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Amperometric biosensor based on laccase immobilized onto a nanostructured screen-printed electrode for determination of polyphenols in propolis

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ABSTRACT

This work describes the preparation of an electrochemical biosensor for polyphenols determination in propolis samples. The biosensing scheme is based on a nanocomposite film of laccase enzyme (Lac) immobilized on gold nanoparticles (AuNPs) electrodeposited in a screen-printed carbon electrode (SPCE) modified with polypyrrole (Ppy) through an in-situ electropolymerization. The electrodeposition of the AuNPs increases the available area for Lac immobilization. The nanocomposite film (Ppy/Lac/AuNPs/SPCE) was characterized by scanning electron microscopy, energy dispersive X-ray spectroscopy and cyclic voltammetry. Polyphenols were detected in ethanolic extracts of propolis (EEP), where in presence of the Lac oxidized to the polyphenols, and so they can be reduced on the Ppy/Lac/AuNPs/SPCE by amperometry at −450 mV vs Ag/AgCl. The calibration plot showed a linear response in the concentration range from 1 to 250 μM expressed as caffeic acid, with a limit of detection of 0.83 μM. The time required for analysis was 15 min, compared to the time (85 min) by spectrophotometric methods, especially the so-called Folin-Ciocalteu method. The method exhibited good selectivity, stability and reproducibility for detecting polyphenols in propolis samples.

1. Introduction

Polyphenols are a broad class of compounds that are present in many fruits, vegetables and their products [1]. In recent years, numerous studies have associated the consumption of foods rich in polyphenols with the prevention of cardiovascular diseases, certain types of cancer and other diseases because of their antioxidant properties [2].

Propolis is a resinous mixture that honey bees produce by mixing saliva and beeswax with exudate gathered from tree buds, sap flows, or other botanical sources. Polyphenols and flavonoids are considered the main bioactive components of propolis whose chemical composition varies according to the flora of each region [3,4]. The determination of the polyphenols content is not an easy task because of their chemical complexity, the difficulty of extraction and the presence of different interferents in the samples. One of the determinations widely applied for its measurement is the total phenolics content, obtained by spectrophotometric methods, especially the so-called Folin-Ciocalteu method [5]. However, this spectrophotometric approach yields an overestimation of the total polyphenolics content [6].

Alternatively, the bioanalytical sensor appears to be suitable for their detection and exhibits advantages such as easy sample preparation, selectivity, sensitivity, reproducibility and low cost [7,8] Electrochemical biosensors, in particular amperometric ones, are an attractive strategy to current used analytical methods. Commonly used amperometric biosensors are based on tyrosinase [9,10], peroxidase [11], pyrroloquinoline quinine dependent glucose dehydrogenase (GDH) [12] or cellobiose dehydrogenase (CDH) [13].

Electrochemical methods have attracted increasing attention because of their high sensitivity, fast response, simplicity, low instrumental costs, small sample volumes, and portability. Among the many electrochemical systems that can be applied for analytical purposes, the combination of amperometric detection (A) with screen-printed carbon electrodes (SPCE) can add mass production capabilities and represents one of the most convenient alternatives [14,15].

Moreover, the surface of the SPCE is amenable for modification by electrodeposition with a variety of metallic nanoparticles (NPs) such as copper, gold, platinum, palladium, or silver. Most of these modifications can provide increases in surface area and make sensors with

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enhanced limits of detection and improved electrocatalytic characteristics [16,17]. Following this line, gold nanoparticles (AuNPs) have great relevance, because of their good biocompatibility, excellent conducting capability and high surface to volume ratio. The incorporation of gold nanoparticles into electrochemical interfaces has infused new vigor in electrochemistry [18–20].

SPCE surface could be functionalized by electropolymerization of conductive polymers [21–25] and non-conductive polymers [26,27]. This process has been well studied and is one of the emerging additive method of biofabrication that may be used to guide and deposit biological entities such as enzymes, antibodies, and even whole cells to metallic or semi-conducting electrode sites of more complex devices [28,29]. Polypyrrole (Ppy) is a conductive electroactive polymer, due to its simple deposition of dopant and entrained macromolecules onto electrode surfaces, has been applied for the fabrication of amperometric, voltammetric, and impedimetric biotransducers [30–32].

