



“In-situ” antimony film electrode for the determination of tetracyclines in Argentinean honey samples



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ABSTRACT

The main goal of this work is the application of an antimony film electrode (SbFE) prepared for the first time “in-situ” to determine organic compound as tetracyclines commonly used in Argentina for hive treatment. The SbFE was prepared on a glassy carbon electrode (GCE) and square wave cathodic stripping voltammetry (SWCSV) technique was used. The SWCSV parameters were optimized based on a Draper Lin small composite design. The SWCSV response was linear in the TCs concentration range from 0.40 to 3.00 μM with a limit of detection (LOD) of 0.15 μM . The proposed method is a good and simple alternative to the determination of tetracyclines in Argentinean honeys samples. Recovery experiments were performed using spiked honey samples with standard deviation values from 0.75% and 9.69%.

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1. Introduction

Tetracyclines, a family of antibiotics with broad-spectrum activity, are frequently used to treat bacterial infections [1,2]. The TCs are widely used in many areas of veterinary, such as animal food production [3,4], apiculture [5–7], etc. Its use as veterinary drug is banned in the European Union, but is still widely used in countries like United States, Canada, Australia and India [8]. In Argentina, is common the use of tetracycline and oxitetracycline (TCs) in activities such as beef cattle raising and beekeeping. The community apiary employs these antibiotics due to its action against bacterial diseases (American and European foulbrood), which severely affect bee larvae and bee products production [9]. Commonly in beekeeping, the application of antibiotics over the hive must take place when the honey harvest was finished. Otherwise, antibiotics can persist mostly in honey and others bee products like propolis, pollen and royal jelly. These antibiotics have a significant residual effect on human foodstuff and it can cause allergic reactions in susceptible persons, chronic toxicity and antimicrobial resistant [10–12].

Currently there are several methods to determine tetracycline in honey. The most common method is high performance liquid chromatography (HPLC) with mass spectrometry, chemiluminescence, UV–vis or fluorescence detection. These methods require expensive equipment, extensive sample preparation, experimental analysis

skills and consume high amounts of reactive, not compatible with the green chemistry philosophy [13–16]. Capillary electrophoresis is also applied to determine tetracyclines in honey samples, nevertheless requires overpriced equipment and a sample pretreatment process [17]. Other methodologies as immuno-sorbent assay (ELISA) used for this purpose, have drawbacks such as the presence of organic and inorganic interference in honey samples, which may be reduced by a biotin–avidin mediated ELISA method [18].

Voltammetric techniques such as differential pulse, square wave and adsorptive stripping voltammetry are used as a simple and low cost alternative for tetracycline determination in sewage, river water, artificial urine, pharmaceutical and milk samples [19–21]. Therefore, few works determine tetracyclines in a complex matrix as honey. Lian et al. modified a gold electrode with cyclodextrin-multiwalled carbon nanotube composites, gold nanoparticles-polyamide amine dendrimer nanocomposites and chitosan derivative, in order to improve the analytical signal of chlortetracycline in these samples [22]. Furthermore, to determine tetracycline in the same matrix, a gold electrode modified with gold nanoparticles and molecularly imprinted polymers was performed [23]. A commercial graphene screen-printed carbon electrodes has been employed to quantify tetracycline in honey [24]. The disadvantages of these methods is that surface electrodes modifications requires laborious steps and employs expensive electrodes and reactives. This is a drawback for a routine quality control.

