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Enzyme sensor based on carbon nanotubes/cobalt(II) phthalocyanine and tyrosinase used in pharmaceutical analysis

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ABSTRACT

A multicomponent enzyme sensor was used to evaluate phenolic compounds in plant-based pharmaceutical formulations. Multiwall carbon nanotubes were used as carbonaceous material used for the electrode construction. Cobalt phthalocyanine was used as electron mediator and tyrosinase as biocatalyst. The enzyme sensor performance was analyzed by cyclic voltammetry and amperometry in model solution of catechol and catechin. The cyclic voltammograms and the amperometric curves show an intense cathodic peak depending on the concentration of thephenolic compound. The cathodic peak was attributed to the reduction of enzymatically produced o-quinone at the enzyme sensor surface. For the model phenolics analyzed, the kinetics of the enzymatic reaction fitted into a Michaelis–Menten type kinetics, as confirmed by the *h* parameter close to 1 obtained from the Hill's plot. The detection limits were in the range of 1.66–6.32 μM demonstrating good quality performances of the enzyme sensor. Pharmaceutical samples were analyzed with multi-component biocomposite enzyme sensor to evaluate its real feasibility in pharmaceutical analysis.

1. Introduction

Polyphenolic compounds are naturally presents in fruits and vegetables and have a great influence in product quality, storage period and human health [1]. Nevertheless, the effect of polyphenolic compounds on human health is somehow contradictory. Various polyphenols are important in prevention of cardiovascular diseases, cancer and diabetes due to antioxidant character [2–4]. Other polyphenolic compounds are considered as endocrine disrupting compounds [5–7]. Therefore, it is of great importance to determine polyphenolics when studying natural products quality, healthcare and environmental monitoring [8,9].

Numerous determination methods of phenolic compounds are accessible, such as high-performance liquid chromatography (HPLC) [10,11], capillary electrophoresis [12], electrochemical sensors [13], and UV–vis spectrophotometry [14]. However, most of these methods are complex and expensive as they necessitate several operations including pre-treatment of the sample and expensive equipments, which make them time-consuming as well [15]. An alternative to those methods are the enzyme sensors. Many amperometric enzyme sensors employing polyphenol oxidase (PPO) for evaluation of polyphenolic compounds have been developed [16–18].

Tyrosinase (that is a PPO) is widely distributed in nature [19]. It is a copper containing metalloprotein, which is known to catalyze two types of reactions: (i) the o-hydroxylation of monophenols to o-diphenols and (ii) the oxidation of o-dihydroxyphenols to o-diquinones [20–22]. Immobilization of enzyme in a substrate is the key factor for obtaining high quality enzyme sensors. Various matrices were used to immobilize PPO such as carbon paste [23,24], conducting polymers [25,26], silica sol–gel composite films [27], Langmuir–Blodgett (LB) thin films [28–30], Layer-by-layer (LbL) films [31], and self-assembled monolayers [32]. Multi-component carbonaceous biocomposites are promising materials for preparing enzyme sensors with high quality performances. In addition, the use of electron mediators can improve the sensibility of the enzyme sensors [33].

In this work, enzyme sensors formed by carbon nanotubes and containing tyrosinase as biocatalyst and cobalt phthalocyanine as electron mediator were prepared and their capability to detect polyphenolic compounds was evaluated. For this purpose, catechin and catechol detection was carried out in aqueous solutions. The amperometric characteristics including kinetics, calibration curves and limits of detection in the detection of polyphenolic compounds

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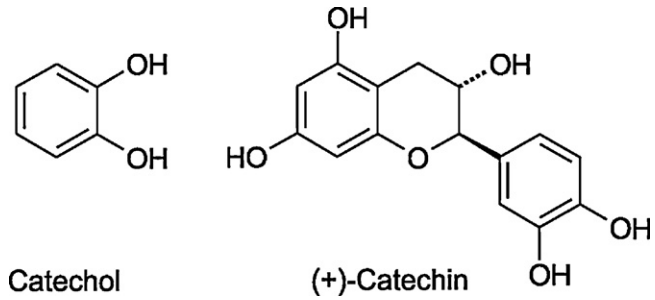


Scheme 1. Chemical structures of catechol and (+)-catechin.

were investigated. The capability of enzyme sensor to detect and quantify the phenolic compounds in pharmaceutical formulations was studied.