Laccases (Lac) (*p*-diphenol: oxygen oxidoreductase, E.C.: 1.10.3.2) are copper containing oxidoreductases detected in many plants [33] and secreted by numerous fungi [34]. They can oxidase many different substrates, e.g. phenols and anilines, with the concomitant reduction of oxygen to water [35,36]. Therefore, Lac has been applied to many industrial processes including discolouration of dyes [37] pulp delignification [38], oxidation of organic pollutants [39], microbial transformation of natural products [40] and the development of biosensors [41,42].

In this work, we report the development of a nanostructured electrochemical biosensor, where the laccase enzyme was immobilized on AuNPs/SPCE modified with polypyrrole through in-situ electropolymerization. The nanocomposite film was characterized by scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), and cyclic voltammetry (CV). This biosensor was applied to the polyphenolic compounds determination in different propolis samples.

2. Materials and methods

2.1. Reagents and solutions

Analytical grade reagents and high purity solvents were used. Tetrachloroauric (III) acid ($HAuCl_4$), potassium chloride (KCl), pyrrole, caffeic acid (CA), potassium ferrocyanide/potassium ferricyanide ($K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$), sodium chloride (NaCl), monosodium phosphate (NaH_2PO_4), ethanol, sodium acetate, acetic acid, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and laccase commercial were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polytetrafluoroethylene membrane (PTFE) (0.2 μm) was obtained from Millipore, USA. Aqueous solutions were prepared using ultrapure water from a Milli-Q® integral water purification system. Working Standard solutions were daily prepared by appropriate dilution of the stock solutions with ultrapure water.

2.2. Apparatus

Amperometric measurements were performed using a BAS LC-4C potentiostat, and a BAS 100 B/W electrochemical analyzer (Bioanalytical System, West Lafayette, IN, USA) was used for voltammetric analysis in unstirred solutions, employing positive feedback routine to compensate the ohmic resistance. Cyclic voltammograms (CVs) and amperograms were obtained by employing a SPCE (DropSens C110, Asturias, Spain). It was made up of a graphite circular working electrode (Ø = 4 mm). Silver (Ag) and graphite electrodes were used as the pseudo-reference and the auxiliary electrode, respectively. The temperature for the electrochemical experiments was set at 25 ± 1 °C.

All pH measurements were made with an Orion expandable ion analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.). The morphology of AuNPs was studied by LEO 1450VP scanning electron microscope (SEM). The elemental composition of the nanostructured film was determined by energy dispersive X-ray spectroscopy (EDS) using a Genesis 2000 spectrometer (LABMEM, Argentina).

2.3. Determination of enzymatic activity

The activity of the Lac was quantified using the assay based on the ABTS oxidation. The assay reaction mixture consisted of 2.5 mM ABTS, 100 mM acetate/acetic acid buffer pH 3.50 and a suitable amount of enzyme to create a total reaction volume of 1 mL, which was incubated at 25 °C. The oxidation of ABTS was followed by monitoring the increase in absorbance at 420 nm. One unit of enzymatic activity (U) was defined as the amount of enzyme that is oxidized at 1 μM ABTS per minute under the assay conditions.

2.4. Modification of SPCE

An electrode pretreatment was carried out before each voltammetric experiment to improve the sensitivity and reproducibility of the results. For the AuNPs electrodeposition, the SPCE was immersed in a solution containing 0.01% $HAuCl_4$ in 0.1 mol L^{-1} KCl and applying a constant potential value of −400 mV for 120 s. Then, the modified electrode was rinsed by stirring at 250 rpm for 30 s in purified water and finally, carefully dried under a N_2 stream. Finally, 10 μL of 10 μg mL^{-1} Lac solution in 0.01 mol L^{-1} PBS pH 7.00 was placed on the surface of the AuNPs/SPCE working electrode and incubated overnight at 4 °C. After that, the modified electrode was immersed in 0.2 mol L^{-1} pyrrole solution in 0.1 mol L^{-1} KCl. In order to obtain the nanocomposite film on the electrode (Ppy/Lac/AuNPs/SPCE), the electropolymerization was performed at +700 mV for 600 s. The Ppy/Lac/AuNPs/SPCE was immersed and stored in PBS pH 7.0 at 4 °C when not in use. Fig. 1 shows the procedure of the biosensor preparation. Finally, the AuNPs/SPCE was optimized and characterized by SEM, EDS and CV.

