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Determination of Tetracycline Residues by Liquid Chromatography Coupled with Electrochemical Detection and Solid Phase Extraction

Innocenzo G. Casella* and Fabio Picerno

Dipartimento di Chimica dell'Università degli Studi della Basilicata, Via N. Sauro 85, 85100 Potenza, Italy

A liquid chromatography based on the reverse phase separation and a solid phase extraction (SPE) procedure using a phenyl-silica sorbent for the simultaneous separation and extraction/preconcentration of six tetracyclines was studied and optimized. An amperometric detector using a polycrystalline gold electrode operating under both DC and IPAD conditions was tested for the determination of the selected tetracyclines. Under optimal SPE and chromatographic conditions, the limit of detection of the investigated tetracyclines comprised between 50 nM (25 μg L^{-1}) and 75 nM (37 μg L^{-1}), and the dynamic linear range spanned generally over 3 orders of magnitude, when the electrochemical detector was used under DC conditions at a constant applied potential of 1.6 V vs Ag/AgCl. The analytical method was successfully tested for the determination of tetracyclines in milk samples ((UHT low-fat, fresh pasteurized, and powdered milk) with reproducibility and recovery levels ranged between 3.1%−5.6% and 70%−118%, respectively.

KEYWORDS: Tetracyclines; electrochemical detection; SPE; liquid chromatography; milk

1. INTRODUCTION

The tetracyclines (TCs) are a family of broad-spectrum antibiotics effective against a remarkably wide variety of organisms and are routinely used in veterinary medicine for the prevention and control of disease. They show a broad antibacterial spectrum and bacteriostatic activity and have good performance against acute disease caused by Gram-positive and Gram-negative bacteria. Because of their wide prophylactic effects, TCs are often used in veterinary contexts as feed additives to promote growth in livestock (*1*). The extensive usage of these drugs in dairy husbandry and the failure to follow good veterinary practices can lead to unsafe residue levels in various tissues and milk, with potential adverse effects on human health. In fact, the occurrence of antibiotic residues in human food, arising from its veterinary use, is a cause of concern to consumers worldwide because of possible toxic or allergic reactions and the possibility that pathogenic organisms could become resistant to these drugs (*2*). Moreover, these antibiotic residues could be carcinogenic, and human exposure in hypersensitive individuals should be reduced to a minimum possible levels. Because large amounts of milk or meat products are consumed all over the world, good quality control programs are very important to maintain the minimum level of TCs in these matrixes in order to guarantee their maximum good health benefits. Thus, in order to prevent any health problems with consumers of meat and milk products, the US Food and Drug Administration has set tolerances for the residues of TCs in bovine milk of 300 μg kg^{-1}, while in the European Union, the maximum residue limits have been established as 100 μg kg^{-1}. In this respect, it is of great interest to develop analytical procedures capable of determining with good accuracy and sensitivity, the animal tissue concentrations of TCs and to evaluate their presence in edible animal products to protect human health. Thus, quantitative and qualitative routine determinations of tetracyclines and their degradation products, derived by epimerization processes, are necessary in the pharmaceutical, environmental, biotechnological, and foods industries.

Several analytical methods have been successfully proposed for the determination of TCs and their major degradation products in various matrixes. In particular, liquid chromatography operating under reverse phase mode (*3*, *4*) and coupled with various detection schemes such as spectrophotometry (*5*−*8*), fluorescence (*9*−*11*), or mass spectrometry (*7*, *11*−*13*) is widely used as a routine method for the determination of TCs in real matrixes. Detection schemes based on the electrochemical methods have attracted much interest owing to their low cost, nonderivatization procedures, high degree of selectivity, and good sensitivity. Thus, amperometric or coulometric methods using carbon electrodes (i.e., carbon fiber, glassy carbon, etc.), have been proposed to develop electroanalytical methods for TCs analysis (*14*, *15*). In addition, mixed-valent films of ruthenium oxide−ruthenium cyanide were electrodeposited on the glassy carbon electrode substrates and used as amperometric sensors for the analytical determination of tetracycline antibiotics at sufficiently high applied potentials (*16*). Tetracyclines have previously been oxidized on noble metals such as Au and Pt electrodes with high sensitivities, but their major disadvantage is associated with the electrode surface deactivation caused by irreversible adsorption of intermediate and/or reaction products. Therefore, pulsed amperometric detection (PAD) or complex multicycle potential waveform (IPAD) using polycrystalline gold electrodes were

*Corresponding author. E-mail: innocenzo.casella@unibas.it.

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described and proposed in the literature (*17*, *18*). The electrochemical pulsed techniques, which combine amperometric detection with alternate cleaning potential steps to remove the fouling species adsorbed on the electrode surface, efficiently overcome the surface deactivation phenomenon of the noble metals when used as sensing probes. However, some oxidation reactions were still possible with good sensitivity and temporal stability at the oxide gold polycrystalline electrodes in acid or neutral solutions without the need to apply detection strategies that employ potential-time waverforms (*19*).