2. Materials and methods

2.1. Reagents and solutions

Carbon paste was made were prepared by mixing carbon nanotubes (CNT) (multi wall nanotubes, Nanoledge Inc., Canada) and cobalt phthalocyanine (Fluka) with high purity mineral oil (Nujol, Fluka). Mushroom tyrosinase (from mushroom EC 232-653-4), with a noted activity of 5370 U/mg of solid (product T3824-250KU), was purchased from Sigma Chemical Co. (USA). Catechol and (+)-catechin were purchased from Sigma–Aldrich and Fluka, respectively. The chemical structures for these compounds are given in Scheme 1.

Glucose (Sigma–Aldrich), caffeine (Fluka), tartaric acid (Sigma), citric acid (Sigma–Aldrich) and L-ascorbic acid (Riedel-de Haën) were used in interference studies. All chemicals were of high purity and used without further purification. All solutions were prepared with ultrapure water (18.3 MΩ cm). A 50 μg μL^{-1} solution of tyrosinase in buffer phosphate 0.01 M (pH = 7) was used for enzyme immobilization. The buffer phosphate solution (PBS) 0.01 M (pH 7) was prepared from potassium monobasic and dibasic phosphate sodium salts (Sigma).

2.2. Carbon nanotubes paste modified based enzyme sensor

Carbon paste electrodes were prepared, as previously reported [13,34], by mixing carbon nanotubes and the cobalt phthalocyanine (15%, w/w). Nujol was used as the conglomerant of the multi-component composite mixture. Paste was packed into the body of a 1 mL PVC (polyvinylchloride) syringe and compacted. A metallic copper wire was used as an electrical contact. The length of entire enzyme sensor is 100 mm. The length of the tube containing carbon nanotubes – cobalt (II) phthalocyanine paste is 10 mm with an inner diameter of 1 mm. The shape of active area is a disk with a diameter of 1 mm. Geometrical active surface of enzyme sensor is 0.785 mm^2.

The enzyme, tyrosinase (Ty), was immobilized on the above cobalt phthalocyanine carbon nanotubes paste electrodes (CoPc-CNTPE) by casting technique followed by cross-linking. 5 μL of 0.01 M phosphate buffer (pH 7.0) containing 50 μg μL^{-1} of enzyme, was added onto carbon paste electrode surface. After drying, the enzyme sensor was immersed in a glutaraldehyde solution (2%) for 20 min followed by drying in air at room temperature [35].

The enzyme-immobilized enzyme sensor (Ty/CoPc-CNTP) was washed with phosphate buffer solution thrice to remove any unbound enzyme. The enzyme sensor was additional dried at 10 °C and stored at 4 °C.

2.3. Electrochemical measurements

Electrochemical measurements were performed using a three electrode system with an Ag/AgCl reference electrode (Princeton Applied Research), a platinum (Pt) wire as counter electrode (Princeton Applied Research) and a enzyme sensor as working electrode. The potentiostat used was an EG&G, Model 263 potentiostat/galvanostat controlled by means of Echem Software. All measurements were carried out in 0.01 M phosphate buffer adjusted to pH 7.0.

2.4. Pharmaceutical analysis

Applicability of multi-component based enzyme sensor was studied by analyzing commercial plant pharmaceutical formulations. Three pharmaceutical samples Dyma-burn (Dymatize) – extract from *Salvia sclarea* and green tea – *Camelia sinensis*, Green tea (Jarrow Formulas (US), Green tea extract (Bio-Synergie) were purchased from local pharmacies.

A simple pretreatment of the pharmaceutical sample was made. The samples were dissolved in water. After that, pharmaceutical samples were centrifuged for 10 min (2500 rpm) and the solutions were mixed with 0.1 M phosphate buffer solution pH = 7.0. The final concentration of PBS was 0.01 M. All pharmaceutical samples were analyzed by triplicate.

3. Results and discussion

3.1. Optimization of the enzyme sensor preparation

For the fabrication of carbon paste electrodes, an optimal ratio carbon nanotubes/Co-phthalocyanine (15%, w/w) was used, in agreement with previously reported results [13].