2.5. Sample pretreatment and electrochemical behavior of propolis samples

Propolis samples were collected from Argentina (A1, A2) and Venezuela (V1, V3 and V7). Samples of Argentina were obtained by Apiculture Research Center (CEDIA) of Santiago del Estero, Argentina, while samples of Venezuela were obtained by the country beekeepers.

Samples were pulverized, homogenized, and stored in freezer at 4 °C when not in use. 1 g of each sample was extracted separately with 100 mL ethanol with magnetic stirring at 50 °C for 30 min [43]. Ethanolic extracts of propolis (EEP) were filtered and stored in the dark in hermetically closed bottles at 4 °C. These ethanolic solutions were directly used for the analysis. CVs of EEP was obtained using a modified SPCE from −750 to +1500 mV vs Ag/AgCl at 75 mV s^{-1} in a 0.1 mol L^{-1} acetate buffer pH 3.50 as supporting electrolyte (Fig. 2). The insert graph shows the voltammogram of the standard compound CA, which represents a typical quasi-reversible oxidation process with an anodic peak at +412 mV and a cathodic peak at +217 mV. CA is one of the widely used standards in the study of total phenolic compounds in different matrices [44,45], exhibiting a greater relative sensitivity for biosensors that use laccase [8,10]. It is also one of the main constituents of propolis [46].

The same quasi-reversible electrochemical behavior was observed for EEP, although shifted to cathodic potentials; which are around −250 mV and +250 mV, respectively. However, other authors found an irreversible electrochemical behavior of same EEP indicating that the diversity of each propolis also affect its electrochemical behavior [47]. The observed anodic peaks of the extracts were broader with respect to the standard. This may be due to the responses of several antioxidants e.g. flavonoids, phenolic acids and water-soluble vitamins with different oxidation potentials [48].

