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ORIGINAL PAPER

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Reagentless biosensor for phenolic compounds based on tyrosinase entrapped within gelatine film

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Abstract A new simple reagentless phenolic compound biosensor was constructed with tyrosinase immobilized in a gelatine matrix crosslinked with formaldehyde. The morphologies of gelatine and gelatine/tryosinase were characterized by SEM. The tyrosinase retains its bioactivity when it is immobilized by the gelatine film. Phenolic compounds were determined by the direct reduction of biocatalytically liberated quinone at −0.1 V vs SCE. The process parameters were studied during the fabrication of the enzyme electrode. Optimization of the experimental parameters has been performed with regard to pH, operating potential, temperature and storage stability. This biosensor exhibits a fast amperometric response to phenolic compounds. The linear ranges for catechol, phenol, and *p*-cresol determination were from 5×10^{-8} to 1.4×10^{-4} M, 5×10^{-8} to 7.1×10^{-5} M, and 1×10^{-7} to 3.6×10^{-5} M, with detection limits of 2.1×10^{-8} M, 1.5×10^{-8} M, and 7.1×10^{-8} M, respectively. The enzyme electrode retained ~77% of its activity after seven days of storage at 4 °C in a dry state. The proposed sensor presented good repeatability, evaluated in terms of the relative standard deviation (RSD=8.6%) for eight different biosensors, and was applied to determine the phenolic compounds in water samples. The recoveries for the samples ranged from 99.0% to 99.8%.

Keywords Tyrosinase · Phenolic compounds · Biosensor · Gelatine

Introduction

Phenolic compounds are industrial chemicals widely used in the manufacture of products. Most of them are generated artificially and are found in the wastewaters from chemical plants, exhaust gases from incinerators, the sidestream smoke from cigarettes, and so on [1]. They are easily adsorbed in humans, regardless of their form. High levels of phenols have been shown to have detrimental effects on animal health, and some phenolic compounds are reportedly carcinogenic [2, 3]. There is considerable interest in determinations of phenolic compounds in industrial, environmental or food samples. Many methods of instrumental analysis have been used for this, such as colorimetry, gas chromatography, liquid chromatography, capillary electrophoresis and spectrophotometric analysis [4–8].

One promising and relatively simple approach to the quantification of phenolic compounds, both in vitro and in vivo, is based on electrochemistry, due to its many advantages, such as good selectivity in the presence of phenol oxidases, relatively low cost of realization and storage, and the potential for miniaturization and automation [9, 10]. Tyrosinase (Tyr) is a copper protein that contains a coupled binuclear copper-active site, which catalyzes the hydroxylation of monophenols to form *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, using molecular oxygen [11, 12]:

$$\text{Phenol} + \text{Tyrosinase}(O_2) \rightarrow \text{Catechol} \quad (1)$$

$$\text{Catechol} + \text{Tyrosinase}(O_2) \rightarrow o\text{–Quinone} + H_2O \quad (2)$$

o-Quinones can be electrochemically reduced to *o*-diphenols using a low over-potential without any electron transfer mediator via the following equation:

$$o\text{–Quinone} + 2H^+ + 2e^- \rightarrow \text{Catechol} \quad (3)$$

Therefore, the detection of phenols is based upon monitoring the liberation of the quinone products or the consumption of the oxygen cofactor [13].

Tyrosinase-based biosensors for the determination of phenolic compounds have been reported extensively; for example, the entrapment of tyrosinase in carbon paste [14,

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15], Eastman-AQ [16], polyhydroxyl cellulose "cryohydrogel" [17], clay [18] and electrogenerated polypyrrole derivatives [19, 20]. Due to the difficulties involved with producing these materials or the complexity of the fabricating process, there is still great interest in the development of a simple and reliable method to stabilize the enzyme.