In order to determine the optimal quantity of enzyme on the carbon nanotubes modified paste electrode surface different enzyme sensors were prepared. Fig. 1 shows the effect of the amount of enzyme tyrosinase on the carbon nanotubes modified paste electrode surface.

The cathodic current increases as the enzyme amount is increased. For higher amounts than 376 U of enzyme a decrease of the enzyme sensor sensitivity is observed. This behavior indicates that at low concentrations the increase of the amount of enzyme produces increase in the rate of enzymatic reaction. High concentrations can cause diffusion limitation. Therefore, 268 U of enzyme was chosen to prepare the enzyme sensor, because no significant variation on the sensitivity between 268 and 376 U of enzyme was observed.

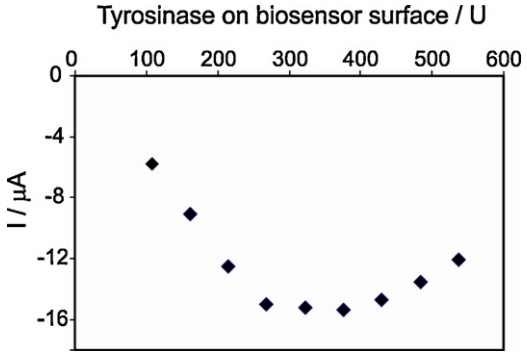


Fig. 1. Effect of the tyrosinase amount added on the carbon nanotube modified paste electrode surface on the enzyme sensor sensitivity for catechol (10^{-4} M catechol, supporting electrolyte 0.01 M phosphate buffer, pH 7.0). Applied potential −50 mV vs. Ag/AgCl.

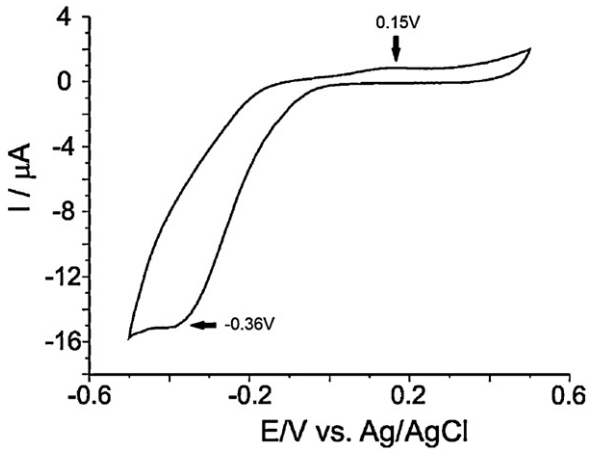


Fig. 2. Cyclic voltammogram of CoPc-CNTPE immersed in 0.01 M phosphate buffer, pH 7.0.

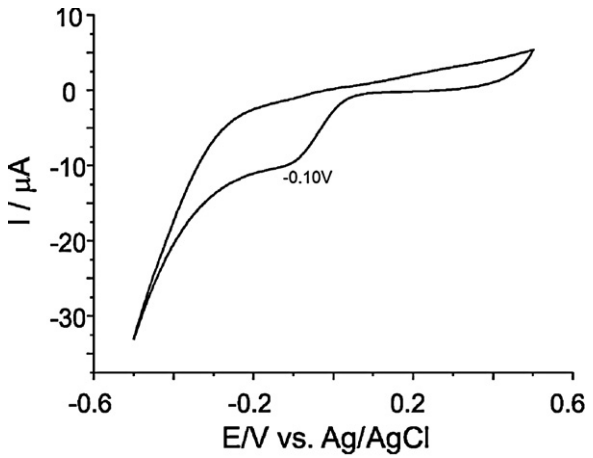


Fig. 4. Cyclic voltammogram of Ty/CoPc-CNTP enzyme sensor immersed in 10^{-4} M catechol (supporting electrolyte 0.01 M phosphate buffer, pH 7.0).

3.2. Cyclic voltammetric studies

In preliminary studies, cyclic voltammograms were recorded for different scan rates from 0.025 to 1.0 V s^{-1}; for scan rates below 0.025 V s^{-1} an excessive noise was obtained for the background signal. Therefore, the optimal scan rate was 0.05 V s^{-1} because ensure an equilibrium between faradaic and non-faradaic currents.

