ANALYTICA CHIMICA ACTA 612 (2008) 198–203

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Phenol biosensor based on Sonogel-Carbon transducer with tyrosinase alumina sol–gel immobilization

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ARTICLE INFO

Article history:
Received 31 October 2007
Received in revised form
14 February 2008
Accepted 16 February 2008
Published on line 6 March 2008

Keywords:
Biosensors
Sonogel-Carbon electrode
Tyrosinase
Alumina sol–gel
Phenolic compounds

ABSTRACT

A new biosensor for detection of phenols, based on tyrosinase immobilization with alumina sol–gel on Sonogel-Carbon transducer, has been developed. The electrode was prepared using high energy ultrasounds directly applied to the precursors. The alumina sol–gel provided a microenvironment for retaining the native structure and activity of the entrapped enzyme and a very low mass transport barrier to the enzyme substrates. Phenols are oxidized by tyrosinase biosensor to form a detectable product, which was determined at −300 mV vs. Ag/AgCl reference electrode. For phenol, the sensor exhibited a fast response which resulted from the porous structure and high enzyme loading of the sol–gel matrix. The linear range was from 5×10^{-7} M to 3×10^{-5} M, with a detection limit of 3×10^{-7} M. The stability of the biosensor was also evaluated.

1. Introduction

The determination of phenolic compounds is very important in chemical manufacturing process and environmental monitoring. Several methods including gas chromatography and spectrophotometry have been used to determine phenolic compounds [1]; the heavy treatment of the sample limits their use for on site applications. Amperometric biosensor based on tyrosinase has been considered to be a promising method since its effectiveness and simplicity [2]. Many immobilization methods have been developed to stabilize the tyrosinase. Wang et al. [3] obtained good results encapsulating the enzyme in carbon paste and Cosnier and Innocent [4] reported its immobilization by electropolymerization in amphiphilic pyrrol. Different kinds of hydrogels have been recently synthesized for the construction of tyrosinase biosensors [5]; however the swelling and solubility of the hydrogel in aqueous solution may limit its practical application. Therefore, the research of a simple and reliable method to immobilize tyrosinase is nowadays of great interest.

Due to the compatibility between the inorganic support and the immobilized biochemical species, the employment of sol–gel technology to produce ceramics based biosensitive materials has received increasing interest in recent years [6–11]. Special advantages, including the relative porosity, chemical inertness, simplicity of preparation, negligible

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0003-2670/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.aca.2008.02.029

swelling in solutions, low temperature encapsulation, and high sensitivity, have been recognized for sol–gel electrode based sensors and biosensors.

The entrapped species, such as chemical and biological molecules, preserve their chemical properties and bioactivities. It has been demonstrated that many of the chemical and biochemical reactions that occur in solution can be accomplished in the pores of sol–gel host [9–13], and this is of the major interest in sol–gel sensing. The optimization of the conditions of fabrication of the sol–gel can increase the stability and sensitivity of a biosensor. Various methods including modification of precursors, sol–gel synthesis conditions or silane:solvent ratio have been proposed. All these strategies include the use of relatively high concentrations of alcohol, and the later evaporation of this component involves an inevitable shrinkage of sol–gel matrix with the time thus affecting its porosity [14]. We proposed a novel sol–gel procedure based on the use of sonocatalysis to obtain solid carbon composite electrodes, called by us Sonogel-Carbon electrodes [14–16]. High energy ultrasounds are applied directly to the precursors, ultrasonic cavitation is achieved, the material shrinkage is avoided, and the pore size can be controlled. In addition, this strategy allows the hydrolysis with acidic water in the absence of any supplementary solvent in only few seconds.

The so-called Sonogel-Carbons are of high density, exhibiting fine texture and homogeneous structure; the presence of spectroscopic grade graphite renders them conductive. This matrix offers an alternative route for developing new composite electrodes with a large variety of structures and shapes. These electrodes show very favourable electroanalytical properties for their use as amperometric sensors and, furthermore, they can easily permit the incorporation of numerous receptor molecules at the Sonogel-Carbon materials, notably with an improvement in the sensitivity and the selectivity compared to classical electrodes [17,18].

In the present study, sensitive biosensors based on Sonogel-Carbon electrode with tyrosinase, were developed. The objective designed for these biosensors was to seek new electrochemical biosensors for phenols determination. The influence of additive-protective, such as alumina sol–gel, PVA-AWP and Titania sol–gel on the surface of the biosensor was explored. The experimental parameters affecting the response of all resulting biosensors are discussed. Sensing performances and kinetic characterisations of the developed biosensors were investigated toward seven phenolic compounds.

