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Indirect determination of sulfite using a polyphenol oxidase biosensor based on a glassy carbon electrode modified with multi-walled carbon nanotubes and gold nanoparticles within a poly(allylamine hydrochloride) film

Elen Romão Sartori [a], Fernando Campanhã Vicentini [a], Orlando Fatibello-Filho [a,b,*]

[a] Departamento de Química, Universidade Federal de São Carlos, C.P. 676, 13560-970 São Carlos, SP, Brazil
[b] Instituto Nacional de Ciências e Tecnologia em Bioanalítica, 13083-970 Campinas, SP, Brazil

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ABSTRACT

The modification of a glassy carbon electrode with multi-walled carbon nanotubes and gold nanoparticles within a poly(allylamine hydrochloride) film for the development of a biosensor is proposed. This approach provides an efficient method used to immobilize polyphenol oxidase (PPO) obtained from the crude extract of sweet potato (*Ipomoea batatas* (L.) Lam.). The principle of the analytical method is based on the inhibitory effect of sulfite on the activity of PPO, in the reduction reaction of *o*-quinone to catechol and/or the reaction of *o*-quinone with sulfite. Under the optimum experimental conditions using the differential pulse voltammetry technique, the analytical curve obtained was linear in the concentration of sulfite in the range from 0.5 to 22 μmol L^{-1} with a detection limit of 0.4 μmol L^{-1}. The biosensor was applied for the determination of sulfite in white and red wine samples with results in close agreement with those results obtained using a reference iodometric method (at a 95% confidence level).

1. Introduction

Carbon nanotubes (CNTs) and nanoparticles (NPs) have become the focus of many scientific research studies as they exhibit interesting properties, such as narrow size distribution, provide significant reduction in overpotential, can increase electrode surface area, increase voltammetric response magnitude, and facilitate electron transfer between electrode and analyte. All of these characteristics make them potential candidates to play a catalytic role in the development of sensors and biosensors for applications in electroanalysis [1–4].

A major barrier for developing CNTs-based sensors or biosensors is the insolubility of CNTs in most solvents such as ethanol, methanol, isopropanol and water. Chemical oxidation with strong acids is the most common treatment for CNTs activation [6,7]. This pretreatment eliminates metallic impurities, removes the end caps and adds oxide groups (primarily carboxylic acids) to the tube ends and defect sites [2]. The integration of CNTs and NPs has received increasing attention because they often possess electrochemical, electromagnetic and structural features that are not available to the individual component alone. The coupling of CNTs–NPs can produce a synergic effect as they combines the excellent properties of gold nanoparticles (AuNPs) in terms of the immobilization of biomolecules (enzymes) with high retention of their biological activity and increase of signal transduction, and the electrocatalytic activity and/or amplification of analytical signal promoted by the CNTs.

Several examples of CNTs-AuNPs modified electrode have been reported previously [5,8–10], in which the AuNPs were grown on the surface of functionalized CNTs by chemical reduction of $AuCl_4^-$ ions [11] or by the electrodeposition method [12] and directly adsorbed on the CNTs surface [6,13].

One polyelectrolyte that has been used in the development of sensors and biosensors through the layer-by-layer technique is poly(allylamine hydrochloride) (PAH) [14,15]. It is a weak cationic polyelectrolyte with many ionizable amine groups in its backbone, being fully protonated in neutral and acid solutions but partly deprotonated in slightly basic solutions [15,16]. In general, this cationic polymer is used in combination with an anionic polyelectrolyte to form assembled multilayers. For example, sulfite oxidase (SO) was co-immobilized together cytochrome c using a sulfonated polyaniline and PAH interlayer in gold wire electrodes [14].

Sodium sulfite, sulfur dioxide, sodium and potassium bisulfite and sodium and potassium metabisulfite, among others, are used as additives (E220-228) in foods and beverages to ensure stability of color, taste, appearance and nourishing benefits during preparation, storage and transportation [17]. They are all chemically equivalent (SO_2, HSO_3^-, SO_3^{2-} and S_2O_5) after incorporation into

* Corresponding author at: Departamento de Química, Universidade Federal de São Carlos, C.P. 676, 13.560-970 São Carlos, SP, Brazil. Tel.: +55 16 33518098; fax: +55 16 33518350.
E-mail address: bello@ufscar.br (O. Fatibello-Filho).

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foods and beverages at a given pH. In particular, sulfur dioxide is widely used in winemaking to prevent oxidation and inhibit bacterial growth, which often leads to the deterioration of the quality of the wine. The level of sulfur dioxide permitted in wine is 350 mg L^{-1}. This is because SO_2 is toxic to some people and may cause allergic reactions, such as nausea, diarrhea, gastric irritation, nettle rash or swelling, and asthmatic attacks [18]. Thus, the development of an accurate analytical procedure for monitoring SO_2 concentration in wine is required in the beverage industry to verify whether the product meets quality requirements.

Several methods for the analytical determination of sulfite have been reported in the literature, such as spectrophotometry [19], capillary electrophoresis [20] and chromatography [21]. However, these methods suffer from some disadvantages, such as high cost, long analysis times, the need for sample pretreatment, and in some cases low sensitivity, making them unsuitable for routine analysis. Distillation with titrimetric quantification of the distilled sulfur dioxide and iodometry [22] is the traditional official method recommended by the Association Official Analytical Chemistry for sulfite determination in foods and beverages. The most common and widespread method for sulfite analysis is that originally developed by Monier-Williams [23], but this classical titration method is a rather time-consuming procedure, and requires an analyst that is detail oriented to ensure accuracy. One major limitation of iodometric titration protocols is that they are only suitable for uncolored samples, since the end-point is detected by the formation of the blue-like starch–iodine complex. Thus, unsophisticated methods are currently and continuously being studied for sulfite determination in foods and beverages, in particular the development of electroanalytical procedures using sensors and biosensors [24].