Herein, we demonstrate a reagentless biosensor for phenolic compounds obtained by entrapping tyrosinase in gelatine crosslinked matrix. Gelatine (Gel), a natural biopolymer, is a linear polypeptide that consists of different amounts of 18 amino acids and offers an unique set of characteristics in terms of gel-forming properties and hydrophilicity, biocompatibility, biodegradability to harmless products, nontoxicity, and remarkable affinity to proteins [21]. Additionally, it is abundant and inexpensive, making it suitable for commercial application. Such factors are important when preparing biosensors and functional devices. To the best of our knowledge, there are only a few reports where gelatine is used as the matrix.

In our work, the prepared biosensor exhibited high sensitivity and a wide linear range for voltammetric and amperometric measurements of phenolic compounds. Such behavior is attributed to the affinity of the gelatine matrix, which provides a friendly environment to the enzyme. This method provides a general, inexpensive, simple and effective route to the immobilization of tyrosinase. Factors such as buffer pH, operational potential and enzyme loading were studied and optimized. In addition, the analytical performances of the biosensor with respect to response time, sensitivity, repeatability and stability were evaluated.

Experimental

Reagents

Mushroom tyrosinase (from mushroom. EC.1.14.18.1), noted activity of 2590 units mg^{-1} solid (Catalog No. T-7755), was obtained from Sigma Chemical Co (St Louis, MO, USA) and used as received. Phenol, catechol and *p*-cresol were purchased from Shanghai Chemical Reagent Company (Shanghai, China). All other reagents were of analytical grade and were used without further purification. 0.1 M phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4 and then adjusting the pH with 0.1 M H_3PO_4 or NaOH. All of the solutions were prepared with doubly distilled water.

Apparatus and measurements

All electrochemical experiments were carried out in a three-electrode cell controlled by a CHI 660 Electrochemical workstation (CH Instruments, Austin, TX, USA). A Tyr biosensor was used as the working electrode. Reference and counter electrodes were SCE and platinum wire, respectively.

The standard solutions of phenols were prepared with doubly distilled water. Aliquots of phenol standard solution were added to the solution in succession. Current–time data were recorded after a steady-state current had been achieved. Scanning electron micrographs (SEM) of gelatine and gelatine/tyrosinase film were taken on a SEM (JSM-5610LV, Jeol, Tokyo, Japan) instrument.

Preparation of enzyme electrodes

Glassy carbon electrodes were polished before each experiment with 1, 0.3 and 0.05 μm alumina powders, respectively, rinsed thoroughly with doubly distilled water between each polishing step, ultrasonicated in 1:1 nitric acid, acetone and doubly distilled water in succession, and then allowed to dry at room temperature.

Gelatine solution (1%) was prepared by dissolving gelatine flakes in tepid doubly distilled water.

To prepare the tyrosinase enzyme electrode, a suitable amount of tyrosinase was added to 0.2 ml gelatine solution, and then 10 μl of formaldehyde solution was added to the enzyme solution. The mixture was hand-mixed thoroughly. Finally, 10 μl of the new mixture was dropped onto the surface of a glassy carbon electrode and allowed to dry at 4 °C for 24 h. All of the enzyme electrodes were washed thoroughly with doubly distilled water before use and stored at 4°C in a dry state.

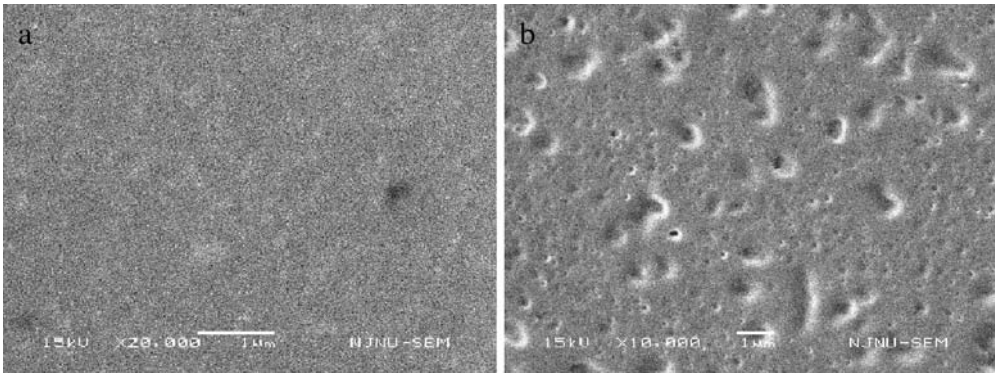

Fig. 1a–b SEM photos of gelatine film before (**a**) and after (**b**) enzyme immobilization