Additionally, the total polyphenol contents were determined using

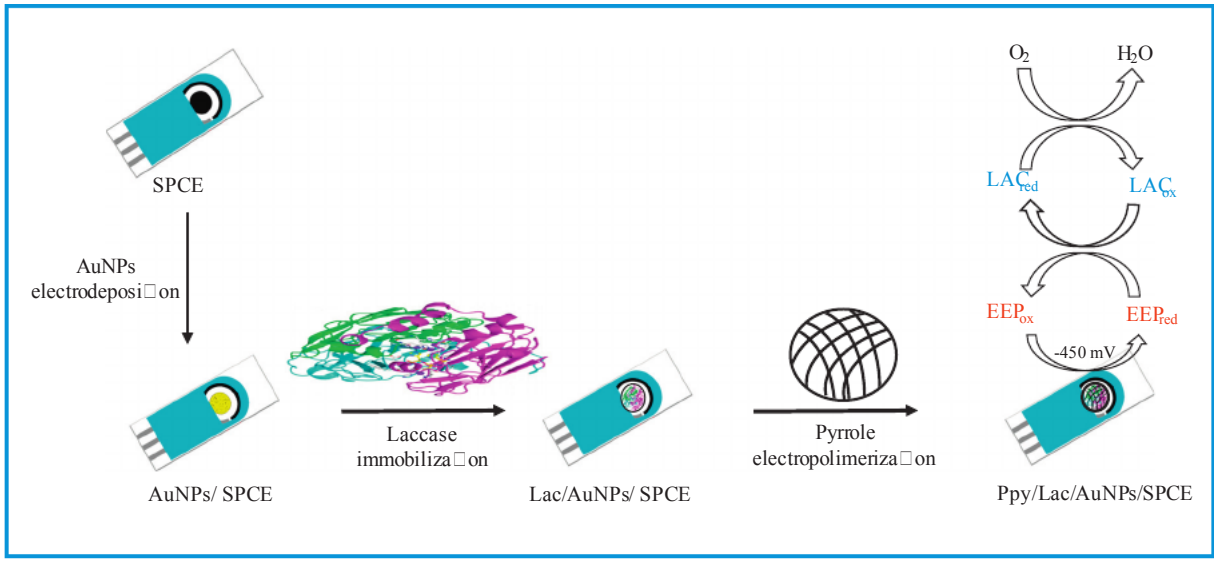

Fig. 1. Schematic representation of the SPCE modification with the nanocomposite film (Ppy/Lac/AuNPs) for the quantification of polyphenols in propolis samples.

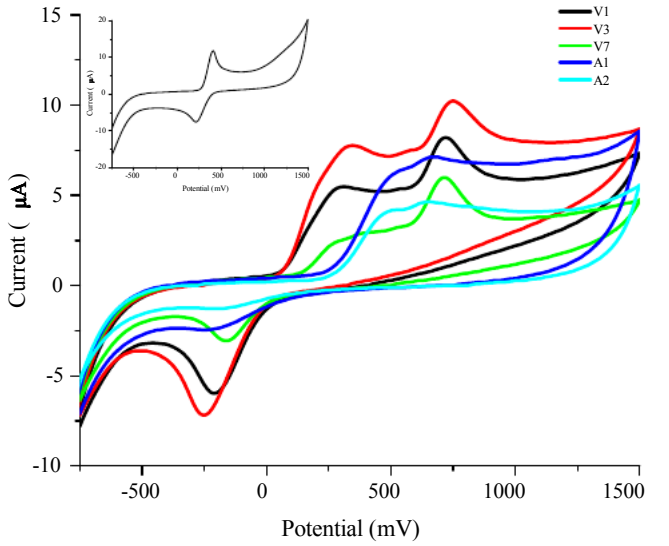

Fig. 2. Cyclic voltammograms of EEP solution in acetate buffer 0.1 mol L^{-1} pH 3.50 from −750 to +1500 mV vs Ag/AgCl at scan rate 75 mV s^{-1}. Insert: Cyclic voltammogram of 1 mmol L^{-1} caffeic acid in acetate buffer 0.1 mol L^{-1} pH 3.50 from −750 to +1500 mV vs Ag/AgCl at scan rate 75 mV s^{-1}.

the Folin–Ciocalteu method [49] in order to compare the results with the developed electrochemical biosensor. The samples were read at 765 nm by a spectrophotometer. The results were expressed in mg of CA per g of propolis.

2.6. Amperometric determination of polyphenols in propolis samples in Ppy/Lac/AuNPs/SPCE

Ppy/Lac/AuNPs/SPCE was conditioned with 0.1 mol L^{-1} acetate buffer pH 3.50 before the quantification step. EEP was added at different concentrations on the electrochemical biosensor in drop mode. The Lac immobilized onto the modified electrode (Fig. 1) oxidized the EEP; subsequently, the EEP was reduced over Ppy/Lac/AuNPs/SPCE by amperometry at −450 mV in 0.1 mol L^{-1} acetate buffer pH 3.50 as supporting electrolyte. Finally, the electrochemical biosensor was washed several times with 0.01 mol L^{-1} PBS pH 7.0 and stored at 4 °C when not in use.

3. Results and discussion

3.1. Characterization of AuNPs/SPCE

Fig. 3 shows the SEM images for the surface of unmodified SPCE (a), AuNPs/SPCE (b), and Ppy/Lac/AuNPs/SPCE (c). AuNPs with a diameter ranging from 30 to 50 nm were obtained. Then, a uniform nanostructured film of Ppy/Lac/AuNPs/SPCE was showed. The elemental composition of the nanocomposite film was determined by EDS. Fig. 3d shows the four peaks of interest at 0.3, 0.4, 2.3 and 9.8 keV, corresponding to the C, N and Au atoms, respectively.