Fig. 2 illustrates the cyclic voltammogram of CoPc-CNTPE at a scan rate of 0.05 V s^{-1} for the background electrolyte (0.01 M phosphate buffer, pH 7.0). Two peaks were observed when cyclic voltammogram of enzyme sensor was recorded in electrolyte support solution, one small anodic peak at 0.15 V and an intense cathodic peak at −0.36 V. The peaks are related with the oxido-reduction of CoPc inside of carbon nanotubes paste matrix.

When the Co-CNTPE is immersed in 10^{-4} M catechol solution (in 0.01 M phosphate buffer, pH 7.0) the voltammogram recorded is more complex (Fig. 3).

The peaks related to redox processes associated to CoPc are observed, but shifted to lower potentials. For instance, the anodic peak appears at 0.00 V and the cathodic peak is present at −0.30 V. This fact is related to antioxidant character of the catechol [34].

The peaks related to redox processes of catechol at CoPc-CNTPE surface appear at +0.25 V (anodic peak) and +0.05 V (cathodic peak). The currents of those peaks are in relation $I_{cathodic}/I_{anodic} = 1$ demonstrating the reversibility of the process at the electrode surface.

The cyclic voltammogram of the Ty/CoPc-CNT enzyme sensor in 10^{-4} M catechol solution do not show well-defined peaks related with CoPc. As is shows in the Fig. 4, only a peak corresponding to reduction of the enzymatically formed o-quinone to catechol at −0.10 V was observed.

The presence of enzyme tyrosinase has a great influence in the electrode response. The enzymatic related peak is shifted to lower potentials comparing with cathodic peak observed in the case of CoPc-CNTPE (with 0.15 V) and the current increase considerable (from −2 μA to −10 μA). Therefore, in the presence of tyrosinase the sensibility increase 5 times based on increase of the peak current and the selectivity is improved by lowering the potential of cathodic peak.

The electrochemical and enzymatic reactions occurring during the measurements in presence of diphenols are shown in Scheme 2 [36].

In the case of catechin solutions the results are similar. The peaks corresponding to cobalt phthalocyanine are not observed; only the reduction peak of the enzymatically formed o-quinone at enzyme sensor surface is observed at −0.05 V. The shape of the curve is somehow different, the reduction peak being more defined in the case of catechol comparing with catechin. The difference between cathodic peak potentials (0.05 V) could ensure a selective determination of catechol and catechin in dual solution.

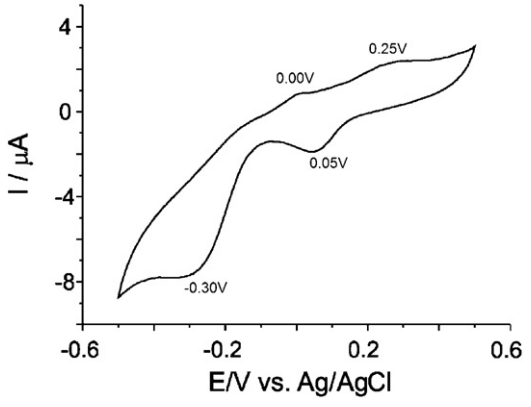
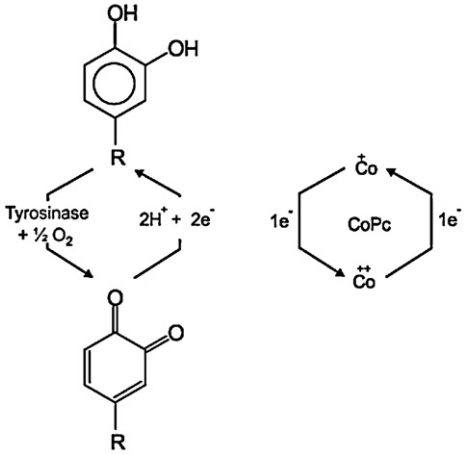


Fig. 3. Cyclic voltammogram of CoPc-CNTPE immersed in 10^{-4} M catechol (supporting electrolyte 0.01 M phosphate buffer, pH 7.0).