2. Experimental

2.1. Materials

Mushroom tyrosinase (from mushroom, EC 1.14.18.1), 4800 units mg^{-1} of solid was purchased from Sigma Chemical Co. (France), Aluminum isopropoxide (Al(i-PrO_3), catechol, phenol, *p*-cresol, caffeic acid and chlorogenic acid were obtained from Sigma Chemical Co. (France). Titanium butoxide was obtained from Aldrich (France). Photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinium groups (PVA-AWP) was provided by Toyo Gosei Kogyo Co. (Chiba, Japan). Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm, Millipore, Bedford, MA).

2.2. Instrumentation

Chronoamperometric measurements were performed with an Autolab PGSTAT20 (Ecochemie, Ultrecht, The Nederlands) potentiostat/galvanostat interfaced with a personal computer, using the AutoLab softwware GPES for waveform generation and data acquisition and elaboration.

A 600-W model, 20 kHz ultrasonic processor (Misonix Inc., Farmingdale, NY) equipped with a 13 mm titanium tip was used. The ultrasonic processor was enclosed inside a soundproof chamber during operation.

All electrochemical experiments were carried out in a cell containing 10 mL of an aerated 0.2 M pH 7 phosphate buffer solutions at 30 °C. The three electrodes system consisted of tyrosinase-Al_2O_3-modified Sonogel-Carbon electrode as working electrode, an Ag/AgCl (3 M KCl) as reference electrode and a platinum wire as auxiliary electrodes. A magnetic stirrer and stirring bar were used to provide continuous convective transport during the measurements.

2.3. Procedures

2.3.1. Amperometric measurements

Amperometric experiments were carried out in an electrochemical cell containing 10 mL of 0.2 M pH 7 phosphate buffer. A magnetic stirrer and a stirring bar provided the convective transport in the amperometric experiments. The working electrode potential was fixed at −0.3 V vs. Ag/AgCl, and the background current was allowed to decay to a steady state. Aliquots of catechol standard solution were added to the cell. Current–time curves for the amperometric experiments were recorded at 30 °C for optimized conditions.

2.3.2. Tyrosinase immobilization methods

Al_2O_3 Sol–gel was prepared as indicated in the litterature [19]. A typical method for the preparation of Al_2O_3 sol–gel (with the molar ratio of Al:H_2O:HCl = 1:100:0.05) is described as below. Al(i-PrO_3 (2 g; chemical grade) was added to 44.0 mL of distilled water. After the mixture was stirred for 45 min at 80 °C, 1.2 mL of 1 M HCl was added. The mixture was heated to 90 °C, and the vessel was opened for 2 h to allow evaporation of the 2-propanol (i-PrOH), which was produced from hydrolysis. Then the mixture was refluxed for 16 h under 90 °C. A stable and homogeneous Boemite sol (γ-AlOOH) was obtained. This formulation could be changed when certain experimental parameters (such as the ratio of Al to H_2O and the pH of the sol–gel) were investigated.

Tyrosinase immobilization in the alumina sol–gel matrix was carried out by addition of 50 μL of 3.8 mg mL^{-1} tyrosinase (0.2 IU) into 50 μL of stock standard sol–gel solution; 1 μL of the mixture was deposited on the surface of the working electrode and allowed to gel and dry for one hour under room conditions for the polymerization of the sol–gel solution. The biosensors were stored in dry state during four days at 4 °C in order to complete the polymerization.

Tyrosinase immobilization in the PVA matrix: 100 μL of a tyrosinase solution (12.8 mg mL^{-1} of 0.2 M pH 7 phosphate buffer was mixed with 100 μL of PVA-AWP. A 1 μL aliquot of the mixture was deposited on the surface of the working electrode. The electrodes were exposed to long wave neon light (365 nm, at a distance of 10 cm from the electrode surface) for 3 h to allow polymerization and dried for 12 h at 4 °C. All electrodes were stored in the dark at 4 °C after preparation.

Titania sol–gel was prepared as indicated in the literature [20]; 50 μL of 3.8 g L^{-1} of tyrosinase (0.2 IU) was mixed with 50 μL of sol–gel solution. A 1 μL aliquot of the mixture was deposited on the surface of the working electrode.