The aim of this study is focused on the electroanalytical characterization of the gold polycrystalline electrodes toward the direct amperometric detection of several common TCs at constant applied potential and under acid medium. At the same time, a liquid chromatography method based on the reverse phase separation and a solid phase extraction (SPE) procedure for the simultaneous separation and extraction/preconcentration of six TCs, was optimized. The proposed analytical procedure is applied to the determination of tetracycline residues in various bovine milk and powder milk samples.

2. EXPERIMENTAL PROCEDURES

2.1. Apparatus. Voltammetric experiments were performed with an Autolab PGSTAT 30 Potentiostat/Galvanostat (Eco Chemie, Utrecht, The Netherlands), and data were acquired using an Autolab GPES software package, version 4.9. A three-electrode cell consisting of a working polycrystalline gold electrode, an SCE reference electrode, and a counter Pt electrode purchased from Amel (Italy) were used. Amperometric measurements in flowing streams were performed by using an Amperometric Detector Model ED 40 (Dionex, Sunnyvale, CA). A thin-layer electrochemical cell consisting of a 1.0 mm-diameter gold working electrode, an Ag/AgCl reference electrode, and a stainless steel serving as the counter electrode was also purchased from Dionex. All experiments were performed using a Mod. PU-1580 (Jasco Corporation, Tokyo, Japan) equipped with a rotary injection valve Mod. 7125i (Rheodyne, Cotati, CA, USA) with a 20 μL sample loop.

Chromatographic separations of selected TCs were achieved with a reverse phase C18 analytical column Synergi Hydro-RP 4 μm (Phenomenex, 250 mm $\times$ 4.60 mm i.d., Torrace, CA, USA). A personal computer equipped with an in-house software allowed the acquisition and processing of chromatograms. Weekly, the column was washed and reconditioned using about 10 column volumes (0.6 mL min^{-1}) each of (1) water, (2) 95%/5% water/acetonitrile, (3) acetonitrile, (4) 95%/5% acetonitrile/water, and (5) water.

The mobile phase was protected from oxygen and other gaseous dissolved species by an online degasser system (Waters In-Line Degasser).

2.2. Chemicals. All selected tetracyclines were of analytical-reagent grade and were purchased from Sigma-Aldrich (Steinheim, Germany). LC grade acetonitrile, methanol, and other chemicals were also purchased from Sigma-Aldrich. Stock standard solutions of considered analytes were prepared in 20 mM $HClO_4$ solution−acetonitrile (80:20) or in McIlvaine/EDTA buffer solutions and stored in the dark at 4 °C for some days. The chemicals were used without further purification, and solutions were prepared by using ultrapure water supplied by a Milli-Q RG unit from Millipore (Bedford, MA, USA). The working solutions were prepared fresh each day.

2.3. McIlvaine Buffer at pH 2. McIlvaine/EDTA solutions used for the precipitation of proteins and extraction of TCs from real matrixes were prepared by mixing 0.07 M citric acid, 0.1 M Na_2HPO_4, and 0.01 M EDTA (as Na_2H_2Y). The pH of the resulting solution was adjusted at 2 by adding an appropriate amount of H_3PO_4.

2.4. Solid Phase Extraction (SPE). Solid phase extraction was performed using various cartridges such as Strata SCX (500 mg 3 mL^{-1}), Strata C18-E (500 mg 6 mL^{-1}), Strata CN (500 mg 3 mL^{-1}), and Strata Phenyl (500 mg mL^{-1}) purchased from Phenomenex (Torrance, CA, USA). The SPE cartridges were conditioned/activated by drawing 3 mL of methanol and 1 mL of water. Aliquots of treated samples or standard mixtures of TCs in McIlvaine/EDTA buffer solutions were transferred to the cartridges. The retained TCs were eluted with absolute methanol. The conditioning solvents, the standard TCs, and the extracting solvent were passed through the cartridges at about 1.0 mL min^{-1}.

2.5. Sample Preparation Procedures. Commercial bovine milk (UHT (ultrahigh temperature processing), low-fat, fresh pasteurized whole milk) and powder milk samples (Neolatte 1, Töpfer GmbH, D-87460 Dietmannsried/Allgäu), were purchased from local stores and opened and/or treated only just before analysis. Powdered milk was reconstructed by dissolving 14 g of powder in 90 mL of heated water. Aliquots of 20 mL of milk were treated with 10 mL of McIlvaine/EDTA buffer solutions, and the suspension was centrifuged at 4 °C and 4000 rpm for 15 min. Ten milliliters of the resulting solutions was transferred into a SPE cartridge previously conditioned. The retained TCs were eluted with 20 mL of methanol, and the resulting alcoholic solution was evaporated to dryness under reduced pressure at 45−50 °C using a rotovapor Laborota 4000 (Heidolph). The residue was dissolved in an appropriate volume of mobile phase (i.e., 80% 20 mM $HClO_4$ and 20% acetonitrile) and injected into the analytical column. The entire precipitation extraction−cleanup and chromatographic analysis should be completed in about 1.3 h.