The development of biosensors for the sulfite determination is of considerable interest that allows a fast, selective and accurate determination, while minimizing costs and time-consuming sample pre-treatment [25–32]. These biosensors are commonly based on electrochemical monitoring of oxygen consumption or the hydrogen peroxide produced and the regeneration of electron-transfer mediators during the sulfite enzyme catalyzed reaction which converts sulfite to sulfate [33]. There are three types of sulfite enzymes presently known, and among them are sulfite oxidase (SO), found in animals or plants, and sulfite dehydrogenase (SDH), found in bacteria. Commercial availability of sulfite oxidase from animals has been restricted.

In this work, the modification of a glassy carbon electrode with multi-walled carbon nanotubes and gold nanoparticles within a poly(allylamine hydrochloride) film for the development of a biosensor is presented, in which the PPO obtained from crude extract of sweet potato (*Ipomoea batatas* (L.) Lam.) is immobilized in gold nanoparticles by using cystamine and glutaraldehyde. The principle of this analytical method is based on the inhibitory effect of sulfite on the activity of PPO, in the reduction reaction of *o*-quinone to catechol and/or the reaction of *o*-quinone with sulfite.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and the solutions were prepared with water (resistivity >18 MΩ cm) from a Milli-Q system (Millipore®). Sodium sulfite, catechol, poly(allylamine hydrochloride), multi-walled carbon nanotubes (of 20–30 nm in diameter and 0.5–2 μm in length; purity: ≥95%), hydrogen tetrachloroaurate (III) hydrate, cystamine sulfate hydrate, polyvinylpyrrolidone and 25% (v/v) glutaraldehyde were obtained from Sigma–Aldrich. Na_2HPO_4 and NaH_2PO_4 salts, used to prepare the supporting electrolyte, were purchased from Merck. All other chemicals were of analytical grade. The sweet potatoes and the samples of wine were purchased from a local supermarket.

A 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) was used as supporting electrolyte for the sulfite determination. An aqueous 0.1 mol L^{-1} sulfite stock solution was daily prepared in this supporting electrolyte solution and standardized by iodometry [22] just before use. Standard sulfite solutions were prepared from stock solution in the phosphate buffer supporting electrolyte and bubbled with ultrapure N_2 gas to prevent its chemical oxidation.

2.2. Apparatus

The voltammetric measurements were carried out using an Autolab Ecochemie model PGSTAT-12 (Utrecht, Netherlands) potentiostat/galvanostat controlled with the GPES 4.9 software. All the electrochemical experiments were conducted in a three-electrode single-compartment glass cell and degassing facilities for bubbling N_2(g), including a PPO-AuNPs-MWCNTs-PAH/GCE biosensor as working electrode, a Pt wire as auxiliary electrode, and an Ag/AgCl (3.0 mol L^{-1} KCl) reference electrode to which all electrode potentials hereinafter are referred. All experiments were carried out at an ambient temperature of 25.0 ± 0.5 °C.

The pH was measured at 25.0 ± 0.5 °C using an Orion pH-meter, Expandable Ion Analyser, model EA-940, employing a combined glass electrode with an Ag/AgCl (3.0 mol L^{-1} KCl) external reference electrode.

Spectrophotometric measurements were carried out using a spectrophotometer Femto model 435, employing a quartz cuvette with a 1.0 cm optical path.

FEG-SEM images were recorded using Supra 35-VP equipment (Carl Zeiss, Germany) with electron beam energy of 25 keV. A cut disk from a glassy carbon electrode was used for immobilizing the dispersion of AuNPs-MWCNTs in PAH solution, using the same drop-coating method employed for electrochemical analysis.

2.3. Preparation of biosensor

2.3.1. Functionalization of MWCNTs

MWCNTs were purified to remove metallic impurities from nanotubes with 2.0 mol L^{-1} HCl solution and then followed by treatment with acid from a mixture of HNO_3:H_2SO_4 (3:1, v/v) for 12 h at room temperature to allow the introduction of polar hydrophilic surface groups, mainly carboxyl group at the ends or at the sidewall defects of the nanotubes structure. After this, the suspension was centrifuged, and the solid was washed several times with ultrapure water until pH 6.5–7.0, and then dried at 120 °C for 6 h.

2.3.2. Preparation of AuNPs

AuNPs were synthesized through the citrate-mediated reduction of hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4$). The AuNPs solution was obtained by adding a volume of 4.0 mL of 0.5 mmol L^{-1} hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4$) in 200 mL of water at 85 °C under stirring. Next, to this solution was added 2.0 mL of 0.3 mol L^{-1} citric acid solution under stirring for 4 min. After that, the solution was placed in an ice-bath to room temperature. The color of the solution changed from a pale yellow to deep red and it was stored in an amber flask at room temperature.

2.3.3. Preparation of the crude extract of sweet potato

Polyphenol oxidase (PPO) was obtained from sweet potatoes, as reported previously [16], by using the crude extract as enzymatic source. Healthy sweet potatoes (*Ipomoea batatas* (L.) Lam.) were washed, hand-peeled and chopped. Subsequently, twenty-five grams of sweet potato were homogenized in a blender with 100 mL of the 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) containing 2.5 g of polyvinylpyrrolidone, for 2 min at 4–6 °C. The

suspension was filtered with four layers of cheesecloth and centrifuged at 18,000 rpm for 30 min at 4 °C. It was stored at this temperature in a refrigerator and used as the enzymatic source of PPO after the determination of the polyphenol oxidase activity. The activity of PPO was determined as described elsewhere [17], by measurement of absorbance at 410 nm of *o*-quinone produced by the reaction between the crude extract containing PPO and catechol solution. The total protein concentration was determined in triplicate by the method of Lowry et al. [34] using serum albumin as standard.

