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A thionine-modified carbon paste amperometric biosensor for catechol and bisphenol A determination

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ABSTRACT

A thionine-modified carbon paste electrode for catechol and Bisphenol A (BPA) detection is presented. Graphite powder was modified by adsorbing thionine as electrochemical mediator. The electrochemical response of the modified carbon paste electrode (CPE) was determined before electrode modification with tyrosinase. Then, tyrosinase was added in order to assemble a biosensor. Once established the best operative conditions, an interelectrode reproducibility around 7% was obtained and the resulting biosensor showed improved sensitivities and ($S = 139.6 \pm 1.1$ nA/μM for catechol and $S = 85.4 \pm 1.5$ nA/μM for BPA) in comparison with the biosensor constructed without thionine ($S = 104.4 \pm 0.5$ nA/μM for catechol and $S = 51.1 \pm 0.6$ nA/μM for BPA) and low detection limits (0.15 μM for both the electrodes and analytes). Also the comparison with the results reported in the literature showed higher sensitivity and lower detection limit for our biosensor. Moreover the functioning of the thionine-tyrosinase CPE was validated following a biodegradation process of water polluted by BPA and comparing the time changes of BPA concentration inferred by the biosensor calibration curve and those determined by means of HPLC measurements.

1. Introduction

Concerns are growing among the public and the scientific community owing to the awareness that a group of chemicals, known collectively as endocrine disrupting compounds (EDCs), are suspected of interfering with the normal function of the endocrine system causing adverse effects for humans and wildlife. EDCs include a range of synthetic estrogens, pesticides, plasticizers and phenol compounds (Colborn et al., 1996). Concerns have been raised, in particular, about the contribution of alkylphenols (breakdown products of detergent alkylphenol polyethoxylates) which have been found in drinking water supplies and waste streams (Bruchet and Janex-Habibi, 2004). These chemicals have phenolic rings, a structure also found in natural estrogens. A similar structure is present also in Bisphenol A (BPA) and catechol.

Bisphenol A [BPA, 2,2-(4,4-dihydroxydiphenyl) propane; CAS Registry No. 80-05-7] is produced by combining acetone and phenol and is an important organic chemical owing to its wide use as an intermediate in the manufacture of polycarbonate plastics, epoxy resins, and flame retardants (Staples et al., 1998; Mutou et al., 2006). Its main use is for coating of cans, powder paints, dental fillings, and antioxidant in plastics. It has also been used as an inert ingredient in pesticides, antioxidants and polyvinyl chloride stabilizer (Staples et al., 1998; Rodriguez-Mozaz et al., 2005). BPA shows estrogenic potential both in vitro and in vivo (Matthews et al., 2000; Segner et al., 2003) and also acute toxicity towards aquatic organisms (Staples and Davis, 2002; Suzuki and Hattori, 2003) and human cultured cells (Nakagawa et al., 2000) In particular it has been found that BPA binds to the estrogen receptor (Matthews et al., 2001) produces meiotic aneuploidy in the female mouse (Hunt et al., 2003) and increases cell proliferation in the male and female sexual organs. Additionally, although still controversial, BPA may even induce a decrease of sperm quality in humans (Maffini et al., 2006).

BPA pollution occurs owing to its release from many commodities, such as the inside cans coating, polycarbonate baby bottles, and plastic waste, as well as accidental spills (Fukazawa et al., 2001; Fromme et al., 2002; Sajiki and Yonekubo, 2004; Maragou et al., 2006).

The results cited above suggest that it is necessary to remove BPA from the environment and to determine its presence also in trace.

Concerning the first priority, we have demonstrated that it is possible, through enzymatic processes carried out with non-isothermal bioreactors, to remove BPA and other phenol compounds from the aquatic environment (Diano et al., 2007;

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Georgieva et al., 2008; Mita et al., 2009). The success in the removal of BPA has been demonstrated not only analytically through high-performance liquid chromatography (HPLC) determination but also by means of the disappearance, after enzyme treatment, of some of the biological effects previously observed in the absence of bioremediation (Ricupito et al., 2009). These last experiments also showed that the enzyme reaction products were not estrogenic.

Concerning the second priority, i.e. the necessity of determining BPA even in trace, this is currently done using classic analytical techniques such as gas chromatography (GC) and HPLC (Watabe et al., 2004; Sun et al., 2006). Immunochemical techniques were recently reported (Ballesteros-Gomez et al., 2009) using monoclonal and polyclonal mammalian (Ohkuma et al., 2002) and chicken (De Meulenaer et al., 2002) antibodies in enzyme linked immunosorbent assays (ELISA's). Mass spectrometry is also considered a useful technique. However, all these technologies are expensive and time-consuming. Electrochemical biosensors, on the contrary, represent a rapid and less expensive method and have the advantage of high sensitivity, potential for miniaturization, and the possibility of in situ analysis (Rogers et al., 1999). The use of carbon paste as electrode material was initially reported in 1958 by Adams (Adams, 1958). Carbon paste offers high versatility, surface renewability, low noise, low background currents, and low cost.