Bismuth and antimony films electrodes were developed as an alternative to the use of mercury drop and mercury film electrodes since 2000 and 2007, respectively [25,26]. These films electrodes have the advantage of being prepared “in-situ” or “ex-situ” on

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many electrodes surfaces, such as glassy carbon, carbon paste, carbon fiber or boron-doped diamond [27–30]. Moreover, they have a wide potential window, no effect of dissolved oxygen and fast and simple film preparation steps, especially on the “in-situ” way. Sattayasamitsathit et al. employed an “ex-situ” bismuth film electrode as an alternative to the use of mercury drop and mercury film electrodes to determine tetracycline in commercial capsules [31]. Unlike bismuth electrode, antimony film electrode (SbFE) allows the use of acidic buffers with $\text{pH} \leq 2.0$ with a wide potential window. This electrode was employed successfully in electrochemical stripping analysis to determine metals [28–30]. However, there were only a few works that determine organic compounds with SbFE. In 2011 a SbFE prepared “ex-situ” was used for the first-time for the determination of sulfasalazine, a pharmaceutical compound, in commercial delayed-release tablets [32]. Few years later, Nigovic and Hocevar reported the determination of pantoprazole in pharmaceutical preparations, with successful analytical results using this electrode [33].

It is important to highlight that to the best of our knowledge, the use of SbFE prepared “in-situ” into organic compounds it has not been even employed. Some of the advantages of “in-situ” film preparation, is that improves sensitivity, selectivity and reproducibility for stripping voltammetry technique. Furthermore, this procedure consists of adding directly Sb (III) ions into the sample solution and a new reproducible surface is generated in the electrochemical cell at each analysis.

In order to identify the best variables operating conditions, is commonly and usefully used the response surface methodology as Draper Lin small composite design [34,35]. This method allows the simultaneous analysis of more than one factor at the same time and their statistical significance study to reduce the number of experiments, minimize reagent consumption and assess the interaction between the factors.

Then, in this paper an antimony film electrode, prepared “in-situ” to determine organic compounds as tetracyclines (tetracycline and oxitetracycline) is used for the first-time. The SbFE is prepared on a glassy carbon electrode and square wave cathodic stripping voltammetry to perform the antibiotic determination in Argentinean honey samples, was employed. A Draper Lin small composite design has been used for the first time to optimize the SWCSV parameters.

2. Material and methods

2.1. Honey samples preparation

Honey samples were obtained from beekeepers at different localities of Buenos Aires province, Argentina such as: Coronel Dorrego (M1), 30 de Agosto (M2), Sierra de la Ventana (M3), Pedro Luro (M4), Coronel Suarez (M5) and Villarino (M6). In these localities, *Diplotaxis tenuifolia* (L.) DC. (commonly known as “Flor amarilla”, Brassicaceae family), *Centaurea* sp. (Asteraceae family) and *Eucalyptus* sp (Myrtaceae family) are the predominant species [36]. Tetracyclines were extracted from honey samples by liquid extraction with acetone [37]. For this purpose, 1.0 g of honey was mixed with 6.0 mL of acetone and immerse in an ultrasonic bath for 30 min. This step was performed twice. Then, the solvent was evaporated and the dry extract suspended with supporting electrolyte solution (10.0 mL).

2.2. Reagents and solutions

All reagents were of analytical grade and solutions were prepared using ultra-pure water (18 M Ω). Antimony working solutions were prepared by a proper dilution of the atomic absorption stan-

dard solution (1.001 mg mL⁻¹, Merck). The waste, which contains antimony, is placed into a hermetically sealed recipient for further treatment. Hydrochloridric acid (Merck) solution 0.01 M was adjusted to pH 2.5 with sodium hydroxide (Merck) and was used as supporting electrolyte 0.01 M HCl/NaCl. Tetracycline and Oxytetracycline ($\geq 98.0\%$ w/w Sigma-Aldrich) stock solutions (500 $\mu\text{g mL}^{-1}$) were prepared in methanol and kept refrigerated in dark. These solutions are stable for a month [38]. All the experiments were carried out at room temperature and without removing oxygen.

2.3. Instrumentation

All voltammetric measurements were performed using an Epsilon potentiostat (BASi-Bioanalytical System, USA) and controlled by electrochemical analysis software. The RDE-2 Rotating Disk Electrode (BASi-Bioanalytical System, USA) module was employed for experimental measurements with rotating control.