2.3.4. Preparation of PPO-AuNPs-MWCNTs-PAH/GCE biosensor

A mass of 1.0 mg of MWCNTs and 1.0 mg of PAH was added to 500 μL of AuNPs solution and subjected to ultrasonication for 10 min to give a stable black AuNPs-MWCNTs-PAH suspension. This suspension was stored in a refrigerator at 4 °C. The GCE (5-mm diameter) was used as base electrode. Prior to modification, the electrode was carefully polished to a mirror finish, sequentially with metallographic abrasive paper (# 6) and slurries of 0.3- and 0.05-μm alumina. After being rinsed with doubly distilled water, the polished GCE was sonicated for 5 min with acetone and then with ultrapure water, and dried at room temperature. A smooth glassy carbon surface is important to support efficient the AuNPs-MWCNTs-PAH suspension. The biosensor was prepared by first dropping 20 μL of the AuNPs-MWCNTs-PAH suspension on the cleaned surface of the GCE using a micropipette and allowing it to dry for 2 h. Afterwards, the modified GCE was placed in the electrochemical cell containing 0.1 mol L^{-1} KCl and fifty cycles in the potential range from −0.3 to 0.7 V at a scan rate of 100 mV s^{-1} were applied using cyclic voltammetry (CV) method. The electrode was rinsed carefully with water. After that, 20 μL of 10 mmol L^{-1} cystamine (CYS) solution was dropped on the modified surface of GCE with AuNPs-MWCNTs-PAH and the electrode was allowed to dry. The electrode was rinsed carefully with water. Then 20 μL of 2.5% (v/v) of glutaraldehyde (GA) in the phosphate buffer solution (pH 7.0) was dropped on the surface and allowed to dry for 1 h. The electrode was rinsed carefully with 0.1 mol L^{-1} phosphate buffer solution (pH 7.0). Finally, 20 μL of PPO was dropped on the top of the electrode and allowed to dry for 1 h. Fig. 1 shows a schematic view of the mechanisms carried out in each step of the biosensor preparation. The PPO-AuNPs-MWCNTs-PAH/GCE biosensor was stored in a flask containing 0.1 mol L^{-1} phosphate buffer in a refrigerator when not in use.

2.4. Analytical procedure

After optimizing the differential pulse voltammetry (DPV) parameters for the proposed methods, the analytical curve was obtained by adding different aliquots of the sulfite standard solutions into the electrochemical cell containing 50 μmol L^{-1} catechol standard solution in 10 mL of the 0.1 mol L^{-1} phosphate buffer solution (pH 7.0). DP voltammograms were obtained after the addition of each aliquot. All measurements were carried out in triplicate ($n = 3$) for each concentration.

For the recovery studies, an aliquot of the wine sample and three consecutive aliquots of standard solutions of sulfite were added to 10 mL of 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) containing 50 μmol L^{-1} catechol standard solution. Set enrichment analyses were carried out in triplicate with the wine sample and with increasing concentration of the sulfite.

For the determination of sulfite in wine samples, an aliquot of each sample was transferred directly to the electrochemical cell containing 50 μmol L^{-1} catechol in 10 mL of the 0.1 mol L^{-1} phosphate buffer solution (pH 7.0), and consequently the DP voltammograms were obtained. The concentration of sulfite in each sample solution was determined by the standard addition method. Three determinations were carried out for each sample, and the standard deviation was calculated.

2.5. Reference method

The iodometric titration method [22] was employed in order to compare the results obtained using the proposed DPV method. An accurate sample volume (20 mL) was transferred into a 125 mL conical flask and an aliquot of 5 mL of standard iodine solution was added. The excess of iodine was titrated with sodium thiosulfate standard solution using starch as indicator. These titrations were carried out as quickly as possible and the end point was indicated by a light blue color.

3. Results and discussion

3.1. Determination of PPO activity

Initially, the PPO activity, total protein concentration and specific activity in the sweet potato crude extract were determined. The values of PPO activity and total protein concentration were 1305 units of PPO per mL (U mL^{-1}) and 0.442 mg mL^{-1}, respectively. The specific activity of 2952 U mg^{-1} of protein was calculated as the ratio between the PPO activity and total protein concentration.

3.2. Electrochemical characterization of the AuNPs-MWCNTs-PAH modified electrode

When the AuNPs-MWCNTs were sonicated with PAH for 10 min, the formation of a stable and homogeneous black suspension was observed, due to the electrostatic interaction between carboxyl groups on the chemically oxidized nanotubes surface and polyelectrolyte chains. The gold nanoparticles were anchored to the surface of the nanotubes through the electrostatic interaction between the

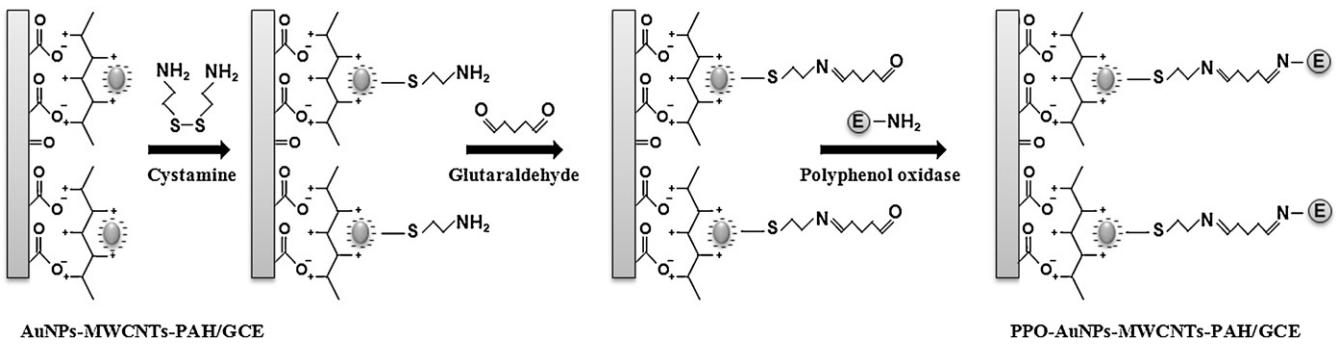

Fig. 1. Schematic illustration of the different steps of preparation of the PPO-AuNPs-MWCNTs-PAH/GCE biosensor. Not drawn in to scale.