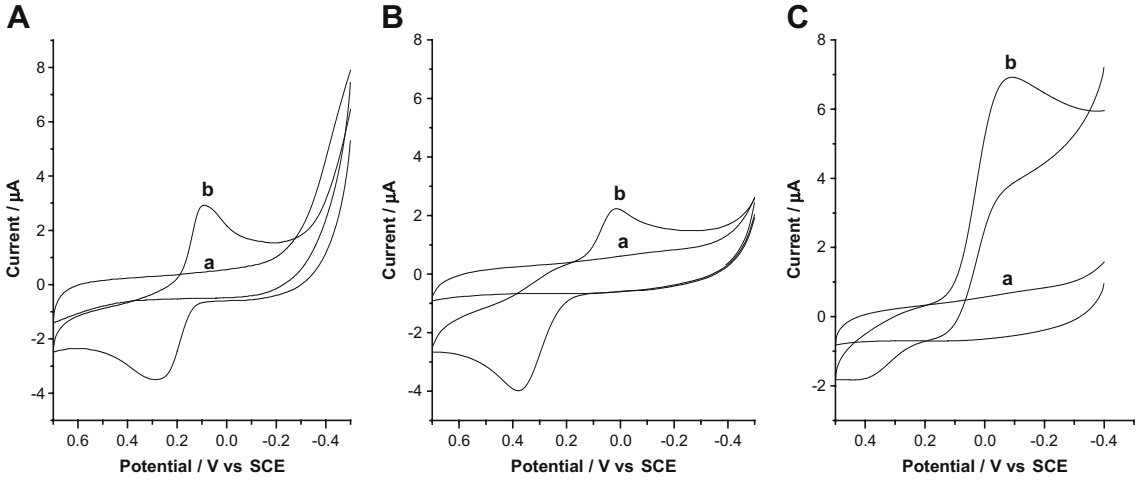

Fig. 2A–C Cyclic voltammograms of the bare GC electrode (**A**), the gelatine-formaldehyde GCE (**B**) and the Tyr-gelatine-formaldehyde GCE (**C**) in the absence (*a*) and presence (*b*) of 0.1 mM catechol (in pH 7.0 PBS at scan rate of 50 mV/s)

Results and discussion

Morphologies of gelatine and gelatine/tyr films

SEM images, as shown in Fig. 1, characterized the micrographs of the gelatine and gelatine/tyrosinase films. Obviously, the membrane of gelatine is a homogeneous structure (Fig. 1a) and the surface is symmetrically flat. Compared to the gelatine film, the membrane of the gelatine/tyrosinase blend is a bumpy structure (Fig. 1b). The sizes and shapes of the bumps are regular and uniform, which was suggested that the enzyme was successfully immobilized in the gelatine network film.

Cyclic voltammetric behavior of the tyr/gelatine modified electrode

Typical cyclic voltammograms of different electrodes in 0.1 M PBS (pH 7.0) at 50 mV/s are shown in Fig. 2. In the absence of catechol, the bare GCE (Fig. 2A, curve a), the gelatine modified electrode (Fig. 2B, curve a) and the enzyme electrode (Fig. 2C, curve a) showed a low background without a detectable signal. Upon addition of catechol to the PBS, at a potential of 0 V, a small reduction peak current was observed at the gelatine modified electrode (Fig. 2B, curve b) as well as the bare electrode (Fig. 2A, curve b). In contrast, a substantially larger response was observed at the gelatine modified enzyme electrode. This modified electrode resulted in a large reduction peak (around −100 mV, Fig. 2C, curve b), which was attributed to the reduction of the enzyme when catalyzing catechol to form *o*-quinone in ambient oxygen. Current–time experiments were also carried out (curves not shown). The results indicated that no response current was seen for the bare electrode and the gelatine modified electrode when catechol stock solution (10^{-4}M) was added to the buffer. For the gelatine/enzyme electrode, in contrast, the response current increased rapidly as the same concentration of catechol was added. Therefore, we can