A standard three-electrodes configuration was used with an antimony film prepared “in-situ” on a glassy carbon electrode as a working electrode (0.0707 cm² exposed area). The reference and counter electrodes were Ag/AgCl (3 M NaCl) and a platinum wire respectively. The glassy carbon surface was polished manually with 0.3 μm and 0.05 μm alumina, sonicated and thoroughly rinsed with ultra-pure water. All electrodes were provided from BASi-Bioanalytical System. Scanning Electron Microscopy (SEM) images were performed on a LEO EVO-40 XVP microscope (detector SE1).

2.4. Square wave cathodic stripping voltammetry measurements (SWCSV)

The square wave cathodic stripping voltammetry (SWCSV) were performed with “in-situ” deposition of antimony prepared on a glassy carbon electrode (GCE). The voltammograms were carried out in an electrochemical cell of 25.0 mL with the three electrodes immerse in 0.01 M HCl/NaCl solution (pH 2.5) as supporting electrolyte and 2000 $\mu\text{g L}^{-1}$ of Sb(III). The experiments were performed with a deposition potential of -0.600 V for 7 s and the working electrode rotation rate was kept at 6000 rpm. After 10 s of quiet time, voltammograms were recorded from -0.600 V to -1.000 V. In order to remove unwanted compounds from the electrode surface, a clean step was carried out by applying 0.20 V during 30 s under stirring conditions (6000 rpm) and polished after each measurement. Prior each experiment, cyclic voltammograms were performed at 100 mV s⁻¹ between -1.000 and -0.600 V in the electrolyte solution to stabilize the surface of the working electrode. The optimized square wave voltammetry parameters were: step potential (ΔE_s) 4 mV, frequency (f) 130 Hz and amplitude of the square wave (ΔE_{sw}) 160 mV. All experiments were performed in the presence of oxygen due it have no effect on the antimony film formation [30,39,40].

3. Results and discussion

The reduction response of tetracycline and oxitetracycline were studied with SbFE as working electrode. For this purpose, 10 μM of each antibiotic solution was used to scan cyclic voltammetry at 100 mV s⁻¹ from -1.10 to -0.600 V in 0.01 M HCl/NaCl (pH 2.5). As can be seen in Fig. 1a), the electrochemical reduction process is irreversible for both cases and presents a reduction peak at (-0.980 ± 0.05) V and the peak current intensities are similar. For this fact tetracycline is used for further studies. In order to evaluate the adsorption behavior of tetracycline on the electrode surface, the electrode was submerged in a 10 μM tetracycline solution 0.01 M HCl/NaCl (pH 2.5) and after one minute (under stirring condition) four successive cyclic voltammograms