Tyrosinase-modified carbon paste electrodes have been described earlier (Marko-Varga et al., 1995; Lutz et al., 1995; Lindgren et al., 1996; Petit et al., 1996; Wang et al., 1997). Tyrosinase is a copper containing metalloprotein which catalyses the hydroxylation and oxidation of mono and diphenols to *o*-quinones. It can exist as a mono and diphenolase (Hedenmo et al., 1997). The mono form can be inactive (met) or active (oxy) and the formation of a significant amount of the active form depends on the presence of catechol. When phenol is used as substrate, only a small part of the enzyme is active due to the absence of the diphenol 'activator'.

In this paper we report the results obtained with tyrosinase based carbon paste electrode modified with the addition of thionine as a mediator. Thionine is an artificial organic dye derivative of phenothiazine, and it is used as a mediator since its formal potential is between 0.08 and −0.25 V, near the redox potential of many biomolecules (Shobha Jeykumari et al., 2007). Thionine was first employed for the electrocatalytic oxidation of NADH by crosslinking it onto a graphite electrode (Ohtani et al., 1997; Hajizadeh et al., 1991). Cosnier (Cosnier et al., 2001) reported the mediated electrochemical detection of catechol using thionine covalently bound to a poly(dicarbazole) backbone. Thionine has also been used as polythionine by means of electropolymerisation of the monomer (Yang et al., 1999; Liu et al., 2008). Later on, Dempsey (Dempsey et al., 2004) developed a biosensor using thionine electropolymerised on a glassy carbon electrode in the presence of tyrosinase for the detection of synthetic estrogens (BPA) and phenolic compounds.

In the present work thionine was used in the construction of a carbon paste electrode after its adsorption onto the carbon powder. The carbon paste was then modified with tyrosinase. Finally, the biosensor was then challenged in amperometric mode. Linear calibration curves have been obtained both for BPA and catechol. Our results have been compared with those obtained by other authors with and without thionine, and with thionine electropolymerised on glassy carbon electrode.

2. Materials and methods

2.1. Materials

Tyrosinase (E.C. 1.14.18.1, 2033 Units/mg) from mushrooms has been used as enzyme. It must be noted that the specific activity of tyrosinase here employed is lower than that we used in our previous work (Mita et al., 2007). It was not possible to find anymore tyrosinase with higher specific activity. It is known that this fact can cause a lower response and thus problems in the reproducibility and in the comparison among different catalytic system (Rescigno et al., 2007).

Catechol and BPA were used as substrate representative of phenolic compounds and endocrine disruptors, respectively. Carbon powder (<0.1 mm in size) was purchased from Fluka. The mineral oil (cod. 69794), employed as pasting material, catechol, BPA and all the other chemicals (of pure pro-analysis grade), were purchased from Sigma (Sigma–Aldrich, Milan, Italy) and used without further purification.

2.2. Methods

Carbon paste was prepared by hand mixing graphite powder, thionine (when it was the case), mineral oil and tyrosinase in an appropriate weight ratio. The resulting pastes were packed into the well of the working electrode as described below. The body of the working electrode was a Teflon tube (3 mm in diameter) with an electrical contact provided by a copper wire. The working electrodes were prepared by packing the Teflon tube with a double layer of carbon paste: an inner layer containing only graphite powder mixed to mineral oil, and an outer layer composed of carbon paste, tyrosinase and thionine (when indicated). The inner layer (about 2/3 of total volume) was prepared by mixing 60% of graphite powder and 40% of mineral oil, for a total weight of 4.8 mg. The outer layer (about 1/3 of the total volume) with a total weight of 2.5 mg, was prepared by a carbon paste obtained from two steps. During the first step, 600 mg of graphite powder are mixed for 10 min with 1 mL of thionine (1 mM or 10 mM), and the thionine-modified powder was put in an oven at 60 °C for about 15 h. In the second step, the thionine/graphite powder was mixed with mineral oil and tyrosinase in the ratio (w/w) of 50:40:10, respectively. For each electrode we used about 0.25 mg of tyrosinase which correspond to about 500 U. To prepare thionine free electrode we followed only the second step, mixing graphite powder, mineral oil and enzyme in the ratio before used. The electrode surface exposed to the solution was polished on weighing paper to give a smooth finish before use. Amperometric measurements were carried out using a PalmSens instrument (Palm Instruments, the Netherlands) coupled to a PC. All experiments were carried out in a three electrode electrochemical cell with a volume of 10 mL (0.1 M sodium phosphate, pH 6.5, containing 0.1 M KCl) with the enzyme modified CPE as working electrode, an Ag/AgCl electrode as reference electrode and a platinum wire as auxiliary electrode. The working electrode was operated at −200 mV vs. Ag/AgCl and the transient currents were allowed to decay to a steady-state value. A magnetic stirrer and a stirring bar provided the convective transport in the electrolytic cell. All measurements were performed at room temperature (25.0 ± 0.5 °C). Each experimental point in Figs. 4 and 5 represent the average of five experiments carried out under the same conditions. The experimental error never exceeded 4.6%.