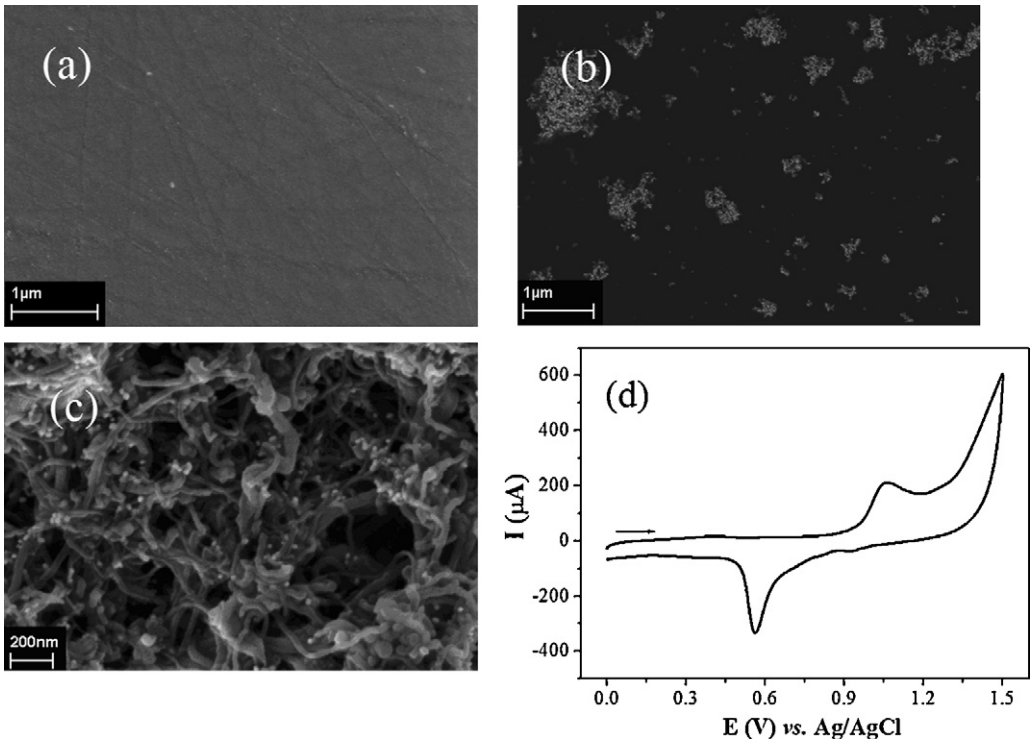

Fig. 2. FEG-SEM images of the surface of: (a) GCE, (b) AuNPs-PAH/GCE, (c) AuNPs-MWCNTs-PAH/GCE, and (d) cyclic voltammogram of the AuNPs-MWCNTs-PAH/GCE in 0.5 mol L^{-1} H_2SO_4 at a scan rate of 50 mV s^{-1}.

polyelectrolyte and the nanoparticles, as reported in a previous work [9].

The utilization of AuNPs in this work is important since it allows the development of a biosensor based on the immobilization of the polyphenol oxidase by using cystamine and glutaraldehyde reagents. The integration of functionalized MWCNTs and AuNPs-PAH film enables a better distribution of AuNPs in the obtained film with high electroactive area, as will be shown below.

Fig. 2 shows the FEG-SEM images of GCE (a), AuNPs-PAH/GCE (b), and AuNPs-MWCNTs-PAH/GCE (c). A clear difference in terms of surface roughness is observed among these electrodes. It can be observed from Fig. 2(c) that a relatively dense film was formed and the GCE surface was completely covered with carbon nanotubes, when compared to unmodified GCE (Fig. 2(a)). It can be seen in Fig. 2(c) that the AuNPs are homogeneously distributed on the MWCNTs and are uniform in size, and that each AuNP has an average diameter of 29 nm. AuNPs dispersed well on the functionalized MWCNTs, when compared to AuNPs-PAH/GCE (Fig. 2(b)). Further characterization of the AuNPs was achieved by recording the CV of the AuNPs-MWCNTs-PAH/GCE in 0.5 mol L^{-1} H_2SO_4 at a scan rate of 50 mV s^{-1}. As shown in Fig. 2(d), the electrode exhibits the characteristic feature of the redox reaction of Au with oxidation and reduction peaks of Au oxide. These results further verify the distribution of AuNPs in the functionalized MWCNTs-PAH film.

The electroactive area of GCE, PAH/GCE, AuNPs-PAH/GCE and AuNPs-MWCNTs-PAH/GCE was calculated in a 1.0 mmol L^{-1} potassium hexacyanoferrate (III) in 0.1 mol L^{-1} KCl solution, using the hexacyanoferrate(III) reduction peaks and applying the Randles-Sevcik equation [35] for a reversible process:

$$I_{cp} = 2.69 \times 10^5 n^{3/2} A D^{1/2} C \nu^{1/2} \quad (1)$$

where I_{cp} is the anodic peak current (A), n is the number of electrons transferred in the redox reaction, A is the electroactive area (cm^2), D is the diffusion coefficient of $[Fe(CN)_6]^{3-}$ in 0.1 mol L^{-1} KCl solution (6.2×10^{-6} cm^2 s^{-1}), ν is the potential scan rate (V s^{-1}), and C is the concentration of $[Fe(CN)_6]^{3-}$ in bulk solution (mol cm^{-3}). The slopes of I_{ap} *vs.* $\nu^{1/2}$ graphs for the oxidation process (data not shown) were: 1.09×10^{-4}, 1.24×10^{-4}, 1.34×10^{-4} and 4.72×10^{-4} A $s^{1/2}$ $V^{-1/2}$ for GCE, PAH/GCE, AuNPs-PAH/GCE and AuNPs-MWCNTs-PAH, respectively. The calculated electroactive area was 0.162 cm^2, 0.185 cm^2, 0.201 cm^2 and 0.705 cm^2 for GCE, PAH/GCE, AuNPs-PAH/GCE and AuNPs-MWCNTs-PAH/GCE, respectively, and AuNPs-MWCNTs-PAH/GCE increased by a factor of 4.3 compared to the GCE. The above results also show that the integration of MWCNTs to AuNPs leads to a higher electroactive area.