3. RESULTS AND DISCUSSION

3.1. Cyclic Voltammetry and Amperometric Measurements. **Figure 1** shows the electrochemical behavior of the tetracycline on the polycrystalline gold electrode in 20 mM $HClO_4$ medium. The main electrochemical characteristics of the gold polycrystalline electrodes in acid solution include a large anodic wave observed during the anodic sweep at about 1.1 V vs SCE corresponding to the hydroxide formation of both Au(I) and Au(III) species. In addition, a massive oxygen formation process is simultaneous with the conversion of surface hydroxide to the higher oxidation state of gold oxide species. On the contrary, a well-defined cathodic wave (Ic) observed during the negative scan at about 0.75 V corresponds to the reduction of the gold oxide species formed during the preceding positive scan. In the presence of increasing concentrations of tetracycline, a developed oxidation wave was observed during the anodic sweep of the potentials, whose results totally overlapped with the gold hydroxide formation and oxygen evolution process. The oxidation currents, measured at 1.3 V, during the anodic sweep, were linearly proportional to the tetracycline concentration up to about 10 mM. The peak potential of the oxidation wave, shifted to higher potentials while the currents of the reduction wave Ic show a slight attenuation on increasing concentration of analyte. Similar voltammetric profiles are obtained with other investigated TCs. On the basis of the electrochemical behavior, the oxidation currents of the TCs are likely controlled by slow surface reactions, and the slight attenuation of the cathodic peak is caused by an irreversible adsorption process of tetracyclines on the gold active sites which inhibits the gold metal formation.

The observation that the gold oxide yields interesting electrocatalytic activity toward TC oxidations suggests the possibility of using this electrode substrate as a sensing probe for the direct amperometric detection (DC) of these molecules at relatively high applied potentials. In order to optimize the choice of the operational applied potential, hydrodynamic voltammograms of tetracycline in the range of potentials comprised between 1.0 V−1.7 V vs Ag/AgCl were evaluated. **Figure 2** shows the resulting hydrodynamic voltammogram of 0.2 mM (89 mg L^{-1}) tetracycline obtained under flow injection conditions at 1.0 mL min^{-1} using 20 mM $HClO_4$ as carrier electrolyte. As can be seen, the peak currents of the tetracycline increase linearly with the applied potentials, while the background currents increase markedly at potential values higher than 1.6 V. The simultaneous and competitive oxidation of the medium at higher potentials is responsible for the marked increase of the background current level. Thus, the detection potential of 1.6 V represents the best compromise

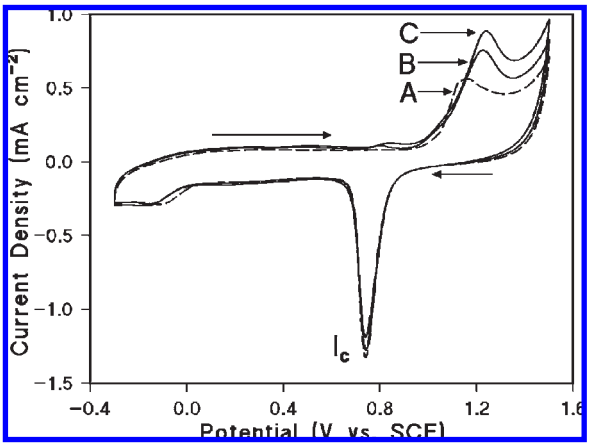

Figure 1. Cyclic voltammograms (5th cycle) at a polycrystalline gold electrode in solution 20 mM $HClO_4$ (A), plus 3.2 mM tetracycline (B), and 6.4 mM tetracycline (C). Scan rate, 50 mV s^{-1}.

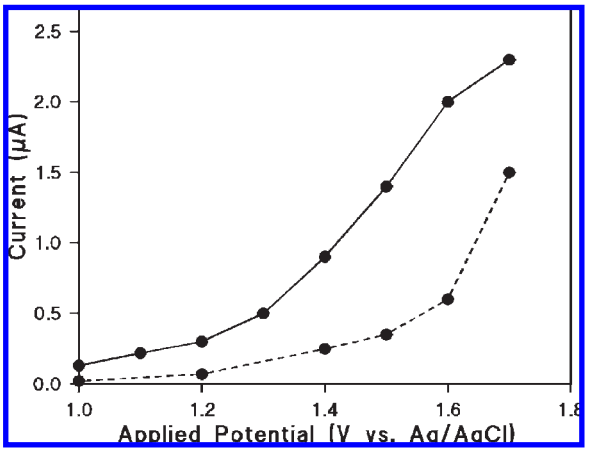

Figure 2. Hydrodynamic voltammogram at an Au polycrystalline electrode of 0.2 mM tetracycline (solid curve) and background currents (dashed curve). Carrier: 20 mM $HClO_4$ at 1.0 mL min^{-1}.

between the maximum amperometric signal, the highest S/N ratio, and a satisfactory analytical reproducibility. The background currents, evaluated at a constant potential of 1.6 V, show a reproducible and constant value of about 0.5 μA $\pm$ 0.1. After 20 repetitive injections of a standard solution containing 0.25 mM tetracycline and over 4 h of operation time, the variation of the amperometric signal ranged between 1.8% and 2.3%.