3. Results and discussion

The hydrophobicity of the immobilization material plays an important role in activity of tyrosinase. Sol–gel materials with variable hydrophobicity were used to fabricate the enzyme electrode, the sol–gel network was highly hydrophilic and water can easily penetrate through the layer.

A tyrosinase-Al_2O_3-modified glassy carbon electrode has been described for the detection of phenols [19]; the performance of this electrode is very similar to tyrosinase-Al_2O_3-modified Sonogel-Carbon electrode. However, the advantages of our device are the very favourable electroanalytical properties of the Sonogel-Carbon material, on which the enzyme film is deposited; these characteristics can be found described in literature [14–16]

3.1. Cyclic voltammetric behavior of tyrosinase/alumina sol–gel/Sonogel-Carbon electrode

Fig. 1 shows cyclic voltametry responses of tryosinase alumina sol–gel modified electrodes in absence of catechol (a) and in presence of catechol (b). A background current without detectable signal is obtained. The catechol shows quasi-reversible peaks at alumina sol–gel modified electrode (c) (Eq. (1)). At tyrosinase/alumina sol–gel modified electrode, anodic peak at the potential of 0.125 V shows a slight decrease in the current height while the reduction current peak shows much

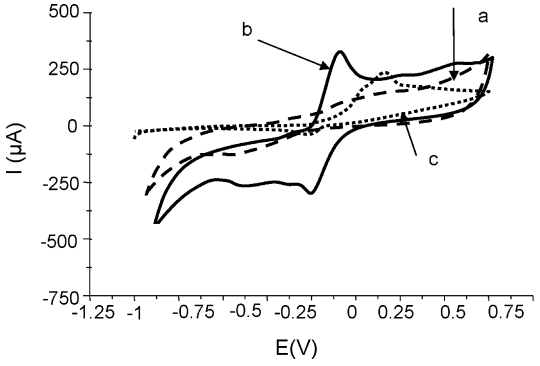

Fig. 1 – Cyclic voltammetry response of tyrosinase/alumina sol–gel modified electrode in absence of catechol (a) and in presence (a), unmodified electrode (without tyrosinase). (c) Scan rate 100 mV s^{-1}. Phosphate buffer at 30 °C.

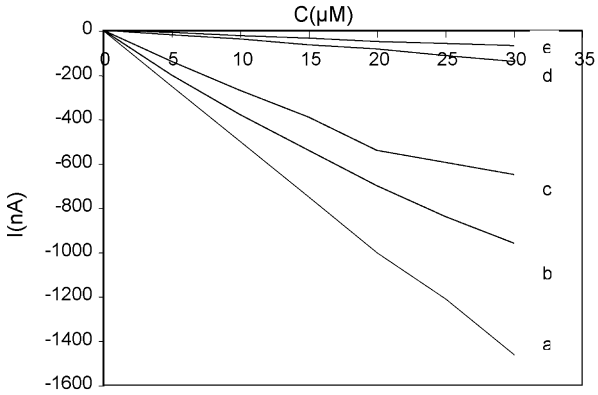

Fig. 2 – Influence of the concentration of electroactive phenols on the steadystate current: (a) catechol, (b) phenol, (c) *p*-cresol, (d) caffeic acid, and (e) chlorogenic acid, in 0.2 M pH 7 phosphate buffer at 30 °C, working electrode potential 0.3 V vs. Ag/AgCl.

higher peak along with a sharp decrease in the peak separation. This could be ascribed to the catalytic chemical oxidation of catechol by tyrosinase in the electrode surface as compared with alumina sol–gel modified electrode (2).

$$\text{catechol (atelectrode)} \leftrightarrows \text{o-quinone} + 2H^+ + 2e^- \quad (1)$$

$$\text{catechol} + \text{tyrosinase}\,(O_2) \rightarrow \text{o-quinone} + H_2O \quad (2)$$

This results indicate that the immobilization process retains the biological activity of tyrosinase in the alumina sol–gel film.

3.2. Response of the sensor for different phenolic compounds

Fig. 2 shows the influence of the concentration of five electroactive phenols on the steady–state current. Their sensitivities followed the sequence catechol > phenol > *p*-cresol > caffeic acid > chlorogenic acid. Table 1 lists the characteristics of the biosensor, including sensitivity, linear range and detection limit, for the phenolic compounds. Responses vary with the substitution group of phenolic compounds. No signal was obtained for o-cresol

3.3. Optimization of enzyme electrode preparation

The amount of enzyme is an important parameter in the preparation of enzyme electrode. Fig. 3 shows the effect of enzyme amount on cathodic peak current for the tyrosinase electrode. The current increases with increasing concentration of enzyme solution deposited onto the electrode surface. The curve shows a plateau at a tyrosinase concentration of 0.2 IU. This corresponds to the maximum amount of tyrosinase which can be encapsulated in the alumina sol–gel thin-film, which has become saturated. Under the optimized preparation conditions, a uniform tyrosinase alumina sol–gel film was obtained with open structure on the electrode surface.