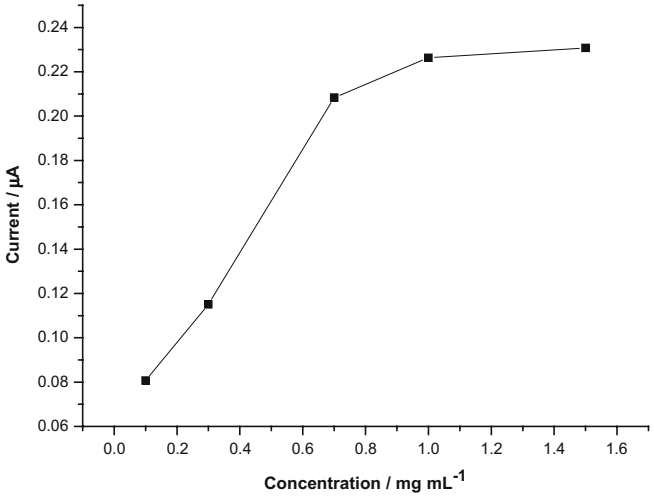

Fig. 3 Effect of the amount of the enzyme on the response of the biosensor. Experimental conditions: 2 μM catechol in pH 7.0 PBS, working potential is −0.1 V

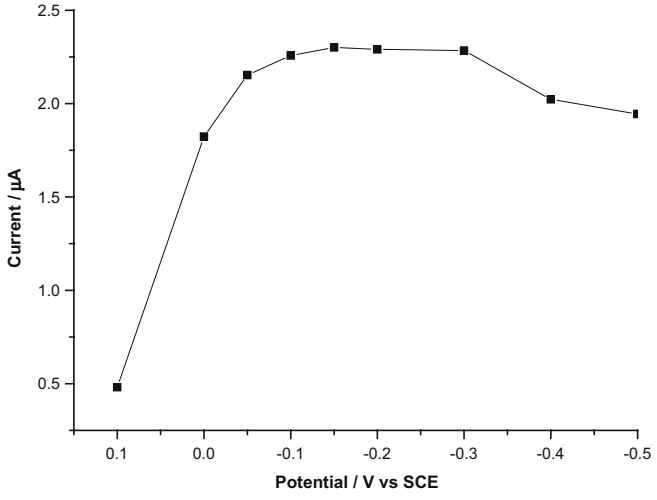

Fig. 4 Effect of working potential on the response of the biosensor to 2 μM catechol

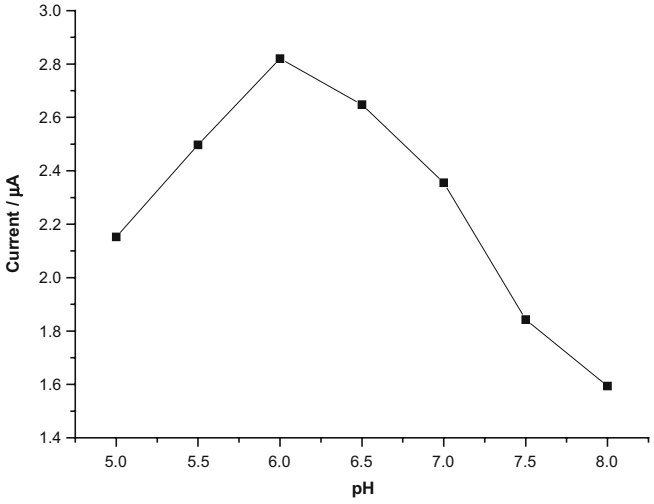

Fig. 5 Effect of pH on the response of the biosensor to 2 μM catechol in PBS at a working potential of −0.1 V

confirm that the increase in current is due to the catalyzing reaction of the enzyme.