3. Results and discussion

3.1. Cyclic voltammetry measurements

In order to investigate the electrochemical behaviour of BPA and catechol at the carbon paste electrode, we first carried out cyclic voltammetry of these molecules at the bare electrode surface. In Fig. 1a and b cyclic voltammogramms of catechol and BPA are respectively shown. The oxidation of catechol exhibits two reversible peaks, as typically reported in literature (Rodriguez and Rivas, 2002). On the contrary, in the case of oxidation of BPA, a non-conducting film (poly(bisphenol A) is formed, which leads to

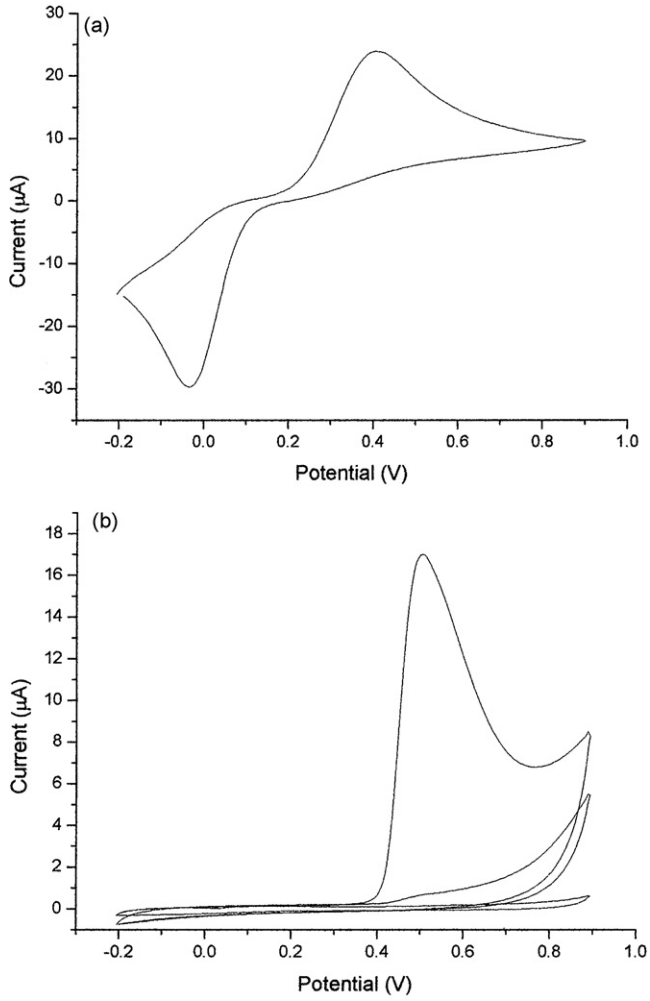

Fig. 1. Cyclic voltammograms of (a) catechol 15 mM and (b) BPA 15 mM in 0.1 M sodium phosphate buffer + KCl 0.1 M, pH 6.5, scan rate 100 mV/s.

the failure of the observed peaks after the second scan. This significant fouling of electrode surface is responsible for the difficulty of a measurement of BPA based on its direct electrochemical oxidation. For this reason, a tyrosinase biosensor was proposed as a successful tool for detecting BPA.

Here, a carbon paste electrode for detecting BPA using the enzyme tyrosinase and the electrochemical mediator thionine as free monomer was realized for the first time. Thionine was initially used as electrochemical mediator for H_2O_2. In this case thionine and Horseradish Peroxidase enzyme were covalently bound to cysteamine self-assembled gold electrode using glutaraldehyde as a bifunctional reagent (Chuanmin et al., 1998). Its use in form of polymeric film obtained by electropolymerisation of the monomer on glassy carbon electrode is also reported in literature for the determination of H_2O_2 (Yang et al., 1999) and of estrogenic compounds (Dempsey et al., 2004). In our work, a stable thionine-modified carbon paste electrode was developed by simply adsorbing thionine onto graphite powder. In this way, the modified CPE was produced using a very simple procedure. Non-covalent functional strategies have generally been considered as more friendly and effective alternatives to chemical processing for obtaining functionalized carbon surfaces (Li et al., 2004). In addition, the physical entrapment of enzyme in the composite electrode matrix provides the advantage of easier, faster and cheaper electrode fabrication and at the same time reduces the possible losses in sensitivity due to covalent attachment.