3.3. *Voltammetric behavior of catechol at AuNPs-MWCNTs-PAH/GCE*

The electrochemical behavior of catechol at AuNPs-MWCNTs-PAH/GCE was studied by cyclic voltammetry (CV). Initially, the AuNPs-MWCNTs-PAH/GCE response was compared to the cyclic voltammetric responses of a GCE, PAH/GCE and AuNPs-PAH/GCE for a 1.0 mmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.5), as shown in Fig. 3. As can be seen, at the GCE (Fig. 3(a)), catechol has an oxidation peak at about 0.380 V and the corresponding oxidation product (*o*-quinone) has a cathodic peak at about 0.004 V, and vice versa. The peak potential separation (ΔE_p), the difference between the anodic peak potential (E_{ap}) and the cathodic peak potential (E_{cp}), is about 0.376 V, which indicates that catechol exhibits an irreversible electrochemical behavior at the GCE. In PAH/GCE (Fig. 3(b)) or AuNPs-PAH/GCE (Fig. 3(c)) similar responses to that obtained using the GCE can be observed. On the other hand, in the AuNPs-MWCNTs-PAH/GCE (Fig. 3(d)), the electrochemical reversibility of catechol is much improved, decreasing the peak potential separation and increasing the associated currents for this electrode, showing that the catalytic activity of the AuNPs-MWCNTs-PAH/GCE towards the electroxidation of catechol. For instance, the reduction peak current goes from 44.5 μA at GCE to 55.8 μA at AuNPs-MWCNTs-PAH/GCE, while the peak separation

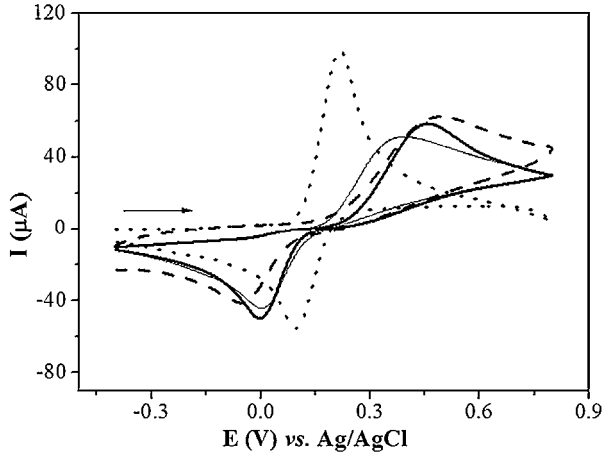

Fig. 3. Cyclic voltammograms, after background subtraction, for 1.0 mmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.5), at (solid line) GCE, (solid black line) PAH/GCE, (dashed line) AuNPs-PAH/GCE, and (dotted line) AuNPs-MWCNTs-PAH/GCE, at a scan rate of 100 mV s^{-1}.

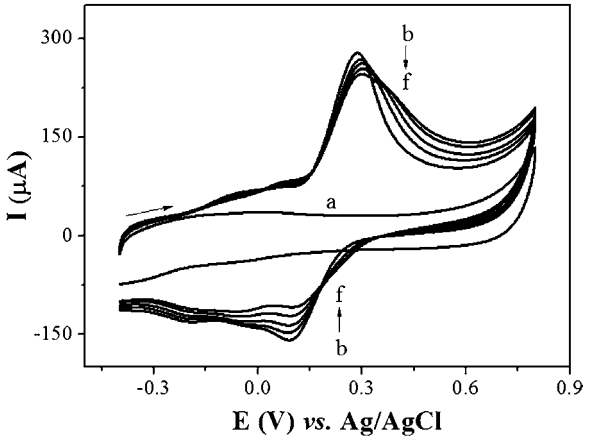

Fig. 5. Cyclic voltammograms obtained using a PPO-AuNPs-MWCNTs-PAH/GCE biosensor as working electrode for (a) 0.1 mol L^{-1} phosphate buffer solution (pH 7.5), (b) 1.0 mmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.5), and in the presence of (c) 0.2 mmol L^{-1}, (d) 0.4 mmol L^{-1}, (e) 0.6 mmol L^{-1} and (f) 0.8 mmol L^{-1} sulfite in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.5), at a scan rate of 100 mV s^{-1}.

decreases from 0.376 V at GCE to 0.155 V at AuNPs-MWCNTs-PAH/GCE. These results demonstrate that there is a significant improvement in the response of the electrode when nanotubes are incorporated in the AuNPs-PAH film, indicating that the modification of GCE with AuNPs-MWCNTs-PAH can greatly improve the electron transfer rate.

3.4. Electrochemical oxidation of catechol on the PPO-AuNPs-MWCNTs-PAH/GCE biosensor in the absence and in the presence of sulfite

The polyphenol oxidase (PPO) was attached to the AuNPs-MWCNTs-PAH film by using cystamine and glutaraldehyde as described in Section 2. The proposed biosensor was used in the present study to evaluate the behavior of catechol. In addition, inhibition of PPO activity by sulfite was investigated for indirect sulfite determination in wine samples. Procedures based on the inhibition of an enzyme usually offer high sensitivity and/or selectivity. Several studies based on the inhibition of oxireductase enzymes such as polyphenol oxidase [19,36–38], peroxidase [39,40] and laccase [41] have been reported in the literature.