Optimization of the preparation of the enzyme electrode

The performance of the enzyme electrode mainly depends upon the amount of enzyme and gelatine entrapped on the electrode surface.

Figure 3 shows how the amperometric response of the enzyme electrode depends on the amount of enzyme entrapped on the electrode surface. The response current increases with the enzyme concentration of the solution dropped onto the surface of the electrode. A plateau can be obtained at an enzyme concentration of 1 mg mL^{-1}. This indicates that the enzyme loading capacity of gelatine thin film has been saturated.

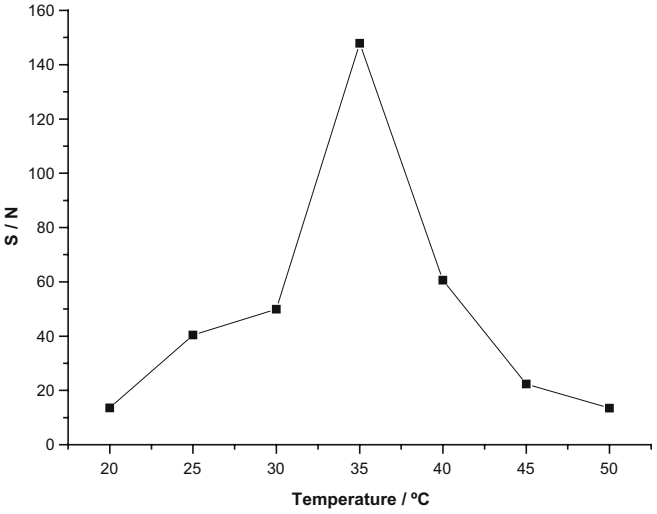

Fig. 6 Effect of the temperature on the response of the sensor. Operating potential: −0.1 V vs SCE. Experimental conditions: 2 μM catechol in 0.1 M PBS (pH 6.0)

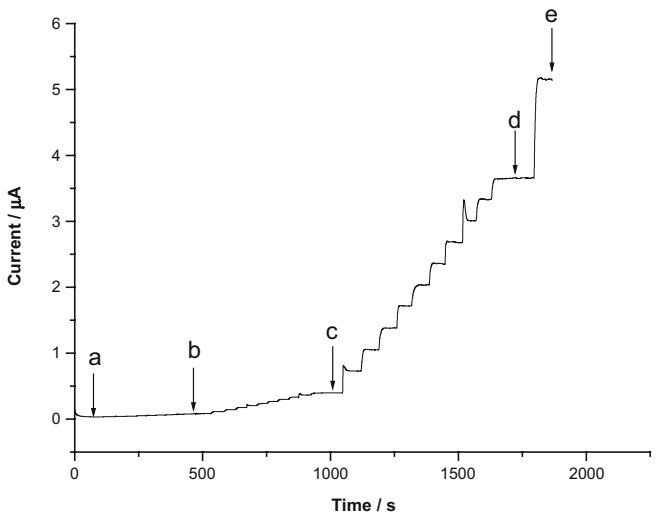

Fig. 7 Dynamic response of the biosensor to successive additions of (*a*–*b*) 1×10^{-7} M, (*b*–*c*) 5×10^{-7} M, (*c*–*d*) 5×10^{-6} M, (*d*–*e*) 2.5×10^{-5} M catechol at a working potential of −0.1 V. *Inset* shows the calibration plot for the current versus the concentration of catechol

Some factors, such as response time, linear range, and stability are affected by the amount of gelatine. When the concentration of gelatine is too high, the response rate is slow and the detected linear range is narrow; and when it is too low, the stability of the biosensor decreases. The optimum concentration of gelatine was found to be 2 mg mL^{-1} according to our experiments.

By constructing eight sensors and determining the peak current obtained for each one, it was possible to evaluate the repeatability of the sensor measurements. The repeatability, expressed as the relative standard deviation (RSD) was 8.6% for n=8.