In general, the chemical nature of the electrode material is the main factor affecting the surface reaction kinetics and plays a key role in amperometric performance in terms of sensitivity and analytical reproducibility. In this respect, we have compared the amperometric performance between the gold and glassy carbon electrodes toward the electrooxidation of TCs under DC conditions and acid medium. **Figure 3** shows an interesting comparison between the amperometric responses of 0.25 mM (111 mg L^{-1}) tetracycline obtained on the polycrystalline gold substrate and a common glassy carbon electrode. As can be seen, the gold electrode shows an amperometric response which is about 130% higher than that observed on the glassy carbon electrode. Considering that the contribution of surface roughness factors at the Au electrode can be ruled out as being responsible for the enhancement of the amperometric response, the gold oxide surface (i.e., $Au(OH)_3$, AuOOH, etc.) can be considered as an effective catalyst for the electrooxidation of TCs at applied potentials

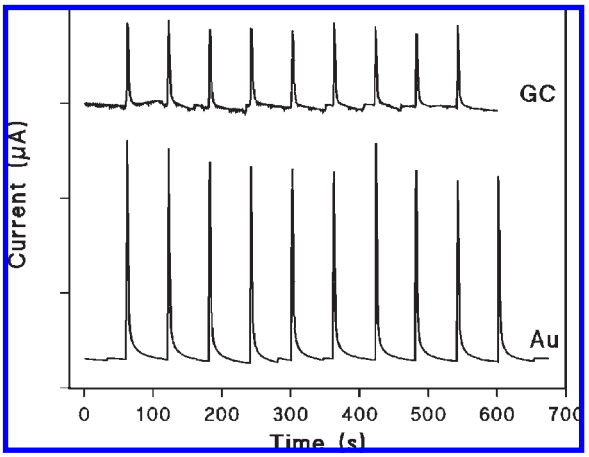

Figure 3. Flow injection analysis of 0.25 mM tetracycline under DC conditions at 1.6 V vs Ag/AgCl. Comparison between Au and glassy carbon electrode. Other experimental conditions are as described in **Figure 2**.

more positive than 1.4 V. In addition, the fast peak current decay and the good reproducibility of the peak height during the consecutive injections of tetracycline confirms the practical absence of any fouling effects caused by irreversible adsorption of reactants and/or reaction products on the gold surface electrode. Thus, although the use of the polycrystalline gold electrode as amperometric probe under DC conditions represents a novelty in electroanalytical applications, because these electrodes are subject to severe fouling processes, this study demonstrates that gold oxide surfaces show an appreciable stability, reproducibility, and sensitivity at a constant applied potential of 1.6 V for the amperometric detection of TCs.

In order to ascertain the effects of some organic modifiers commonly employed in chromatography, the gold electrodes were tested under flow injection conditions using carrier electrolyte containing acetonitrile (ACN) or methanol (MeOH) species. The use of a carrier electrolyte of 20 mM $HClO_4$ containing 20% ACN or MeOH, induces a diminution of the amperometric signal of about 40%−50% of the investigated TCs. This behavior can be associated at a favorable adsorption of ACN or MeOH molecules on the gold oxide surface, with consequent inhibition of the electrooxidation of TCs species. In addition, the presence of 20% MeOH in the carrier electrolyte induces a marked increase of the background current of about 300% with subsequent deterioration of the analytical performance of the amperometric detector.

3.2. Chromatographic and Amperometric Measurements. A Synergi 4 μm Hydro-RP 8A C18 (250 × 4.60) analytical column based on a C18 reverse bonded phase packed with a 4 μm type-B silica was used for the separation of TCs in standard and complex mixtures. Considering that, the strong interaction of TCs with the silanols and traces of metals present in silica packing materials significantly contributes to peak tailing, end-capping on reverse phase columns was preferred for its ability to minimize silanol interactions. In this respect, the C18 phase used here is end-capped with a proprietary polar group to provide good retention of both hydrophobic as well as polar compounds via polar interactions. Thus, the selectivity should allow balanced polar, acidic, basic, and hydrophobic character in order to guarantee a good retention and resolution level. A typical chromatogram of six TCs, such as minocycline, oxytetracycline, tetracycline, democycline, chlortetracycline, and doxycycline, separated by a Synergi 4 μm Hydro-RP 8A and detected under direct amperometric conditions (DC) at 1.6 V vs Ag/AgCl, is shown in **Figure 4**. The separation was performed under isocratic conditions using a mobile phase of 20 mM $HClO_4$ containing 20% ACN at a flow

rate of 1.0 mL min^{-1}. As can be seen, a complete and satisfactory elution of the considered molecules was achieved in 13 min. Under the present chromatographic conditions, the peaks are well resolved and, with the exception of chlortetracycline and doxycycline, show an acceptable level of symmetrical degree. Very recently, oxalic acid as carrier electrolyte was proposed and employed in order to improve the symmetrical nontailed peaks of TCs separated under reverse phase conditions (*6*−*8*, *10*, *11*, *20*−*22*). Thus, in order to reduce the peak tailing effects, oxalic and methanesulfonic acids in place of perchloric acid were also tested as carrier electrolyte in the mobile phase. Unfortunately, the use of the mobile phase containing 10 mM oxalic acid or metansulfonic acid plus 20% ACN did not lead to significant improvement in the symmetrical degree of the chromatographic peak of chlortetracycline and doxycycline. In addition, oxalic acid induces a marked increase of the background current (about 250%), while the use of methanesulfonic acid induces a sensible decrease of the amperometric sensitivity (about 40−50%). Considering that TCs can be easily decomposed to the anhydro-form and converted to their epimers (*3*) (see chromatographic peak of oxytetracycline and tetracycline), the contribution of probable decomposed TCs on the pronounced asymmetrical character of the chlortetracycline and doxycycline peak cannot be excluded.