Scheme 2. Scheme of the reactions that take place at enzyme sensor surface.

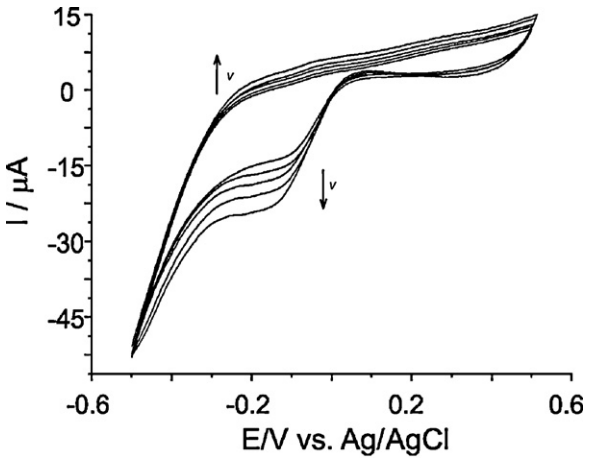


Fig. 5. Cyclic voltammograms of enzyme sensor registered at different scan rates.

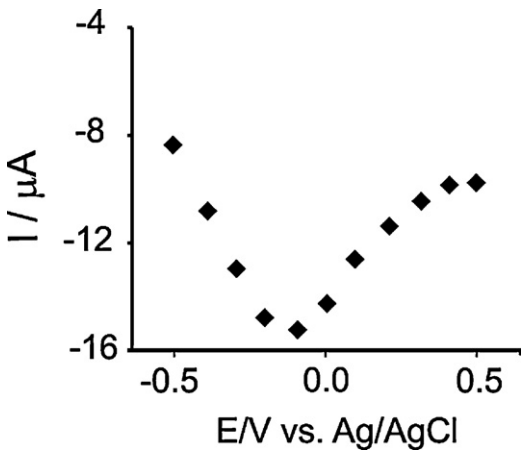


Fig. 6. Current–potential dependence in 10^{-4} M catechin (supporting electrolyte 0.01 M PBS, pH 7.0) under stirring.

The presence of reduction peak indicates that the immobilization process retains the biocatalytic activity of tyrosinase in solid substrates. Therefore, the value of the potential that must be applied to monitor reduction of the species at the electrode surface was in −0.05 to −0.10 V range, potential frame that allows to reach a minimum of possible electrochemical interferences.

3.3. Kinetics of the enzyme sensors

Kinetic studies were performed by registering the cyclic voltammograms of the Ty/CoPc-CNTP enzyme sensor at different scan rates, from 0.02 to 0.50 V s^{-1} in 10^{-4} M catechol solution (Fig. 5). It is obvious the influence of the scan rate; when this parameter increases the residual current increase too, and this reveals an important contribution of charging (non-faradaic) currents to the background current under the conditions used.

The current of the peaks were proportional to scan rates, indicating a charge transfer limited process (due to the electrochemical activity of the enzyme deposited in the electrode surface).

From the relationship of cathodic peak potential and log of scan rate for an irreversible process is given by Eq. (1) [37].

$$E_{pc} = \frac{2.3RT}{\alpha n_\alpha F}\log\ \nu + K \quad (1)$$

where E_{pc} is the potential of cathodic peak (V), α is the electron transfer coefficient, n_α, is the number of electrons involved in the redox process, F is the Faraday constant (F = 96,485 C mol^{-1}), ν is the potential scan rate (V s^{-1}), R is the ideal gas constant (8.314 J K^{-1} mol^{-1}), K is a constant, and T is the temperature (K).

The Tafel slopes of catechol and catechin were found to be 190.2 mV/decade and 180.6 mV/decade for catechol and catechin, respectively, highlighting the issue of enzyme electrode passivation since they are higher than the 60 mV/decade estimated for a two-electron rate determining step [37,38]. Such high Tafel slopes could be associated with either chemical reaction coupled to electrochemical steps or to substrate-biocatalyst interactions in a reaction intermediate [38]. Considering n_α = 2, the transfer coefficient determined from the slope of the representation $E_{pc} = f(\log(\upsilon))$ is 0.310 for catechol and 0.327 for catechin.