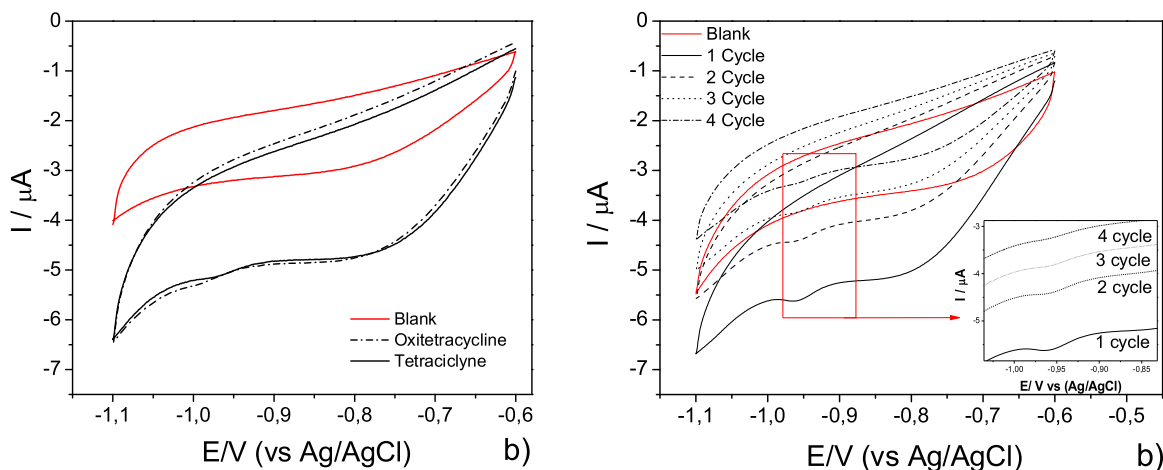


Fig. 1. a) Cyclic voltammograms of tetracycline and oxitetracycline $10 \mu\text{M}$ solutions at a SbFE in 0.01 M HCl/NaCl (pH 2.5). b) Cyclic voltammograms of four successive scans of tetracycline $10 \mu\text{M}$ at a SbFE in 0.01 M HCl/NaCl (pH 2.5), scan rate 100 mV s^{-1} . Red line inset shows a figure amplification of the peak current decrease at the different cycles performed (cycles 1 to 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

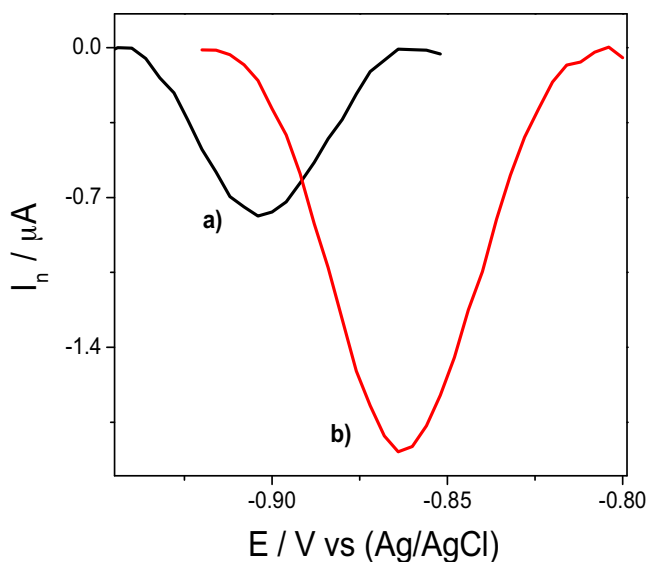


Fig. 2. SWCSV voltammograms of tetracycline $1.0 \mu\text{M}$ in 0.01 M HCl/NaCl pH 2.5, C_{Sb}^* : $2000 \mu\text{g L}^{-1}$, $\Delta E_s = 4 \text{ mV}$, $\Delta E_{\text{SW}} = 160 \text{ mV}$, $f = 130 \text{ Hz}$ and deposition time = 7 s at a) GCE and b) SbFE as working electrodes.

were registered (Fig. 1b)). It can be seen a gradual decrease of the reduction peak with increasing the number of consecutive cycles. This may be due to the adsorption of the reduced tetracycline, which leads to electrode passivation [41]. Furthermore, the dependence of reduction peak current (I_{pr}) on the scan rate (ν) (range of $25\text{--}300 \text{ mVs}^{-1}$) showed an increase in I_{pr} with an increase in ν , suggesting the adsorption-controlled process. The linear relationship between I_{pr} (A) and ν (mVs^{-1}) was: $-I_{\text{pr}} = (4.7 \pm 0.2) \times 10^{-10} \nu - (1.0 \pm 0.3) \times 10^{-8}$, $R^2 = 0.994$.

A comparative study of the SWCSV response of $1.0 \mu\text{M}$ TCs in 0.01 M HCl/NaCl (pH 2.5) at GCE and SbFE was carried out. The obtained voltammograms are shown in Fig. 2. Although both electrodes show a reduction peak, by using antimony electrode a stronger signal is obtained, under the same conditions. As can be seen in this figure, SbFE is suitable to determine TCs when compared to unmodified GCE. Fig. 2 shows that using antimony electrode the peak current is considerably higher than that at GCE. Furthermore, in Fig. 2, it was observed that the TCs reduction peak potential suffers a shift toward less negative values when SbFE was used ($-0.864 \pm 0.005 \text{ V}$) as compared with the GCE ($-0.904 \text{ V} \pm$

0.005 V). This shift is probably as a result of a faster electron transfer reaction due to an electrocatalytic effect caused by the surface modification [33].