Fig. 4 presents the difference in CV responses between the AuNPs-MWCNTs-PAH/GCE and the proposed PPO-AuNPs-MWCNTs-PAH/GCE biosensor under identical experimental conditions. As can be seen, when PPO was further immobilized on the surface of the AuNPs-MWCNTs-PAH/GCE, the electrochemical responses increased greatly. The biosensor (Fig. 4(a)) presents a higher voltammetric response (higher anodic and cathodic current peaks) than the AuNPs-MWCNTs-PAH/GCE (Fig. 4(b)) in the presence of catechol solution.

The effect of sulfite on the electrochemical behavior of catechol was performed at the PPO-AuNPs-MWCNTs-PAH/GCE biosensor in 0.1 mol L^{-1} phosphate buffer solution (pH 7.5) as shown in Fig. 5(a). As can be seen in this figure the cyclic voltammogram of the PPO-AuNPs-MWCNTs-PAH/GCE in pure supporting electrolyte does not show any anodic or cathodic peaks. In Fig. 5(b), the catechol is oxidized by PPO in the presence of molecular oxygen to *o*-quinone and this product is then electrochemically reduced back to catechol. On the other hand, when the sulfite solution in the concentrations of (c) 0.2 mmol L^{-1}, (d) 0.4 mmol L^{-1}, (e) 0.6 mmol L^{-1}, and (f) 0.8 mmol L^{-1} is added to the catechol solution, both the anodic and cathodic peak currents showed a remarkable decrease. A schematic view of the catalytic reaction between catechol and sulfite occurring on the biosensor surface is presented in Fig. 6. According to

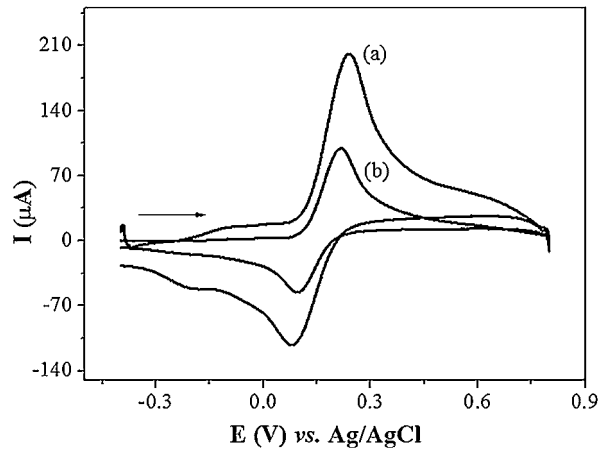

Fig. 4. Cyclic voltammograms, after background subtraction, for 1.0 mmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.5), at (a) AuNPs-MWCNTs-PAH/GCE and (b) PPO-AuNPs-MWCNTs-PAH/GCE, at a scan rate of 100 mV s^{-1}.

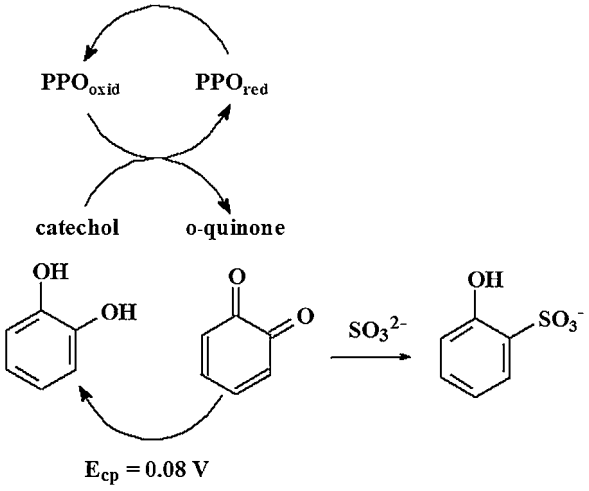

Fig. 6. Schematic representation of the enzymatic reaction between catechol, *o*-quinone, PPO and sulfite on the PPO-AuNPs-MWCNTs-PAH/GCE biosensor. Oxid: oxidized form and red: reduced form.

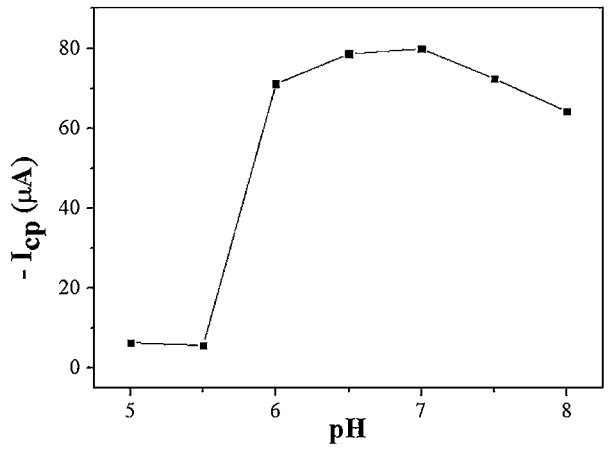

Fig. 7. Effect of pH on the analytical response for a 1.0 mmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution at the PPO-AuNPs-MWCNTs-PAH/GCE biosensor with pH values, at a scan rate of 100 mV s^{-1}.

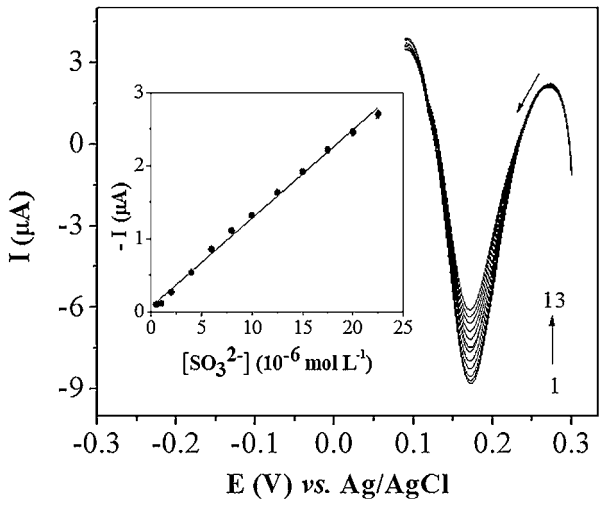

Fig. 8. Differential-pulse voltammetric responses obtained using the PPO-AuNPs-MWCNTs-PAH/GCE biosensor for: (1) 50 μmol L^{-1} catechol; (2) 0.5; (3) 1.0; (4) 2.0; (5) 4.0; (6) 6.0; (7) 8.0; (8) 10; (9) 12; (10) 15; (11) 17; (12) 20; and (13) 22 μmol L^{-1} sulfite in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0). Inserts: analytical curve for the sulfite.

previous related works in the literature [17,36,42,43], the sulfite can act by direct inhibition of the enzyme PPO, in the reduction reaction of *o*-quinone to catechol and/or it may react with the *o*-quinone by the formation of an *o*-quinone-sulfite compound because sulfite is a strong nucleophile [19,42].