$K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ system is a convenient and valuable tool to monitor the surface properties of the electrode during the modification steps. Fig. 4 shows the cyclic voltammograms for (a) blank solution (0.1 mol L^{-1} KCl at pH 6.50), (b) SPCE, and (c) AuNPs/SPCE, which were recorded in 1 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ 0.1 mol L^{-1} KCl pH 6.50 solution (scan rate = 50 mV s^{-1}). Well defined CVs and characteristics of a diffusion-controlled redox process were observed. By studying the electrochemical behavior of the electrodes, we could observe that the peak currents of the AuNPs/SPCE improved due to the increase in the active surface area of the modified electrode [14].

In the same way, during the polymerization stage with Ppy an increase of the peak currents was generated in the presence and absence of enzyme compared to the AuNPs/SPCE (insert graph in Fig. 4). In the case of Ppy/AuNPs/SPCE, this feature is produced by the enhancement in electrical conductivity and the electrocatalytic properties of the nanocomposite deposited on the surface of the SPCE. However, a slight decrease in the generated current was observed for Ppy/Lac/AuNPs/SPCE compared to Ppy/AuNPs/SPCE, because the immobilized enzyme in the nanocomposite decreases the electrical conductivity and presents higher resistance to electronic transfer [44].

3.2. Variables optimization for polyphenols determination

For the optimization steps, 1 μM polyphenol standard solutions were used. As already described above, the electrodeposition of AuNPs on the electrode surface was strongly affected by several parameters, such as the electrodeposition time (t_e) and electrodeposition potential (P_e). Both factors have been optimized to obtain the best analytical performance in our system. For the optimization of the t_e the potential used was −400 mV and the t_e was evaluated in a range from 20 to 140 s. In this case, when the t_e was ranged from 20 to 120 s, this situation leads to