3.4. Optimization of the experimental parameters

The applied potential has an important influence over the enzyme sensor response, because the applied potential contributes to the sensitivity and selectivity of the system. The potential dependence on the enzyme sensor response is shown in Fig. 6 using 10^{-4} M catechin in 0.01 M phosphate buffer (pH 7.0). Different potentials between −0.5 V and +0.5 V was applied to enzyme sensor and the current were measured after equilibrium establishing. The solution was maintained under continuous stirring.

The maximum of the signal is obtained at −0.10 V. Therefore, −0.10 V was used as applied potential in amperometric measurements.

It is well-known that pH is a critical parameter of the enzymatic activity and the stability in aqueous media. The effect of pH in the analysis of catechin with the enzyme sensor is presented in Fig. 7.

The best response was observed at pH 7.0, this value is close to the optimum pH observed for soluble tyrosinase [39]. This behavior shows that the carbonaceous matrix does not affect the optimum pH for the catalytic activity of immobilized tyrosinase, when catechol was used as substrate.

3.5. Amperometric response of enzyme sensor

Fig. 8(a) shows the amperometric response for the enzyme sensor at −0.10 V after the addition of successive aliquots of catechol to the 0.01 M PBS (pH 7.0) under constant stirring. As can be seen, a well-defined reduction current proportional to the concentration

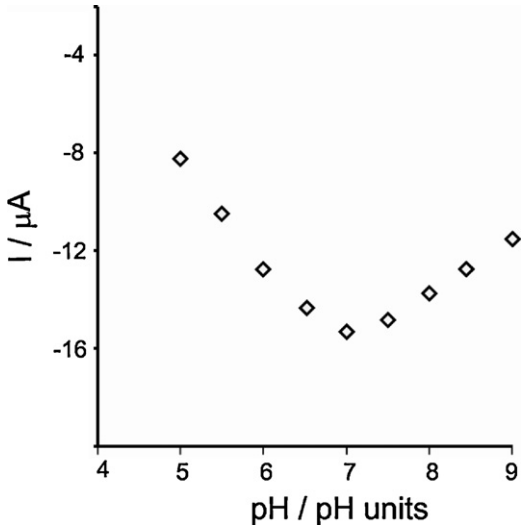


Fig. 7. The effect of pH value in enzyme sensor response. Experimental conditions: 0.01 M PBS containing 10^{-4} M catechin. Applied potential: −0.10 V.

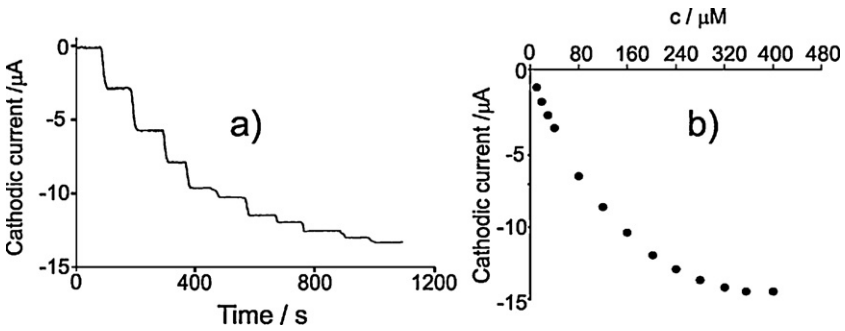


Fig. 8. (a) Amperometric response of Ty/CoPc-CNTP enzyme sensor to catechol (in 0.01 M PBS solution, pH = 7), with levels increasing in 40 μM increments. (b) Calibration curve between the cathodic current and the concentration of catechol in 0.01 M PBS solution (pH 7.0) of Ty/CoPc-CNTP enzyme sensor.

of catechol is observed, which results from the electrochemical reduction of o-quinone species enzymatically formed.