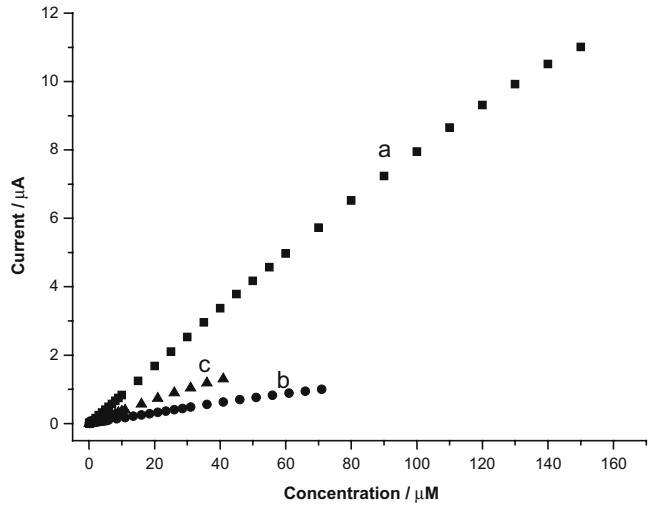

Fig. 8 Calibration plots for the tyrosinase electrode for catechol (*a*), phenol (*b*) and *p*-cresol (*c*) determinations in 0.1 M PBS (pH 6.0) at −0.1 V

Table 1 Detection parameters for tyrosinase/gelatine modified GCE measurements of phenolic compounds

	Phenol	Catechol	*p*-Cresol
Linear range	5×10^{-8}– 7.1×10^{-5}M	5×10^{-8}– 1.4×10^{-4}M	1×10^{-7}– 3.6×10^{-5}M
Detection limit	1.5×10^{-9} M	2.1×10^{-9}M	7.1×10^{-8}M
Sensitivity	0.0546 μA/μM	0.122 μA/μM	0.0201 μA/μM
K_m^{app}	0.039 mM	0.157 mM	0.721 mM

Optimization of the experimental parameters

Various factors influenced the performance of the sensor. In order to obtain a high sensitivity and fast response, the influences of the applied potential, the buffer pH and the temperature were investigated.

Figure 4 shows the effect of the applied potential on the amperometric response of the biosensor to catechol. The reduction current of *o*-quinone was initially observed at around 100 mV, and it increased rapidly as the applied potential shifted from 100 mV to −100 mV, which was due to the increased driving force for the fast reduction of *o*-quinone at low potential. The current approached a plateau at −100 mV. In comparison with other reported results [22], the working potential for phenol reduction in our experiment occurred at a more positive value. −100 mV was selected as the operating potential for the low working potential, which made the sensor more able to minimize any interference [23].

At this potential, the effect of pH on the enzyme electrode response was studied in the pH range 5.0~8.0 in the presence of 2 μM catechol (Fig. 5). It is apparent that the current response of the electrode reached a maximum value at pH 6.0. Therefore, pH 6.0 phosphate buffer solution was chosen throughout this study.

The effect of temperature on the response of the sensor was also studied. After immersing the enzyme electrode in buffer solution at different temperatures for ten minutes, the steady-state current was recorded. As can be seen from Fig. 6, an almost peak-shaped profile was obtained. Increasing the temperature enhanced the sensitivity of the electrode to catechol, but the noise was also enhanced. It reached a maximum value at 35 °C (according to the S/N), and then decreased rapidly to a low value because of partial denaturation of the enzyme and possible dissolution of the gelatine film. However, considering the requirement that it should be possible to use the biosensor real samples, room temperature was selected throughout the study.

Amperometric response of the biosensor

Figure 7 displays a typical current–time plot for the biosensor at room temperature with successive additions of catechol to 0.1 M PBS (pH 6.0) at −100 mV. When an aliquot of catechol was added to the supporting solution, the reduction current rose steeply, reaching a stable value. The enzyme electrode achieved 95% of steady-state-current in less than ten seconds.