3.1. Optimization of analytical parameters

Chemical analytical parameters such as pH and antimony concentration were optimized based on a univariate experimental method. SWCSV response for the reduction of TCs was studied in the pH range of 1.5 to 3.0, in HCl solution, by using an “in-situ” SbFE. A decrease in reduction current could be observed for pH higher or lower than 2.5, thus this value was selected as optimum. Fig. 3a) shows the voltammetric profiles for TCs at pH 1.5, 2.0, 2.5 and 3.0. As regards to Sb(III) concentration, a range between 1000 and $2500 \mu\text{g L}^{-1}$ was investigated. Fig. 3b) illustrates the four different Sb(III) concentrations studied and their respective TCs reduction current values. The stripping signal for TCs increased while increasing the concentration of Sb(III) up to $2000 \mu\text{g L}^{-1}$ and then started to decrease. Consequently, the optimum concentration chosen was $2000 \mu\text{g L}^{-1}$ Sb(III). All the optimization studies were performed in triplicate.

The optimization of adsorptive cathodic stripping voltammetry (AdCSV) and square wave voltammetry (SWV) parameters has been carried out by using experimental designs [42]. Among these methods, Draper Lin small composite design employs a reduced experimental runs [43,44]. Nevertheless, this is the first report where Draper Lin small composite design is used to optimize SWCSV parameters. For this proposed, the deposition time (t_d), amplitude of the square wave (ΔE_{SW}), frequency (f) and step potential (ΔE_s) were studied. The TCs net reduction current was used as response signal. The design was performed in twenty-one experiments, including sixteen experiments for the cube and star points, and five for the central point experiments. The extreme level of axial points selected was $\alpha=1$. Higher values of α , leads to negative values for frequency and deposition time. The levels for all variables were selected based on the screening studies, where the primary conditions and limits of each variable were found. Table 1 shows the levels chosen for each parameter. All the experiments were conducted randomly and the experimental data obtained were analyzed using ANOVA. This statistical method can be used to test the different causes of variation, where the variation due to a controlled factor can be discriminated from one caused for a random error. A graphical representation of the response surfaces applying Draper Lin small composite design was shown in Fig. 4. All the optimiza-