3.6. Effect of catechol concentration

Fig. 8(b) showed the relationship between the cathodic current of the enzyme sensor and the catechol concentration in PBS (pH 7.0) at −0.10 V (calibration curve). It can be seen from Fig. 8(b) that the response current is linear with catechol concentration in the range from 10 to 120 μM. The sensitivity of enzyme sensor is 0.064 μA μM^{-1}. The corresponding detection limits were calculated according to the $3s_b/m$ criterion. In this equation m was the slope of the calibration graph. The s_b was estimated as the standard deviation ($n = 7$) of the amperometric signals from different solutions of the substrate at the concentration level corresponding to the lowest concentration of the calibration plot. The detection limits calculated were 1.66 μM.

From the calibration data, the Hill coefficient (h) can be calculated by representing the $\log[I/(I_{max} - I)]$ vs. log[catechol]. A Hill coefficient of 1.02 ± 0.02 was calculated for the reduction process of o-quinone formed from the enzymatic reaction on the electrode surface ($R^2 = 0.985$). The value obtained for the h parameter, obtained from the corresponding Hill's plot, was close to unity. This result demonstrated that the kinetics of the enzymatic reaction fitted into a Michaelis–Menten type kinetics.

The value almost 1 obtained for enzyme sensor immersed in catechol solution ($h = 1.02 \pm 0.02$) demonstrates a noncooperative effect between the occupied active sites (independent binding of catechol to tyrosinase). A slight negative cooperative effect between the occupied active sites takes place in the case of catechin ($h = 0.96 \pm 0.02$). This result can be related to molecular steric hindrance effects [40].

The parameters for Michaelis–Menten kinetics were calculated from the steady-state currents and the electrochemical version of the Lineweaver–Burk equation (Eq. (2)):

$$\frac{1}{I} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}[S]} \quad (2)$$

where I is the steady-state current after the addition of analyte, [S] is the concentration of analyte, I_{max} is the maximum rate of the enzymatic reaction, and K_M^{app} is the apparent Michaelis–Menten constant.

The I_{max} and K_M^{app} can be calculated from the intercept and slope. In the case of catechol solution, the I_{max} is 15.34 μA, and K_M^{app} is 78.12 μM.

In the case of catechin, similar results were obtained as was summarized in Table 1.

The performance of Ty/CoPc-CNTP enzyme sensor was compared with that of comparable enzyme sensors previously reported. For instance, the linearity range of enzyme sensor is higher than 2.5–25.0 μM reported for enzyme sensor based on composite film of multiwall carbon nanotube (MWCNT)/dimethylditetradecylammonium bromide (DTDAB)/tyrosinase (Tyr) on a Nafion-incorporated carbon paste electrode [41]. The detection limits obtained with enzyme sensor are comparable with those already reported in literature, detection limits in the micromolar range [42]. Detection limit of 1.13 μM was reported recently for catechol using tyrosinase-carbon based enzyme sensors [43].

From the above results could be concluded that Ty/CoPc-CNTP enzyme sensor have good quality performances, comparable with those of previously reported enzyme sensors.

3.7. Effect of interferences

The interferences in this work were considered all compounds that can suffer an electrochemical reaction on the electrode surface. In spite of the enzyme specificity, enzyme sensors may give erroneous results when used in natural samples, because several electroactive species present can be in such complex media, and can be electroactive, affecting the results.

Different molar ratios of catechin and interfering compounds as glucose, caffeine, tartaric acid, citric acid and ascorbic acid (1:0.25, 1:0.5, 1:0.75, 1:1) and their effects on the relative intensity changes in the cathodic current response of catechin was investigated. No significant changes (lower that 4% based on cathodic current modification) were found for any of these compounds, indicating that the proposed enzyme sensor does not present interferences in presence of the compounds analyzed.

3.8. Selectivity of the enzyme sensor

In order to determinate the selectivity of the Ty/CoPc-CNTP enzyme sensor the voltammetric signal were registered in 10^{-4}M catechol – 10^{-4}M catechin dual solution in 0.01 M phosphate buffer

Table 1
Analytical parameters of the biosensors to polyphenolic compounds.