Table 1 – Characteristics of biosensors including sensitivity, linear range and detection limit

Compound	Sensitivity (nA/μM)	Linear rang (M)	R[a]	Detection limit (M)
Catechol	48	10^{-7}–3×10^{-5}	0.999	3×10^{-8}
Phenols	32	5×10^{-7}–3×10^{-5}	0.999	3×10^{-7}
p-Cresol	22.3	5×10^{-6}–3×10^{-5}	0.997	5×10^{-7}
Caffeic acid	4.6	5×10^{-6}–3×10^{-5}	0.999	6.2×10^{-7}
Chlorogenic acid	2.3	5×10^{-6}–3×10^{-5}	0.999	6.1×10^{-7}
o-Cresol	0	–	0	–

[a] Correlation coefficient of the linear rang.

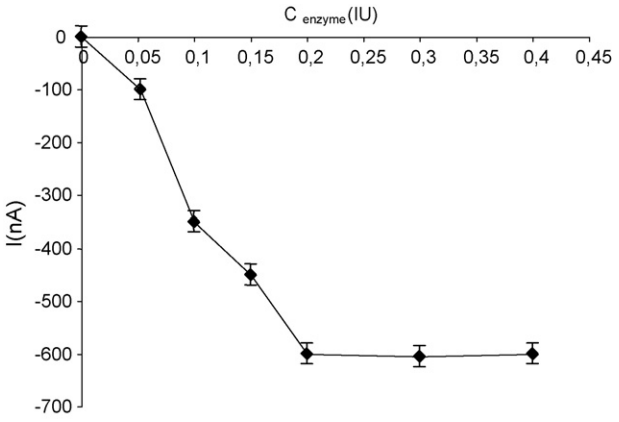

Fig. 3 – Effect of enzyme concentration on cathodic peak current of the tyrosinase electrode, 0.2 M pH 7 phosphate buffer at 30 °C, −0.3 V vs. Ag/AgCl after addition of 10 μM catechol.

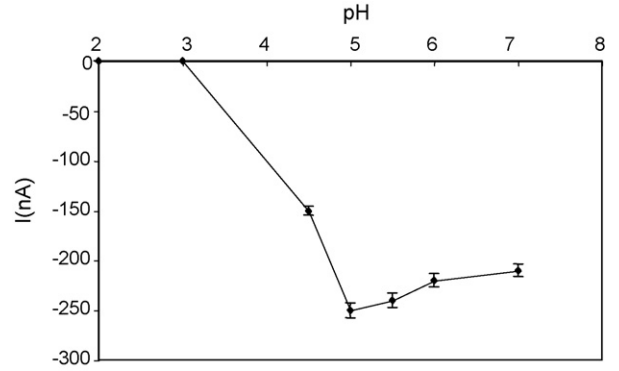

Fig. 4 – Effect of pH on sol–gel matrix, 0.2 M pH 7 phosphate buffer at 30 °C, −0.3 V vs. Ag/AgCl after addition of 5 μM catechol.

3.4. *Effect of pH on sol–gel matrix*

The pH of sol–gel affects directly the performance of the enzyme sensor due to the accumulation of protons. Fig. 4 shows the effect of pH on the reduction peak current of the biosensor for 5 μM catechol concentration. The best response is obtained at pH 5. All subsequent experiments were performed at pH = 5.

Fig. 5 shows the amperometric response of the three tested sol–gel matrixes under the optimal experimental conditions. The alumina sol–gel shows lowest detection limit and the highest stability when compares with Titania sol–gel and PVA-AWP polymerization. This matrix was selected to continue the studies.