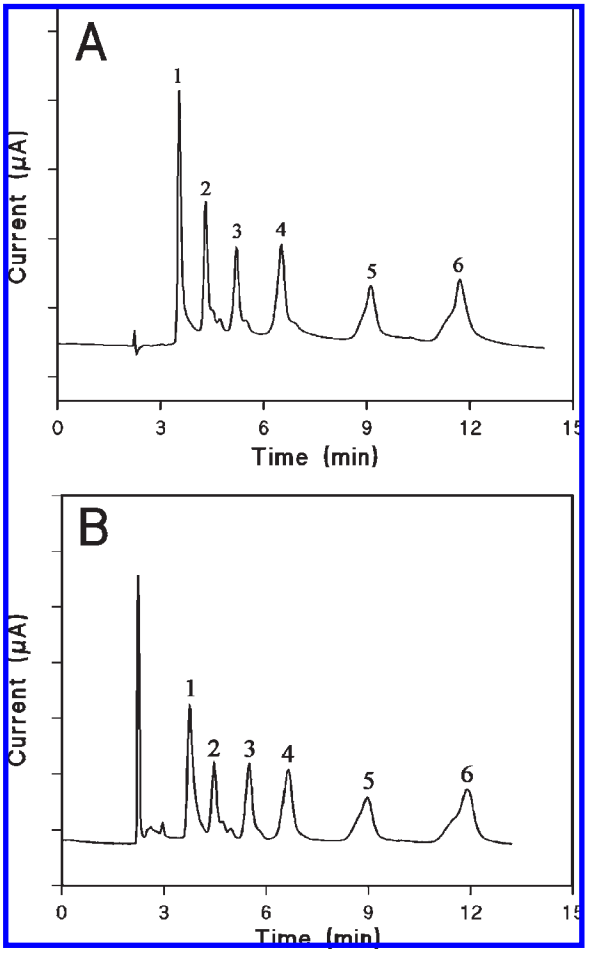

Figure 4. (**A**) Chromatogram of a standard mixture of TCs (10 μM each analyte) obtained under DC conditions at 1.6 V; (1) minocycline, (2) oxytetracycline, (3) tetracycline, (4) democycline, (5) chlortetracycline, and (6) doxycycline. Column: Synergi Hydro-RP 4 μm (250 mm × 4.60 mm i.d.). Mobile phase: 20 mM $HClO_4$−acetonitrile (80:20) at 1.0 mL min^{-1}. (**B**) Chromatogram of a standard mixture of TCs (10 μM each analyte) obtained under IPAD conditions; the waveform was described in the text and detailed in ref *18*. Other experimental conditions are as described in **A**.

The effect of the ACN on the chromatographic profile of the TCs, using a mobile phase of 20 mM $HClO_4$, was also evaluated. In general, the retention times of the analyzed TCs were sensibly reduced on increasing ACN concentration in the mobile phase. As a consequence, a mobile phase containing ACN > 30% led to poor chromatographic resolution with a partial overlapping of some peaks such as minocycline, oxytetracycline, and tetracycline. On the contrary, the retention times of the TCs increased markedly with decreasing ACN concentration in the mobile phase. In addition, the peak asymmetry for all analyzed molecules increased sensibly for mobile phases containing an ACN concentration < 10%.

In order to extend the electroanalytical potentialities of the gold electrode toward TC detection in acid medium, a multicycle waveform detection (IPAD) was tested under the present chromatographic conditions. In this study, an IPAD method previously optimized and proposed for the chromatographic detection of some TCs (*18*), which is composed of a triangular waveform repeated five times between 0.1 and 1.2 V (80 ms for each cycle of potentials) and an activating potential step at −1.5 V, was adopted. **Figure 4** shows the relevant chromatogram of a standard mixture of TCs obtained under IPAD conditions. The chromatographic profile of the standard mixture of TCs obtained under DC conditions at 1.6 V and under IPAD conditions are virtually identical, and the relevant limits of detection (LOD), the dynamic linear range, and the reproducibility (RSD %) of the analyzed molecules are summarized and compared in **Table 1**. The LOD were evaluated as a signal-to-noise ratio of 3 at the lowest injected concentration of analytes, and the reproducibility was obtained from six replicate chromatographic analyses of 2 μM of each analyte. As can be seen in **Table 1**, it is possible to make some analytical considerations between the amperometric detection of TCs on polycrystalline gold electrodes based on DC and IPAD schemes:

- The LOD obtained under IPAD conditions comprised between 80 nM (39 μg L^{-1}) and 150 nM (77 μg L^{-1}) for minocycline and chlortetracycline

Table 1. Quantitative Analytical Results of Investigated TCs by Liquid Chromatography Coupled with Electrochemical Detection[a]

	DC			IPAD		
	LOD (μg L^{-1})	linear range (mg L^{-1})	RSD%	LOD (μg L^{-1}) (mg L^{-1})	linear range (mg L^{-1})	RSD%
minocycline	247	0.25−246	3.3	39	0.04−4.9	3.5
oxytetracycl.	246	0.25−258	2.7	40	0.04−4.9	4.3
tetracycline	267	0.26−248	2.1	53	0.05−4.5	4.5
democycline	261	0.26−250	3.3	44	0.04−5.0	3.9
chlortetracycl.	412	0.41−206	3.9	77	0.08−6.2	4.0
doxycycline	462	0.46−263	3.7	77	0.08−7.1	4.3

[a] Experimental conditions: Synergi Hydro-RP 4 μm (250 mm × 4.60 mm i.d.). Mobile phase: 20 mM $HClO_4$−acetonitrile (80:20) at 1.0 mL min^{-1}. DC conditions: 1.6 V vs. Ag/AgCl. IPAD: as described in the text and detailed in the literature (*18*).

(doxycycline), respectively, showing a favorable sensitivity in comparison with that in the DC detection mode at the gold electrode (i.e., LOD comprised between 0.5 μM (247 μg L^{-1}) and 0.9 μM (462 μg L^{-1})). It is interesting to emphasize that the IPAD mode is generally comparable or more sensitive than other common detection methods based on spectrophotometric or spectrometric techniques (*3*).

- The DC detection on the gold electrode shows dynamic linear ranges generally comprised over 3 orders of magnitude for all analyzed TCs with correlation coefficients (r^2) > 0.99. The dynamic linear ranges for the calibration graphs obtained under IPAD conditions spanned generally, at the most, over 2 orders of magnitude (r^2 > 0.99). Thus, the dynamic linear ranges obtained in IPAD mode, although extending up to 2 orders of magnitude, are generally lower than those obtained under DC mode.
- The intraday reproducibility for both electroanalytical methodologies ranged between 2.1% and 4.5%, confirming the good temporal stability of the signals. Thus, low and sufficiently stable baseline level of the noise is generally observed. Amperometric measurements performed under DC conditions produced an average background current of 230 ± 20 nA, while under IPAD conditions, an average background charge of 200 ± 40 nC was obtained.

Therefore, on the basis of the analytical results, the polycrystalline gold electrode shows an interesting amperometric versatility in liquid chromatography for the detection of TCs and can be favorable used as an amperometric sensor under both DC and IPAD mode with good sensitivity, temporal stability, and wide dynamic linear ranges, in dependence of the particular specific analytical applications required and/or matrixes to be analyzed.

3.3. Solid-Phase Extraction. Solid-phase extraction (SPE) is commonly employed to accomplish simultaneous cleanup and preconcentration processes for the analysis of complicated real biological matrixes. Because of their carbon backbone, aromatic region, and a variety of functional groups, TCs could be extracted from biological matrixes by a wide range of SPEs (*3*−*8*, *10*, *11*, *20*−*24*). Thus, the nonpolar region of the rings allows favorable interactions with the C18 reversed phase, while ionic or polar sorbents allow interactions with TCs through various carbonyl, hydroxyl, and amino groups. In order to establish the better SPE conditions, different type of sorbents such as C18-E silica-based reversed phase, CN silica-based normal phase, phenyl-silica, and polymeric Strata SCX (strong cation-exchanger) were tested and compared among them. The cartridges were previously activated by drawing 3 mL of methanol and 1 mL of water. A standard mixture of 5 mL of 0.25 mM TCs in McIlvaine/EDTA buffered solution at pH 2 was passed at about 1.0 mL min^{-1} through the cartridges, and the relevant eluted solution was analyzed by liquid chromatography and DC amperometry at 1.6 V. **Table 2** shows and compares the loading capacity of the selected sorbents tested here. The percent loading capacity (LC%) was expressed as:

$$LC = ((A_1 - A_2)/A_1) \times 100$$

where the terms A_1 and A_2 represent the peak area of the specific analyte before and after the elution of the standard mixture of TCs through the exchanger, respectively. As can be seen, the best loading capacity was obtained for the cartridges containing phenyl-silica and polymeric Strata SCX, which provided good retention for all investigated TCs. The better performance of these sorbents in comparison with C18-E silica-based reversed phase and CN silica-based normal phase can be attributed to their aromatic structure, which can interact with aromatic rings of the analytes via $\pi-\pi$ interaction. In addition, under strongly acidic solutions (i.e., pH 2), the TCs molecules exist in the fully protonated form, and consequently, strong interactions with the anionic sites of the polymeric Strata SCX are expected to play a fundamental role in the retention process.