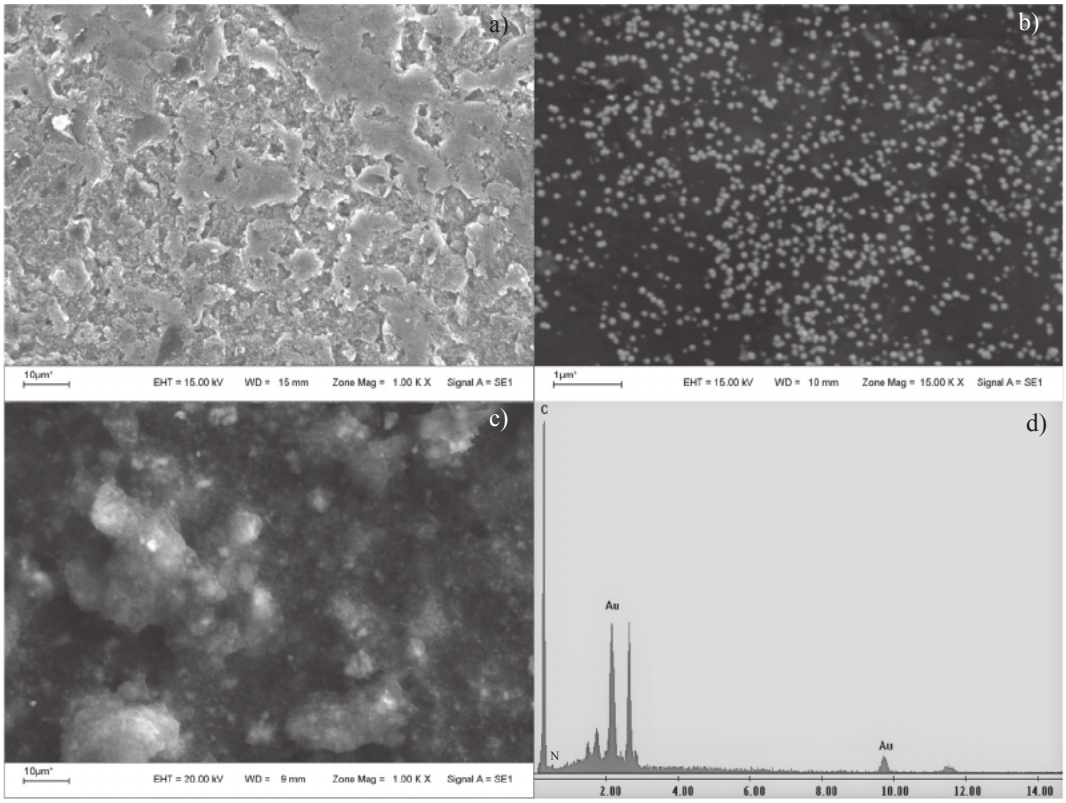

Fig. 3. Surface characterization by SEM of a) SPCE, b) AuNPs/SPCE, c) Ppy/Lac/AuNPs/SPCE and the corresponding elemental composition of d) Ppy/Lac/AuNPs/SPCE by EDS.

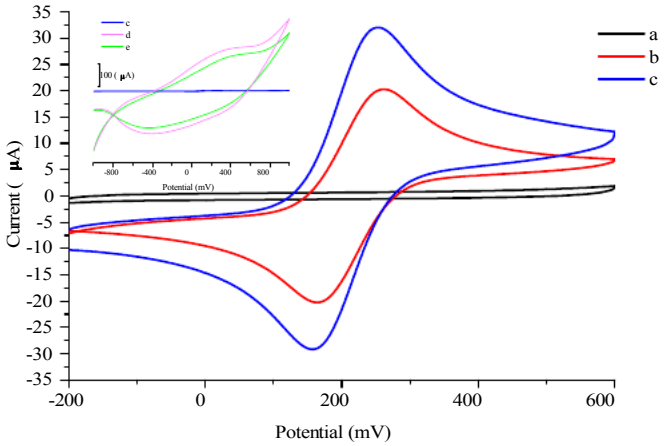

Fig. 4. Electrochemical characterization of a) blank solution (0.1 mol L^{-1} KCl at pH 6.50), b) SPCE, and c) AuNPs/SPCE. Cyclic voltammograms obtained in 1 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in 0.1 mol L^{-1} KCl pH 6.50 solution from −200 to +600 mV vs Ag/AgCl at scan rate: 50 mV s^{-1}. Insert: cyclic voltammograms of d) Ppy/AuNPs/SPCE and e) Ppy/Lac/AuNPs/SPCE in the same conditions.

significant current increases, an effect that plateaued at longer deposition times. Therefore, an electrodeposition time of 120 s was selected as optimum (Fig. 5a). The effect of the P_e was investigated using a deposition time of 120 s and varying the potential applied to the working electrode in the −50 to −500 mV range. As shown in Fig. 5b, significant increases in the current were obtained when the potential applied was changed from −50 mV to −400 mV. Because no further enhancements in the signal were obtained at lower potential values, an electrodeposition potential of −400 mV was selected as optimum.

The enzymatic response was studied in the pH range from 2.00 to 5.00, and showed maximum activity at pH 3.50. The pH value used was 3.50 in 0.1 mol L^{-1} acetate buffer (Fig. 5c).

The effect of the enzyme concentration was also investigated. This factor was evaluated using a range concentration from 2 to 20 µg mL^{-1}. As it can be observed in Fig. 5d, the response of the electrode increased with respect to the solution concentration until reaching a concentration of 10 µg mL^{-1}. Consequently, a laccase concentration of 10 µg mL^{-1} was selected as optimum for the preparation of the biosensor.

The pyrrole concentration to be employed in the immobilization procedure was also considered. This parameter was evaluated in the concentration range from 0.05 to 0.25 mol L^{-1} and reached a maximum at 0.2 mol L^{-1} value. The optimum pyrrole concentration value used was 0.2 mol L^{-1} (data not shown).