3.5. *Reproducibility and stability*

Operational stability was investigated by consecutive measurements of catechol within a 10 h period. After 100 measurements, the biosensor retained 80% of its initial activity. It was observed that the Al_2O_3 sol–gel immobilized enzyme electrode did not swell in the solution during continuous measurements. Additionally, the Al_2O_3 sol–gel derived tyrosinase biosensor exhibited long-term stability. After measurement, the sensors were rinsed with distilled water and stored at 4 °C. Fig. 6a shows data referred to thermal stability and lifetime of the biosensor. The reproducibility of the biosensors was evaluated by mean of 10 repetitive measurement in 0.2 M phosphate buffer, pH = 7, containing 5 μM of catechol; a relative standard deviation of 1.48% was obtained for this biosensor. While the activity of the enzyme after 3 weeks loss 100% of it activity indicating that tryosinase has much better stability when immobilized within alumina sol–gel matrix (Fig. 6b). The tyrosinase enzyme in matrix or in soluble state shows optimal activity at 30 °C, thereafter the activity of the enzyme was decreased in the same manner in both cases.

The relative stability of biosensors can be attributed to two factors. The first of them is the biocompatibility of the sol–gel with the enzyme tyrosinase, probably due to the inorganic–organic structure of the sol–gel. The second is the

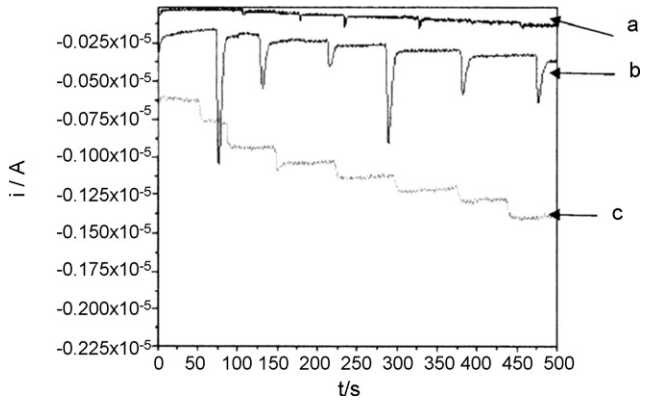

Fig. 5 – Effect of sol–gel matrix on the response of biosensor, 0.2 M pH 7 phosphate buffer at 30 °C, −0.3 V vs. Ag/AgCl (a) PVA, (b) Titania sol–gel, (c) alumina sol–gel.

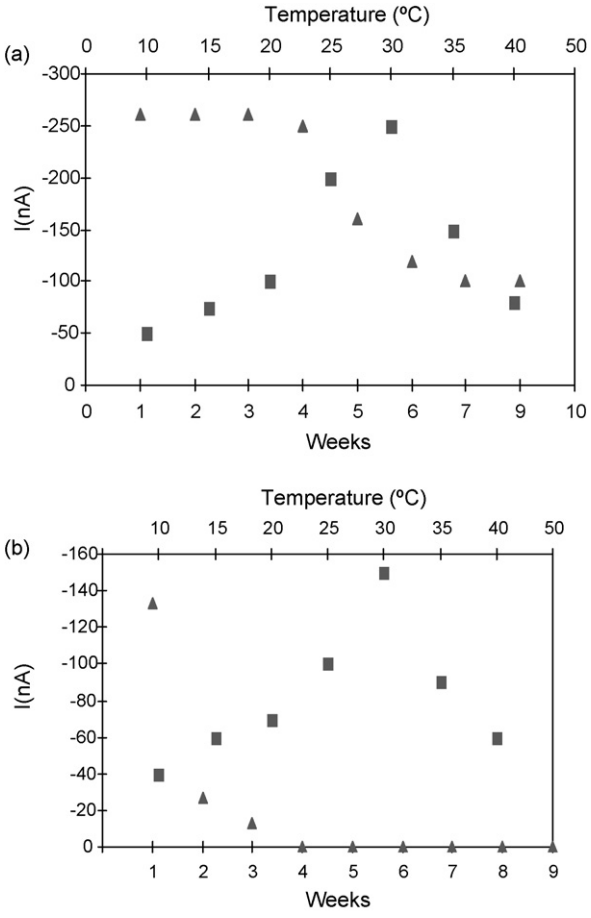

Fig. 6 – Experimental data referred to thermal stability (■) and lifetime of the biosensor (▲) for immobilized enzyme (a) and soluble enzyme (b).