Table 2. Loading Capacity (LC%) and Recovery (R%) of the Investigated Sorbents Used in the SPE Procedure[a]

	C18-E-silica		CN-silica		phenyl-silica		strata SCX	
	LC%	R%	LC%	R%	LC%	R%	LC%	R%
minocycline	77	75	65	66	>95	87	>95	<5
oxytetracycl.	76	78	38	35	>95	101	>95	<5
tetracycline	85	83	83	81	>95	106	>95	<5
democycline	94	91	>95	94	>95	95	>95	<5
chlortetracycl.	>95	98	>95	91	>95	90	>95	<5
doxycycline	>95	96	>95	88	>95	101	>95	<5

[a] Experimental conditions: the cartridges were activated by drawing 3 mL of methanol and 1 mL of water. A standard mixture of 5 mL of 0.25 mM TCs in McIlvaine/EDTA buffer (pH 2) was passed at about 0.5 mL min^{-1}. The retained TCs were eluted from cartridges using 20 mL of absolute methanol.

After the TC loading process, the cartridge must be washed with a suitable solvent that neutralizes the interaction between the analyte and the sites of the sorbent and allows these last to elute efficiently. Considering that the preconcentration step is needed in order to improve the analytical sensitivity of the proposed methodology, absolute methanol is tested as solvent in order to elute the investigated TCs from the cartridges. The retained TCs were eluted with 20 mL of methanol, and the resulting alcoholic solutions were evaporated to dryness and treated with an appropriate volume of the mobile phase. The recoveries were calculated by the percent difference between the standard TC solutions and the relevant extract dryness, and dissolved in the mobile phase. The percent recoveries from the solid-phase extraction of TCs with the four sorbents tested here are summarized in **Table 2**. As can be seen, for all investigated TCs a very good recovery level was obtained for the cartridges containing the phenyl-silica substrate, while very low recoveries were obtained for cartridges containing polymeric Strata SCX, where the tetracyclines are predominantly in their protonated form and remain irreversibly bonded to the sulfonic moieties of the strong cation exchange sorbent. The more complex mechanisms involved in ion-exchange interactions require other elution conditions or the use of weaker sorbents for the loading process of the TCs. Extreme retention of TCs under ion-exchange cartridges was also described in the literature (*24*). Cartridges based on C18-E silica reversed phase and CN silica normal phase provided acceptable recoveries for chlortetracycline and doxycycline and lower values for the other TCs. Best recoveries were obtained for cartridges containing the phenyl-silica substrate, which were therefore used for analytical applications. **Figure 5** shows a typical comparison between the chromatogram of a 0.7 μM standard mixture TC and the relevant extract from phenyl-silica cartridge obtained under DC amperometric conditions operating at 1.6 V.

In order to check the SPE character as a preconcentration technique, the limits of detection of the considered analytes are re-evaluated. The limit of detection using DC amperometry at 1.6 V decreased generally by one factor when the proposed SPE procedure is adopted. Thus, LOD of investigated TCs are comprised between 50 nM (25 μg L^{-1}) and 75 nM (37 μg L^{-1}) for minocycline and oxytetracycline, respectively. The limit of quantification (LOQ) determined at a signal-to-noise ratio of 10

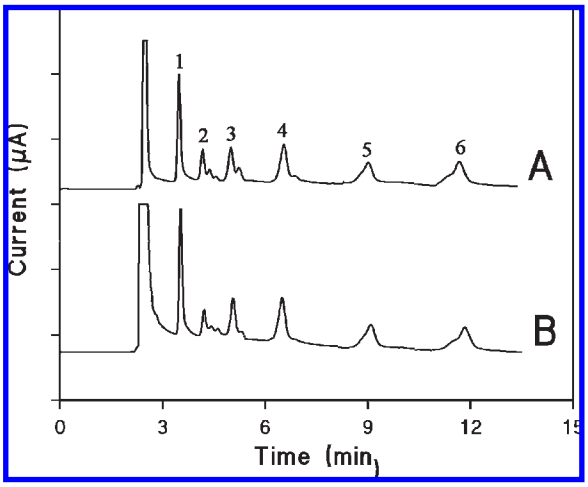

Figure 5. Comparison of chromatograms of a standard mixture of TCs (0.7 μM each analyte) after (A) and before (B) the SPE treatment with phenyl-silica based cartridges. Other experimental conditions are as described in **Figure 4**.

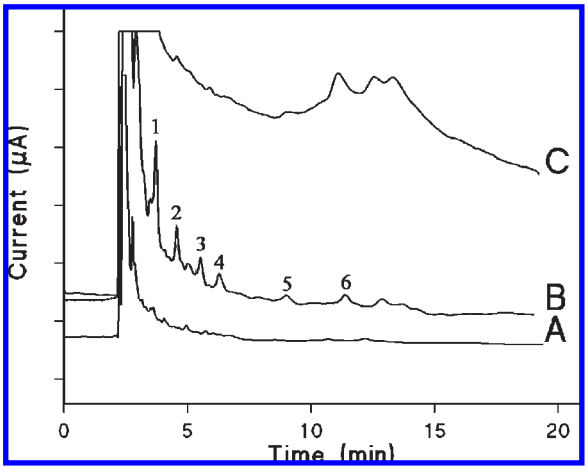

Figure 6. Chromatograms of UHT low-fat milk samples. Unspiked (A) and spiked samples with about 0.1 μM of each tetracycline (B and C), obtained under DC conditions at 1.6 V (A and B) and under IPAD conditions (C). The sample was treated with the McIlvaine/EDTA buffer and successively with SPE (phenyl-silica based cartridges) as reported in the section Sample Preparation Procedures. Chromatographic conditions are as described in **Figure 4**.

evaluated with the proposed SPE procedure, using a preconcentration factor of 10:1, are comprised between 70 nM and 90 nM for all investigated molecules.