3.3. Analytical performance for the polyphenols detection using a Ppy/Lac/AuNPs/SPCE

The polyphenols calibration plot was obtained by plotting current (µA) vs total polyphenols concentration (expressed as µM CA, Fig. 6). In the insert graph of Fig. 6 can be seen the amperometric response observed after successive additions of CA in 0.1 mol L^{-1} acetate buffer at pH 3.50 for Ppy/Lac/AuNPs/SPCE. Applied potential of −450 mV vs Ag/AgCl. A linear relation was observed in the range from 1 to 250 µM CA in propolis. The linear equation is represented by $i = 0{,}020 + 0{,}883 \times C_{polyphenols}$. The correlation coefficient (r^2) for this plot was 0.998. The coefficient of variation (CV) for the determination of polyphenols was below 4.9% (n = 6). The limit of detection (LOD) was 0.83 µM. These values indicate that the proposed method exhibits a wide concentration range and a low LOD.

The precision of the electrochemical assay was tested with standards at 5, 20 and 40 µM CA. These series of analyses were repeated for 3 consecutive days in order to estimate the between-assay precision. The

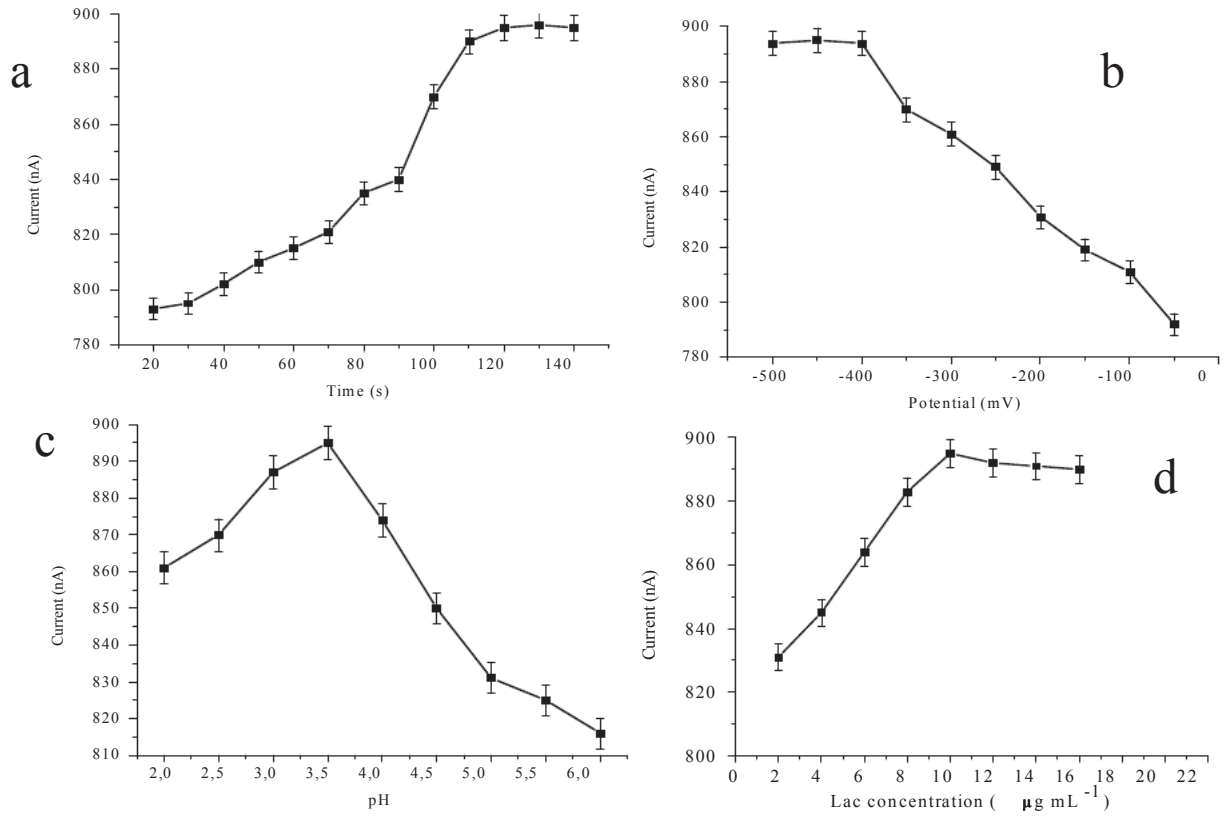

Fig. 5. Optimization of experimental conditions. a) Electrodeposition time of AuNPs, b) electrodeposition potential of AuNPs, c) pH, and d) laccase enzyme concentration.