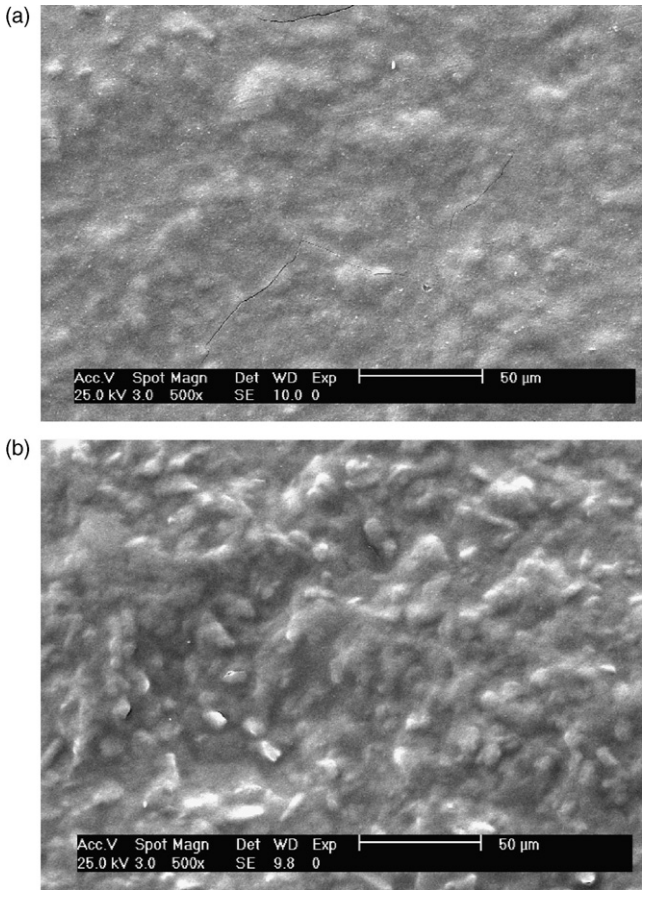

Fig. 7 – SEM measurements obtained at 25.0 KV with a 500× zoom. (a) Alumina sol–gel/Sonogel-Carbon electrode, (b) tyrosinase/alumina sol–gel/Sonogel-Carbon electrode.

inertness of this material; the shrinkage phenomenon was avoided thanks to exclusion of the use of organic solvent in the sol–geling process.

3.6. *SEM measurements*

The results of morphological studies conducted on Al_2O_3/Sonogel-Carbon and tyrosinase/Al_2O_3/Sonogel-Carbon are shown in Fig. 7. The SEM of Al_2O_3/Sonogel-Carbon indicates that the film is uniform. The SEM of tyrosinase/Al_2O_3/Sonogel-Carbon shows granular (porous) structure which confirms the presence of enzyme.

3.7. *Application to real samples*

To study the real feasibility of the tyrosinase based alumina sol–gel/Sonogel-Carbon electrode, this was employed to determine the phenolic content in water real samples. The phenols were estimated by standard addition analysis of cathecol. The samples were taken in the Martil river (located at the north of Marocco), near of a source of industrial waste water dumping. The results, in standards equivalent, are showed in Table 2, and they show that the alumina sol–gel tyrosinase/Sonogel-Carbon electrode retains its stable and reproducible response in real sample. We think that the high amount of hydroxyl groups in the sol–gel film and Sonogel electrode favours the formation of strong hydrogen bonds with tyrosinase, and thus the enzyme loading into the sol–gel mesh; so, it can provide a good protection for the immobilized enzyme against possible inhibitors, such as mercury and pesticides (chlorpyrifos-ethyl-oxon, paraoxon and dichlorvos).

Table 2 – Application of alumina sol–gel-tyrosinase Sonogel-Carbon electrode to real samples

Samples	Total phenols (M)	RSD%	No. of exp.
S1	10^{-7}	1.4	6
S2	5×10^{-7}	1.8	8

4. Conclusion

A new tyrosinase biosensor for phenolic compounds, based on the combination of an alumina sol–gel and the Sonogel-Carbon material, is proposed. Sol–gel hybrid material provides a good matrix for the immobilization of tyrosinase; strong hydrogen bonds and sol–gel networks not only effectively entrap the enzyme molecule, but also increase the thermal and long-term stability of immobilization enzyme. In view

of the remarkable performance of the biosensor, the sol–gel based enzyme electrode could be coupled with separation techniques to realize the respective determination of electroactive phenolic compounds. The good results obtained for LOD, sensitivity, and stability, encouraged us to continue our investigations with these systems in search of new applications.

Acknowledgement

Hanane ZEJLI would like to thank Agence Universitaire de la Francophonie (AUF) for her post-doc grant.

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