveries of the TCs added to milk samples at three concentration levels with three replicates for each level. The recoveries are shown in Table 5 along with the relative standard deviation. The mean recoveries obtained for the four TCs were in the range of 92–103%. The relative standard deviation (RSD) is less than 5% in all cases using the methodology proposed. There was a variation between the recoveries for different analytes, likely related to the structure

**Table 5**
Recovery of the tetracyclines and %RSD ($n = 3$) of TCs from spiked milk samples by MSPE.

| Added [$\mu g\,l^{-1}$] | Analyte | Found [$\mu g\,l^{-1}$] | | | Average recovery (%) | RDS (%) |
|---|---|---|---|---|---|---|
| 100 | CT | 91.3 | 93.7 | 92.4 | 92.5 | 1.3 |
| | TC | 104.1 | 103.7 | 102.9 | 103.6 | 0.6 |
| | OT | 98.1 | 96.6 | 92 | 95.6 | 3.3 |
| | DT | 96.1 | 94.5 | 91.8 | 94.1 | 2.3 |
| 150 | CT | 143.7 | 141.8 | 142.6 | 95.1 | 0.7 |
| | TC | 149.8 | 153.4 | 155.3 | 101.9 | 1.8 |
| | OT | 147.6 | 148.8 | 151.4 | 99.5 | 1.3 |
| | DT | 148.1 | 152 | 148.6 | 99.7 | 1.4 |
| 200 | CT | 189.4 | 192.9 | 190.5 | 95.5 | 0.9 |
| | TC | 198.7 | 199.3 | 197.3 | 99.2 | 0.5 |
| | OT | 200.7 | 211.5 | 210.1 | 103.7 | 2.8 |
| | DT | 208.8 | 209.5 | 201.5 | 103.3 | 2.1 |

**Table 6**
Oxytetracycline content ($\mu g\,l^{-1}$) in real milk samples obtained from different brands.

| Sample | Oxytetracycline concentration ($\mu g\,l^{-1}$) | | |
|---|---|---|---|
| | EC | HPLC | SPE–CE |
| 1 | 142.0 | 141.2 | 129.1 |
| | 141.9 | 134.5 | 133.8 |
| | 142.0 | 137.7 | 132.0 |
| 2 | 127.0 | 135.4 | 129.1 |
| | 136.9 | 136.9 | 133.8 |
| | 131.9 | 131.4 | 132.0 |
| 3 | 97.3 | 98.9 | 93.5 |
| | 98.8 | 102.2 | 97.4 |
| | 98.1 | 99.6 | 94.7 |
| 4 | 137.5 | 139.1 | 139.6 |
| | 143.2 | 136.9 | 137.9 |
| | 139.7 | 136.4 | 141.4 |
| 5 | 207.6 | 204.8 | 199.2 |
| | 218.9 | 211.8 | 208.2 |
| | 212.5 | 208.5 | 205.6 |

of the analyte.

To further ensure the accuracy of the MSPE–CE method, positive samples were also analyzed by SPE–CE and MSPE–HPLC. The results obtained by the proposed method (Table 6) were compared with those obtained by the classical pre-treatment method (solvent extraction, centrifugation, and subsequent clean-up and concentration by SPE). To evaluate differences between the methods, one-way ANOVA and a Tukey multiple comparison test were performed. The calculated $F$ value ($p = 0.05$) did not exceed the critical $F$ value ($F_{2,42} = 3.22$, $p = 0.05$), thus indicating that there are no significant differences between the results due to the analytical method employed. Results of the Tukey test also demonstrated the absence of significant differences between the results provided by the three methods compared ($Q_{calculated} < Q_{critical} = 33.07$). It is therefore confirmed that MSPE is a robust and accurate technique that can be coupled to HPLC or CE for TCs determination in milk.

## 4. Conclusions

The proposed MSPE technique based on the synthesized magnetic adsorbent ($Fe_3O_4$–$SiO_2$–phenyl modified) was demonstrated to be an efficient strategy for the rapid preconcentration of TCs residues in complex matrices such as milk. The methodology described is faster than classical preparation procedures as SPE, with a minimum sample manipulation, lower solvent consumption, and consequently lower cost.

Additionally, this technique provides good results in terms of sensitivity and accuracy. When coupled to CE, the MSPE method yielded LODs of 2–9 $\mu g\,l^{-1}$, according to the more restrictive MRLs established, for the four target TCs determined. Additionally, the MSPE preconcentration technique is also a good alternative for coupling to other analytical methodologies, such as HPLC.

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