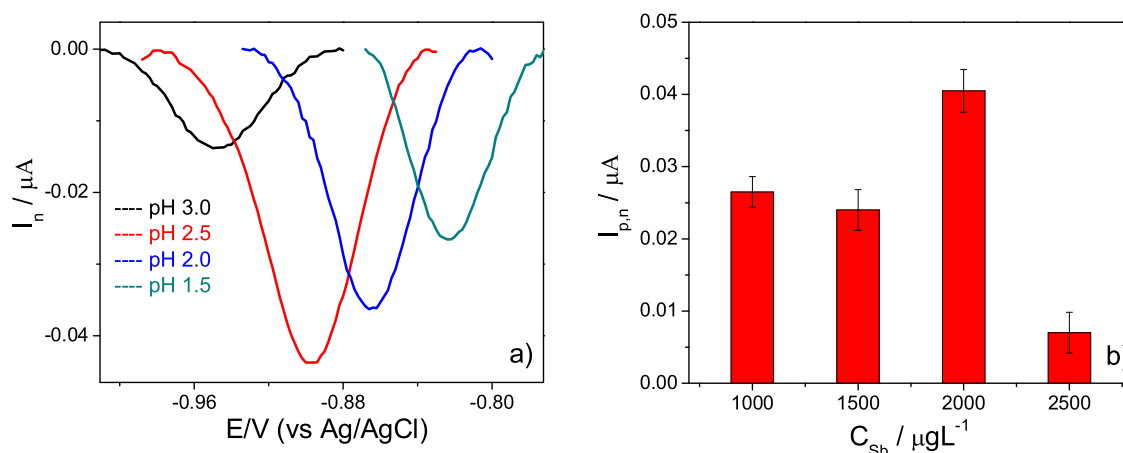


Fig. 3. a) SWCSV voltammograms of 10 μM tetracycline in 0.01 M HCl/NaCl at different pH. Experiments were performed on an “in-situ” deposited antimony film at optimal conditions: 2000 $\mu\text{g L}^{-1}$, $\Delta E_s = 4$ mV, $\Delta E_{\text{SW}} = 160$ mV, $f = 130$ Hz and deposition time = 7 s b) Histograms of response of 10 μM tetracycline in HCl/NaCl 0.01 M pH 2.5 with different concentration of antimony. Experiments were performed at the same SWCSV conditions.

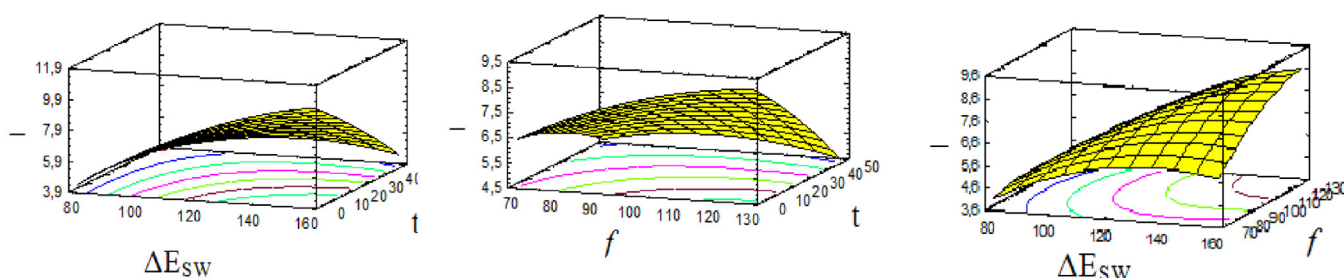


Fig. 4. Response surface diagrams of the Draper Lin model.

Table 1

Experimental values of independent variables in “Draper Lin” small composite design.

| Independent variable | Low | High |
|-----------------------------|-----|------|
| ΔE_s [mV] | 3 | 7 |
| ΔE_{SW} [mV] | 80 | 160 |
| f [Hz] | 70 | 130 |
| time [s] | 5 | 45 |

tion experiments were carried out on a tetracycline 10 μM in 0.01 M HCl/NaCl pH 2.5 solutions.

These surfaces can be represented by the following equation:

$$\begin{aligned}
 I_{p,n} = & (6.94 \pm 0.17) + (1.51 \pm 0.65) \Delta E_s + (3.28 \pm 0.65) \Delta E_{\text{SW}} \\
 & + (1.21 \pm 0.22)f - (2.46 \pm 0.65)t - (1.28 \pm 0.58) \Delta E_s \\
 & - (2.15 \pm 0.73) \Delta E_s \Delta E_{\text{SW}} - (0.31 \pm 0.33) \Delta E_s f + (0.56 \pm 0.76) \Delta E_s \\
 & t - (1.77 \pm 0.58) \Delta E_{\text{SW}}^2 + (1.67 \pm 0.33) \Delta E_{\text{SW}} f - (2.10 \pm 0.73) \Delta E_{\text{SW}} \\
 & t - (1.05 \pm 0.58)f^2 - (1.09 \pm 0.33)ft - (0.31 \pm 0.58)t^2 \quad (1)
 \end{aligned}$$

According Eq. (1), the most significant effects were: ΔE_{SW} , f and t_d . Furthermore, increments in frequency and amplitude of the square wave values provide an increment of the net peak current, but an increase of deposition time cause decreased on the analytical signal (as evidenced in Eq. (1)). Coefficient of determination (R^2) = 0.989 (in concordance with a residuals analysis), indicated a very good fit model.

The corresponding surface responses (Fig. 4) shows that the optimum values ΔE_{SW} and f are outside of the experimental region. Then, ΔE_{SW} and f are set at the extremes of the surface response. Higher values in ΔE_{SW} may cause a splitting of peak signals [45].