	α	h	LD (μM)	I_{max} (μA)	K_M (μM)
Catechol	0.310	1.02 ± 0.02	1.66	15.34	78.12
Catechin	0.327	0.96 ± 0.02	6.32	8.46	107.67

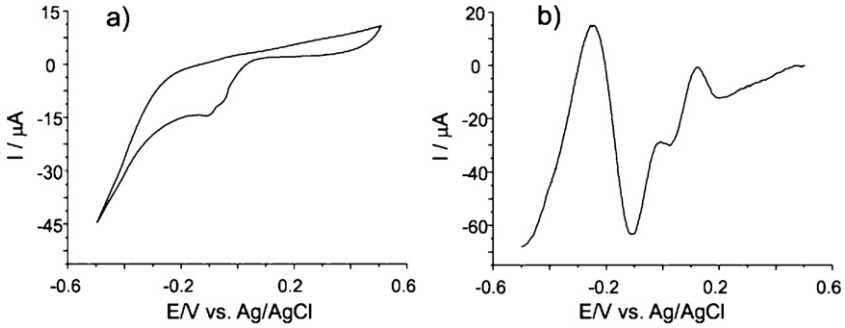


Fig. 9. (a) CV and (b) SWV of Ty/CoPc-CNTP enzyme sensor in 10^{-4}M catechol – 10^{-4}M catechin (in 0.01 M PBS solution, pH = 7).

Table 2
The catechin concentration values (mean of three replicates) founded in pharmaceutical formulations by chronoamperometry.

	Pharmaceutical product Dyma-burn (Dymatize)	Green tea (Jarrow Formulas)	Green tea extract (Bio-Synergie)
Labeled claim (mg catechin)	100	50	20
Amount found (mg)	89	46	16
Standard deviation (mg)	4.24	2.24	1.54

solution pH = 7.0. As observed in Fig. 9(a), the resolution of the peaks corresponding to each component of the solution is reduced. The resolution can be improved by using square wave voltammetry (SWV). Square Wave Voltammetry (SWV) was performed by using optimal conditions: f = 15 Hz; E_{sw} = 90 mV; ΔE_s = 2 mV. Square wave voltammograms presented in Fig. 9(b) demonstrate that catechol and catechin could be selectively identified and quantified by SWV using Ty/CoPc-CNTP enzyme sensor.

By using SWV the intensity of the peaks and the separation between the cathodic peaks increases.

3.9. Enzyme sensor stability study

The stability study is one of major problems that concern enzyme sensor research, because is a significant parameter for the feasibility of any enzyme sensor. The stability of the tyrosinase enzyme sensor was therefore assessed in two ways, first with regard to repeated use in operational conditions and second for storage stability. The enzyme sensor stability under optimal conditions showed a decrease in the cathodic current about 5.5% after 50 successive measurements. The storage lifetime of the tyrosinase enzyme sensor at 4 °C and pH 7.0 showed a decrease in sensitivity about 10% after 1 month of storage.

3.10. Pharmaceutical studies

Different pharmaceutical formulations were analyzed with Ty/CoPc-CNTP enzyme sensor to evaluate its real feasibility. A simple pretreatment of the pharmaceutical samples were made in order to extract the catechin and in phosphate buffer solution 0.01 M of pH = 7.0, optimal conditions for enzyme sensor.

The catechin content of the pharmaceutical samples was estimated by chronoamperometry at −0.10 V under continuous stirring. Three replicates were carried out for each sample. The contents in catechin of pharmaceutical formulations determined with Ty/CoPc-CNTP are presented in Table 2.

As observed in Table 2, the values obtained with the enzyme sensor were smaller than labeled content. These values can be explained considering the heterogeneity of natural catechin present in the pharmaceutical samples. These results are consistent with the literature values determined by tyrosinase-based enzyme sensor comparing with Folin–Ciocalteau method [44].

4. Conclusions

This study has demonstrated the feasibility of developing a multi-component biocomposite based enzyme sensor for monitoring polyphenolic compounds in aqueous medium. Furthermore, it has confirmed that the carbonaceous matrix having cobalt phthalocyanine as electron mediator, can be utilized for the immobilization of enzyme, tyrosinase. The enzyme sensor exhibits high sensitivity and selectivity for the amperometric detection of polyphenolic compounds because of the high loading of tyrosinase and the rapid electron transfer between the enzymatically-produced quinones and the enzyme sensor surface. Possible applications of this type of enzyme sensor include identification and quantification of biologic active compounds from pharmaceutical formulations.

Acknowledgments

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