Figure 8 illustrates calibration plots for the enzyme electrode for catechol, phenol and *p*-cresol in 0.1 M, pH 6.0 PBS under optimal experimental conditions. The analytical performance, including the sensitivity, linear range and detection limit of the enzyme electrode to each substrate is listed in Table 1. The results illustrate that the gelatine matrix is suitable for enzyme loading.

According to the IUPAC definition [24] the detection limit (DL) can be related to the smallest response that can be detected with reasonable certainty. For a given analytical method, the detection limit is given as follows:

$$DL = \frac{kS_B}{b}$$

Where S_B is the standard deviation of the blank measurement, b is the sensitivity of the method (determined as the slope of the calibration curve), and k is a statistical constant (a value of k=3 is strongly recommended by IUPAC, based on the confidence interval). Using a least-squares regression program (for a linear fit, and not forcing zero), the slopes for the data from catechol, phenol, and *p*-cresol voltammetric analysis (Fig. 7) were determined as 0.06844, 0.01532, and 0.03545. Thus, the detection limits for these substances are listed in Table 1.

The sensitivity trend is catechol >phenol >*p*-cresol. This is different from that shown by a tyrosinase/silica sol-gel electrode in aqueous solution [25]. The sensitivity sequence for a biosensor is dependent upon the hydrophobicity characteristics of the immobilization matrix [26]. At high concentrations, plateau responses are observed, showing the characteristics of the Michaelis-Menten kinetic mechanism. The apparent Michaelis-Menten constant (K_M^{app}), generally used to evaluate the enzymatic affinity, were calculated according to the Lineweaver-Burk equation [27] and are also listed in Table 1. Comparing the results for phenol determination with those reported previously [28, 29], the linear ranges, sensitivities and detection limits are better than those in aqueous solution. Thus, the immobilized tyrosinase shows a slightly better affinity to phenol.

Table 2 Recovery (%) obtained for 2 μM catechol in the presence of different interferents at a interferent/catechol molar ratio of 100:1

Interferent	K^+	Ca^{2+}	Mg^{2+}	NH_4^+	Cl^-	Glucose	VitC	SCN^-	SO_3^{2-}	Ac^-
Recovery (%)	101.3	97.3	97.5	99.6	98	99.4	89.6	88	60	72

Stability of the enzyme electrode

The main objective of enzyme immobilization on a transducer, for analytical purposes, is to stabilize the enzyme so that the biosensor can be used repeatedly over a long period of time. The operational stability of the biosensor was investigated by measuring its resposnse to 2.0 μM catechol solution everyday. When not in use, the enzyme electrode was stored in the refrigerator at 4 °C. It retained ~77% of the initial current response after seven days and ~55% after 15 days.

Effect of the interferent

The possible effects of the presence of foreign ions and compounds in the solution were analyzed. When the concentration of catechol was 2 μM, the effects of interferents were investigated by testing the response of the enzyme electrode when the molar ratio of interferent to catechol is 100. Table 2 shows that the results obtained on the proposed sensor exhibit no significant interference in the presence of the tested ions and compounds, except in the cases of SCN^-, SO_3^{2-} and Ac^-. These anions could react with catechol, reducing the response of the detector.

This method was applied to determine the phenolic compounds in a wastewater sample. The recovery values were calculated by applying the standard additions method. The recoveries for the water sample range from 99.0% to 99.8%.

Conclusions

In this work we have developed a simple biosensor for phenols by entrapping tyrosinase in gelatine crosslinked matrix. This provides a very simple way to immobilize tyrosinase. Because gelatine is also a protein, the biosensor has a good loading, high enzyme catalytic activity, and a fast response rate. It can stop the leaching of tyrosinase from the surface of the electrode under normal operating conditions. The resulting biosensor exhibits high sensitivity, good repeatability and a wide linear range, implying that the matrix can provide a suitable microenvironment for the enzyme. Gelatine-based materials, as yet under-utilized, are predicted to be widely exploited in the near future, especially for environmentally benign applications to systems in biological environments, such as enzyme immobilization supports.

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