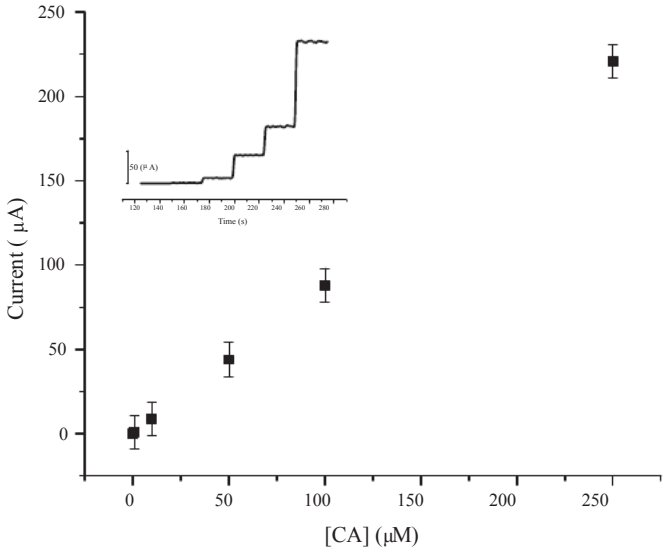

Fig. 6. Calibration curve obtained by plotting current (μA) vs total polyphenols concentration (expressed as μM caffeic acid). Insert: amperometric response observed after successive additions of CA in 0.1 mol L^{-1} acetate buffer pH 3.50 for Ppy/Lac/AuNPs/SPCE. Applied potential of −450 mV vs Ag/AgCl.

polyphenols assay showed a CV within-assay values were below 4.3% and the between-assay values were below 6.6%.

In this work, three propolis samples from Venezuela (V1, V3, V7), two propolis samples from Argentina (A1, A2) and a blank solution by electrochemical biosensor were analyzed. The content of polyphenols of these samples was previously determined by the Folin-Ciocalteu spectrophotometric method, as can be seen in Table 1. As expected, taking into account the completely different analytical methodologies used by

Table 1
Comparison between the electrochemical biosensor and spectrophotometric Folin-Ciocalteu method.

Samples	Electrochemical biosensor	Folin-Ciocalteu
V1	2.04 ± 0.08[a]	4.44 ± 0.62[a]
V3	1.22 ± 0.05	2.33 ± 0.13
V7	6.20 ± 0.10	6.29 ± 0.80
A1	27.40 ± 0.80	28.43 ± 1.90
A2	42.04 ± 0.86	49.60 ± 1.70

[a] Expressed in CA mg g^{-1}.

both types of methods, the absolute values of the polyphenol indices obtained are significantly different. The methodologies involved with the electrochemical biosensor have some advantages over the Folin-Ciocalteu method such as a high simplicity, a shorter detection time. These results prove that Ppy/Lac/AuNPs/SPCE has an excellent selectivity and sensitivity for the specific detection of polyphenols in propolis samples. Additionally, this enzymatic biosensor shows good reproducibility and long-term stability with more than 85% activity retention after one-month storage at 4 °C. Due to its low cost and easy-handling process, our proposed enzymatic sensor has the potential application in polyphenols quantification in propolis samples.

4. Conclusion

This article described the development of an electrochemical biosensor for polyphenols detection using a nanostructured functional platform. Ppy/Lac/AuNPs/SPCE nanocomposite was synthesized using soft conditions and the resulting material showed a good stability. Each nanomaterial used enhance the electronic transference and increase the response of the electrode. On the other hand, the developed method showed many advantages like portability, low cost, wide linear range,

accuracy with excellent LOD. Another important parameter was the analysis time that was only 15 min against the 85 min that takes the spectrophotometric method. Finally, this method could be a very promising analytical tool for the direct determination of polyphenols in the food production, ensuring safety and food quality, as well as consumer's health.

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