Cyclic voltammetric studies for a solution containing only sulfite on the PPO-AuNPs-MWCNTs-PAH/GCE biosensor under these conditions do not show any electrochemical activity for sulfite in the investigated potential range.

3.5. Effect of pH

Given that pH is a critical parameter in the determination of enzymatic activity, the effect of pH on the analytical response of the PPO-AuNPs-MWCNTs-PAH/GCE biosensor was studied over the pH range 5.0–8.0 in 0.1 mol L^{-1} phosphate buffer solution by CV using a 1.0 mmol L^{-1} catechol solution. The plot of cathodic peak current *vs.* pH is shown in Fig. 7. With the increase in pH value, the cathodic peak current of catechol increased until the pH value reached 7.0 and then decreased. Therefore, this pH was selected for further experiments.

3.6. Effect of scan rate

The effect of scan rate on the analytical response was evaluated by varying the scan rate during the electrocatalytic oxidation of 1.0 mmol L^{-1} catechol solution in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) at the PPO-AuNPs-MWCNTs-PAH/GCE biosensor. Cyclic voltammograms were obtained at different scan rates from 0.005 to 0.5 V s^{-1}. With an increase in scan rate, a gradual shift in the redox peak potentials occurred with increasing peak-to-peak separation (not shown). The oxidation peak currents were proportional to the square root of the scan rate, indicating that the catechol oxidation is a diffusion controlled process [44]. The slope of 0.62, obtained from of the plot of the $\log I_{cp}$ *vs.* $\log \nu$, is in close agreement with the theoretical value of 0.5 for a diffusion-controlled process.

3.7. Analytical curve and validation parameters of the method proposed for indirect sulfite determination

The DP voltammograms obtained using the PPO-AuNPs-MWCNTs-PAH/GCE biosensor presented features similar to those initially obtained by CV. The optimization of the DPV parameters was carried out in a 0.5 mmol L^{-1} catechol solution. The experimental parameters influencing the DPV response and their corresponding investigated ranges are: pulse amplitude (10 mV $\leq \alpha \leq$ 75 mV), scan rate (1 mV $s^{-1} \leq \nu \leq$ 7 mV s^{-1}), and modulation time (5 ms $\leq t \leq$ 85 ms). The obtained optimum values for these parameters were: α = 50 mV, ν = 1 mV s^{-1}, and t = 75 ms. The previous optimized DPV experimental parameters were employed to record the analytical curve for sulfite using the PPO-AuNPs-MWCNTs-PAH/GCE biosensor.

When the current reached a steady state after the addition of the 50 μmol L^{-1} catechol to the 0.1 mol L^{-1} phosphate buffer solution (pH 7.0), DP voltammograms were recorded in the presence of various concentrations of sulfite. It was found that the current decreased with increasing of sulfite concentration, as shown in Fig. 8. The insert in this figure depicts the respective analytical curve obtained for concentration range from 0.5 to 22 μmol L^{-1} sulfite in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) (r = 0.9962), for which the corresponding regression equations is $I_{ap}/\mu A = 6.34 \times 10^{-8} + 0.122\ [c/(\text{mol L}^{-1})]$, where I_{ap} is the anodic peak current and c the sulfite concentration in mol L^{-1}. The detection limit value was 0.4 μmol L^{-1}, based on a signal-to-noise ratio of three, low enough for trace sulfite determination.

The result of the analytical determination of sulfite by this method showed a low detection limit, which is much better than the previous reports based on direct determination using biosensors [25–31], as shown in Table 1, or using other chemically modified electrodes [45–47]. Considering the advantages of PPO-AuNPs-MWCNTs-PAH/GCE biosensor such as simplicity of preparation and use, relative low cost, stability, and lifetime of the developed biosensor it can be used for indirect determination of sulfite in wine samples.

The intra-day repeatability of the peak current was determined by successive measurements (n = 10) of 8.0 μmol L^{-1} sulfite solution in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) containing 50 μmol L^{-1} catechol. A relative standard deviation of 1.4% was obtained, showing thus the good repeatability of the proposed method. The inter-day repeatability of the peak current was evaluated by measuring the peak current for similar fresh solutions over a period of 5 days. Compared to the obtained original peak current values, discrepancies of only up to 2.4% were observed in the measurements with fresh solutions prepared daily.

Table 1
Comparison of the analytical parameters obtained using different biosensors for the determination of sulfite.

Electrode	Concentration range (μmol L^{-1})	*LOD* (μmol L^{-1})	Reference
SO/polytyramine/platinized glassy carbon electrode	2.0–300	1.0	[25]
SO/teflon membrane/oxygen probe	200–1800	200	[26]
SO/carbon paste electrode	10–1000	10	[27]
SO/polypyrrole/platinum disc electrode	0.9–400	0.9	[28]
SO/mercury thin film/GCE	200–2800	200	[29]
SO/polyaniline aluminium modified electrode	6.0–5000	2.0	[30]
SO/cyt c/screen-printed electrode	40–5900	40	[14]
SO/cyt c/gold wire electrode/SAMs	1.0–60	1.0	[31]
SDH/cyt c/gold electrode/SAMs	0.5–5.5	4.4×10^{-5}	[32]
PPO-AuNPs-MWCNTs-PAH/GCE	0.5–22	0.4	This work

SO, sulfite oxidase; cyt c, cytochrome c; SAMs, self-assembled monolayers; SDH, sulfite dehydrogenase.