Otherwise, higher frequency values would not cause a significant increase in the peak current. The optimum SWCSV parameters were $\Delta E_s = 4$ mV, $\Delta E_{\text{SW}} = 160$ mV, $f = 130$ Hz and $t = 7$ s.

In order to confirm that the deposition of antimony film was carried out under the optimal conditions, a square wave anodic stripping voltammetry (SWASV) was performed between -0.60 to 0.10 V. The corresponding antimony oxidation peak is shown in Fig. 5a) at 0.00 V. Furthermore, scanning electron micrographs (SEM) of SbFE and GCE surfaces are shown in Fig. 5b) and c) respectively.

3.2. Analytical parameters

Fig. 6a) displays the square wave cathodic stripping voltammograms of tetracycline solution in the range 0.40 – 3.00 μM employing the SbFE, under experimental optimum conditions. The linear regression equation is presented in Fig. 6b) with a slope value of (1.46 ± 0.02) and an intercept of (-0.07 ± 0.02) . $I_{p,n}$ represents the maximum peak current and C_{TC}^* the tetracycline concentration (μM). The correlation coefficient (R) was 0.996. The detection limit (LOD) was 0.15 μM , estimated as 3 times $S_{y/x}/\text{slope}$ [46]. This value is lower than those found in the literature for some electrochemical sensors, as can be seen in Table 2.

The repeatability of SbFE was calculated as the percentage of variation relative standard deviation of six independent measurements of a 1.50 μM tetracycline solution. The obtained value was 8.23% for peak current and 0.41% for the corresponding peak potential.

3.3. Application to honey samples

The electroanalytical method was applied to determine TCs in six honey samples, using the optimum experimental condi-

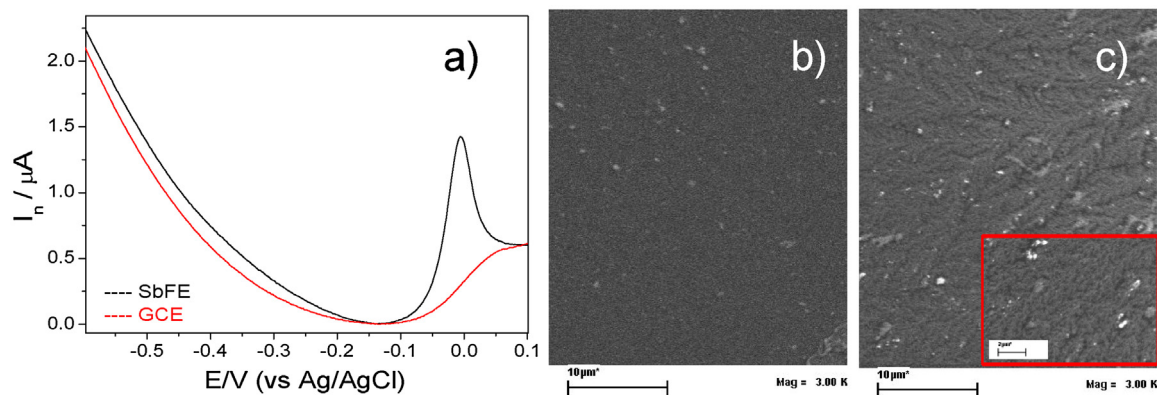


Fig. 5. a) Square wave anodic stripping voltammograms at GCE and SbFE in HCl/NaCl 0.01 M pH 2.5 under optimal conditions. SEM images (3000x magnification) at b) GCE and c) SbFE. Inset: SEM image for SbFE under 8000x magnification.