3.4. Analytical Applications: Milk Analysis. Analytical determination of tetracyclines in milk or powdered milk includes an initial treatment of the samples with McIlvaine/EDTA buffered solution at pH 2 and a centrifugation step, which removes proteins and fat and in addition inhibits complexation of tetracycline with metal species. Subsequently, an aliquot of the resulting solutions were transferred into a SPE cartridge and the extracts, after dryness and dilution with mobile phase, were injected into the analytical column. **Figure 6**, chromatograms A and B show typical chromatograms of unspiked and spiked extracts of a commercial bovine milk (UHT low-fat) after SPE treatment, obtained under DC amperometric conditions at 1.6 V. No TC residues were detected in the unspiked extract of UHT bovine milk (see **Figure 6**, chromatogram A), and the baseline was

Table 3. Analytical Determination of Some TCs in Milk Samples[a]

UHT Low-Fat

	found (μg L^{-1})	added (μg L^{-1})	R%	RSD%	r^2
minocycline	38	47	81	4.1	0.98
oxytetracycl.	49	52	94	3.5	0.97
democycline	51	45	113	4.9	0.97
doxycycline	34	48	71	5.6	0.96

Pasteurized Whole Milk

	found (μg L^{-1})	added (μg L^{-1})	R%	RSD%	r^2
minocycline	61	52	117	5.5	0.99
tetracycline	53	50	106	3.1	0.99
democycline	52	47	111	5.2	0.98
chlortetracycl.	43	49	88	5.1	0.98

Milk Powder

	found (μg L^{-1})	added (μg L^{-1})	R%	RSD%	r^2
minocycline	45	51	88	3.9	0.98
oxytetracycl.	50	55	91	3.5	0.97
tetracycline	54	48	112	4.6	0.98
doxycycline	39	53	73	4.3	0.99

[a] Experimental conditions: chromatographic conditions are as described in **Table 1**. The analytical concentrations were determined by a linear-square regression procedure ($n = 4$) using the method of standard addition. The chromatographic peak areas were obtained using an Au electrode operating under DC conditions (1.6 V). The precision (RSD%) was estimated on five consecutive chromatograms of the extracts containing 0.3 μM TCs. The percent recoveries (R%) were calculated considering the difference between the added and found concentration of TCs.

sufficiently flat in the chromatographic regions where TCs eluted. Similar results are obtained with fresh pasteurized whole milk and powdered milk samples. **Figure 6**, chromatogram C shows the relevant chromatogram of the spiked extract of the milk (UHT low-fat) obtained under IPAD conditions. As can be seen, under IPAD conditions, the probable presence of matrix interferences with pronounced electrochemical activity, under pulsed amperometric conditions, produce an unstable and large baseline in the chromatographic region close to the front of the solvent and where several TCs, such as minocycline, oxytetracicline, and tetracycline are eluted. Virtually, the same chromatographic performance is obtained with fresh pasteurized whole milk and powdered milk samples. Therefore, although a multicycle waveform detection mode (IPAD) allows a good sensitivity and temporal reproducibility when used for the determination of the standard mixture of TCs, it was not possible to use this electroanalytical methodology for milk analysis since the time window where the elution occurs is not interference-free. Thus, additional investigations are necessary in order to define better extraction and cleanup procedures of the proposed SPE technique when it is coupled with the IPAD mode for the analysis of TCs in milk samples.

The relevant analytical results, obtained under DC conditions at 1.6 V, expressed in terms of percent relative standard deviation (RSD%), percent recoveries (R%), and correlation coefficients (r^2) are summarized in **Table 3**. The concentrations of all analyzed TCs in the spiked samples were calculated by ordinary, linear least-squares regression by using the standard addition method. The precision estimated as percent relative standard deviation (RSD%) of five consecutive chromatograms of the extracts containing 0.3 μM of TCs ranged between 3.1% and 5.6%. The percent recovery of TCs from milk samples, spiked at a concentration of 0.1 μM of each tetracycline, ranged from 71% and 117%,

which was within the AOAC acceptable range for trace analysis (*22*). The acceptable level of recovery of each determined tetracycline in milk samples confirms the practical absence of interfering compounds and matrix effects of the proposed analytical methodology based on McIlvaine/EDTA precipitation, SPE extraction, and liquid chromatography coupled with amperometric detection operating under DC conditions.

A fast, sensitive and reproducible analytical method was proposed and successfully applied to the determination of some TCs in milk samples using a SPE procedure followed by reverse-phase liquid chromatography coupled with amperometric detection operating under DC conditions at 1.6 V.

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