3.8. Interference studies

The effect of some possible interferent compounds was investigated by addition of these compounds to a standard solution containing 50 μmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0). Ascorbic acid, sodium sorbate, ethanol, and sucrose present in the analyzed wine samples were tested at the concentration ratios (standard solution:interferent compound) of 1:1, 1:10, and 10:1. The corresponding current signals were compared with those obtained in the absence of each interferent compound. In the case of ascorbic acid, the concentration ratio 1:10 led to an error of approximately 10%, because it is a reducing agent that competes with sulfite in the enzymatic reaction between catechol and PPO. Nevertheless, in the analyzed samples ascorbic acid is not present, and has not any interference effect in the determination of sulfite. Ascorbic acid can be removed from the samples containing it through a glass column packed with cucumber, as previously reported by our research group [19]. Thus, the potential interference of these compounds should not be a significant interference on the proposed methodology; consequently, sulfite in the presence of these concomitants can be accurately determined using the proposed method, since it was confirmed by the addition and recovery studies.

3.9. Application of the proposed method in indirect sulfite determination in wine samples

The optimized PPO-AuNPs-MWCNTs-PAH/GCE biosensor was applied to the indirect determination of sulfite in six wine samples, which comprised four white wines and two red wines. The method of standard additions was employed to determine the sulfite concentration in each wine sample. No interference from other electroactive species in the wine samples was found. Good recoveries were obtained for the investigated wine samples, ranging from 91.8 to 108% for sulfite, indicating that the matrix effect does not present significant interference.

Table 2 presents the values of the amounts of sulfite determined in wine samples employing the proposed DPV method and an iodometric method [22]. Samples A–D comprised white wines and samples E–F comprised red wines. Three determinations were carried out for each sample, and the standard deviations were calculated. As can be seen in this table, no significant differences were observed between the sulfite concentration found in the wine samples employing the DPV and iodometric titration methods. Applying the paired *t*-test [48] to the obtained results using both methods, the calculated t values (0.654) were smaller than the critical value (2.571, $\alpha = 0.05$), one may conclude that the results obtained with the proposed procedure are not statistically different from the comparative methods, at a 95% confidence level. The advantages of the electrochemical method are less time consuming analysis, and also that the color of the sample has no influence upon the analysis. The results illustrate that the present PPO-AuNPs-MWCNTs-PAH/GCE biosensor is suitable for the determination of sulfite in wine samples.

Table 2
Determination of sulfite in wine samples by the proposed differential pulse voltammetric (DPV) method, using the PPO-AuNPs-MWCNTs-PAH/GCE biosensor and by the iodometric reference method [22].

Samples	Sulfite (mg L^{-1})		Relative error[b] (%)
	Reference method[a]	DPV method[a]	
A	175 ± 4	176 ± 2	0.6
B	168 ± 5	166 ± 5	−1.2
C	124 ± 6	129 ± 3	4.0
D	230 ± 5	227 ± 4	−1.3
E	229 ± 6	226 ± 3	−1.3
F	188 ± 7	184 ± 4	−2.1

[a] Average of 3 measurements.
[b] [100 × (DPV value − reference method)]/reference method.

The repeatability of three independent PPO-AuNPs-MWCNTs-PAH/GCE biosensors was evaluated by measuring the cathodic peak current of 1.0 mmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) by three repeated measurements. A relative standard deviation of 3.9% was obtained among the three biosensors, confirming that the results are highly reproducible.

The stability of the AuNPs-MWCNTs-PAH suspension was studied over a 2-week period. In each determination, a GCE was prepared and the analytical response was determined for a 1.0 mmol L^{-1} catechol in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0). It was not observed significantly decrease in the oxidation or reduction peaks. The cathodic current response decreased about 10%, over this period, indicating the good stability of the AuNPs-MWCNTs-PAH suspension over this period. This good stability of suspension can be attributed primarily to the electrostatic interaction between the nanotubes and nanoparticles, which contain negative charges, and the PAH cationic polyelectrolyte, which contains positive charges (amino groups) [9].

The stability of the PPO-AuNPs-MWCNTs-PAH/GCE biosensor was assessed with regards to long-term storage. To available long-term stability of the PPO-AuNPs-MWCNTs-PAH/GCE biosensor, its response was monitored in 1.0 mmol L^{-1} catechol solution during 4 weeks. After each measurement, the biosensor was rinsed and stored at 4 °C in 0.1 mol L^{-1} phosphate buffer solution. From these experiments, the cathodic current response decrease by only 11% after 30 days, indicating that the immobilization procedure of the PPO provides a positive effect in the long-term stability of the biosensor.

4. Conclusions

In this work, PPO was immobilized on the AuNPs-MWCNTs-PAH/GCE to construct a biosensor. Using this biosensor, an amplification of the electrochemical signal of catechol was obtained

with respect to AuNPs-MWCNTs-PAH/GCE. The stable analytical response of the PPO-AuNPs-MWCNTs-PAH/GCE biosensor for catechol is an attractive feature for indirect determination of sulfite using the DPV method. Under these conditions, a detection limit of 0.4 $\mu mol\,L^{-1}$ for sulfite was attained. Satisfactory results were obtained in the addition-recovery tests, and they exhibited values that are similar to those obtained by the iodometric method. This biosensor exhibits good analytical properties, such as fast response, storage stability and a good detection range for sulfite, and is suitable for the routine analysis of sulfite, avoiding the use of expensive equipment or time-consuming sample preparation. Moreover, the use of AuNPs-MWCNTs-PAH film could offer a friendly environment to immobilize other biomolecules in the future works.

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