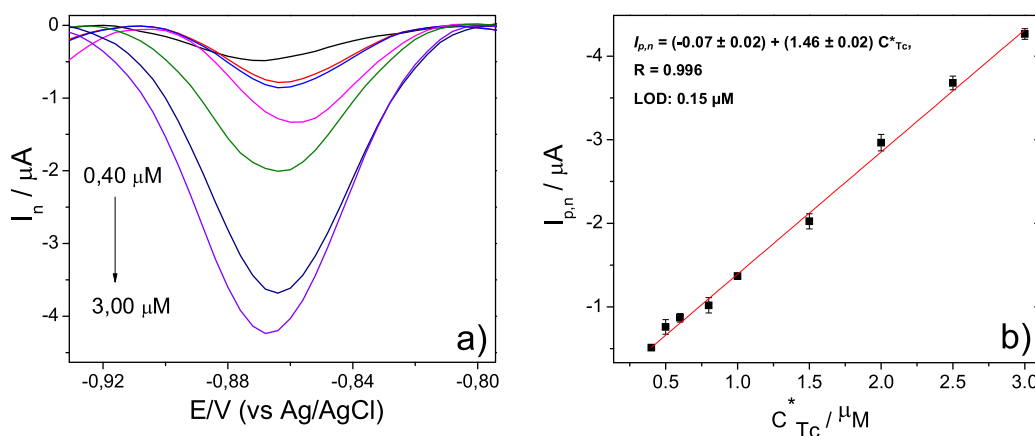


Fig. 6. a) Square wave cathodic stripping voltammograms of tetracycline solution 0.40 to 3.00 μM range at SbFE and b) Calibration curve at optimal SWCSV conditions.

Table 2

Comparison of the proposed method with others electrochemical sensors found in the literature.

| ELECTRODE | Linear range [μM] | Sensitivity [$\mu\text{A } \mu\text{M}^{-1}$] | LOD [μM] | Reference |
|---|--------------------------------|---|-----------------------|-----------------|
| BiFE (Bismuth film electrode) | 500–6000 | 7.70 | 1.2 | [31] |
| SPGE (Screen-printed gold electrode) | 1–500 | 6.09×10^{-4} | 0.96 | [45] |
| CPE GO/MWCNT-COOH (Modified carbon paste electrode) | 20–310 | 1.2×10^{-2} | 0.36 | [46] |
| GPU (Graphite-polyurethane composite electrode) | 4.00–40.0 | 0.03 | 2.80 | [47] |
| GPU (Graphite-polyurethane composite electrode) | 3.8–38.0 | 0.02 | 2.60 | [48] |
| GME (Gold microelectrode) | 2.25–22.5 | 0.21 | 0.20 | [49] |
| SbFE (“In-situ” antimony film electrode) | 0.40–3.00 | 1.46 | 0.15 | Proposed method |

Table 3

Recovery assays of tetracycline in spiked honey samples.

| Samples | Added (μM) | Found (μM) | Recovery % | Relative error % |
|---------|-------------------------|-------------------------|------------|------------------|
| M1 | 1.000 | 0.918 | 91.81 | – 8.19 |
| M2 | 1.000 | 1.097 | 109.69 | + 9.69 |
| M3 | 1.000 | 0.914 | 91.46 | – 8.54 |
| M4 | 1.000 | 1.055 | 105.54 | + 5.54 |
| M5 | 1.000 | 0.971 | 97.12 | – 2.88 |
| M6 | 1.000 | 0.992 | 99.25 | – 0.75 |

tions. Their content was negligible in the analyzed honey samples. Therefore, untreated honey samples were spiked and analyzed in triplicate. The obtained results are shown in Table 3.

4. Conclusion

The present study is the first investigation where an “in-situ” antimony film electrode was used for the determination of TCs using square wave cathodic stripping voltammetry. Some of the advantages of “in-situ” film synthesis are that the film is prepared in seven seconds, in the same solution as the analyte. Then a new surface is generated at each measurement. Under the same experimental conditions, the SbFE response shows a substantial increase in the peak reduction current than with GCE as work electrode, with the consequent increase in the sensitive. The proposed method is low cost analytical procedure, fast, exhibiting low consumption of reagent and became an attractive and widely used alternative to common mercury and bismuth electrodes. Moreover, it can be suc-

cessfully used for the determination of TCs in Argentinean honey samples.

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