Before electrode modification with tyrosinase, an electrochemical investigation of the thionine-modified CPE was carried out by means of the cyclic voltammetry technique. A scan rate study between 5 and 30 mV/s vs. an Ag/AgCl electrode was carried out using the thionine-modified CPE. The current of the anodic and cathodic peaks increases linearly with increasing scan rate up to 30 mV/s as described by the equation $y = (0.5 \pm 0.1)x + (1.3 \pm 0.3)$ ($R = 0.998$), where y is the current (μA) and x is the scan rate (mV/s) for anodic peaks. This behaviour demonstrates that at low scan rates the electrochemical reaction is surface controlled. The ratio Ipa/Ipc is almost equal to 1 and the peak to peak separation (ΔE_p) at low scan rate (5 mV/s) is about 100 mV. This latter value of ΔE_p is higher than the theoretical one which is zero as indicated in the case of ideal Nernstian behaviour of surface-confined species (Wang, 2002). The ΔE_p found in this work should be due to the fact that the mediator thionine is enclosed in the carbon paste. In fact, the presence of the oil, a non-conductor system, hampers the electrochemical communication between the particles of thionine-modified graphite, causing such a high value of ΔE_p.

Investigation of the pH dependence of the formal redox potential (E°) was carried out in the range between 4 and 8 using the Britton–Robinson buffer. The formal redox potential was correlated to the pH by the equation: $E^\circ(\text{mV}) = (-55 \pm 4)\ \text{pH} - (18 \pm 6)$ ($R = 0.994$), showing a negative shift in E° with increasing pH. The slope of 55 ± 4 mV/pH is close to the expected Nernstian value of 59 mV, indicating that the redox reaction of thionine involves one electron and one proton. These results are in agreement with the ones obtained by Dempsey et al. (2004) with electropolymerised thionine.

In order to check the electrocatalytic properties of thionine towards the product of the BPA/tyrosinase reaction, cyclic voltammograms obtained using a CPE modified only with tyrosinase, and having thionine free in solution, have been carried out. Fig. 2 shows the CVs of 0.4 mM thionine in 0.1 M sodium phosphate buffer solution in presence of KCl 0.1 M, pH 6.5, using the tyrosinase carbon paste biosensor before (continuous line) and after (dashed line) the addition of BPA at a final concentration of 3 mM. As shown in Fig. 2, the current due to the thionine reduction increases compared to the scan obtained in the absence of BPA. For the reverse scan, in presence of BPA, the current of the anodic peak due to the thionine oxidation decreases as expected for mediated reactions. The enzymatic reaction following the addition of BPA causes an increase in concentration of the thionine$_{ox}$ at the surface of working electrode, resulting in an increase of the cathodic peak current. On the contrary, the anodic peak current is proportional to the amount of

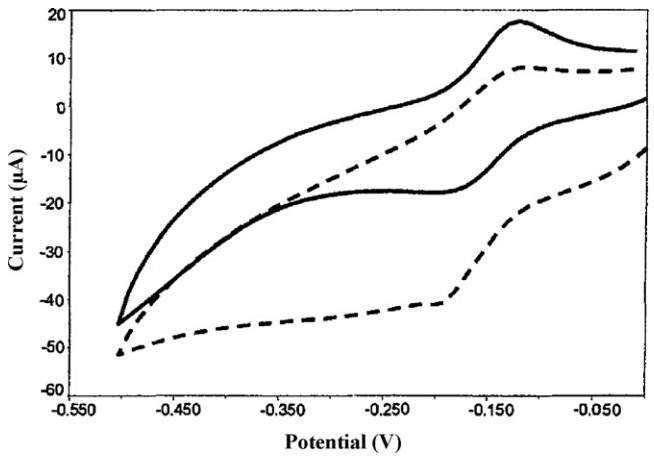

Fig. 2. Cyclic voltammograms using tyrosinase carbon paste electrode and thionine 0.4 mM in 0.1 M sodium phosphate buffer plus KCl 0.1 M, pH 6.5, in absence (continuous line) and presence (dotted line) of BPA 3 mM, scan rate 100 mV/s.

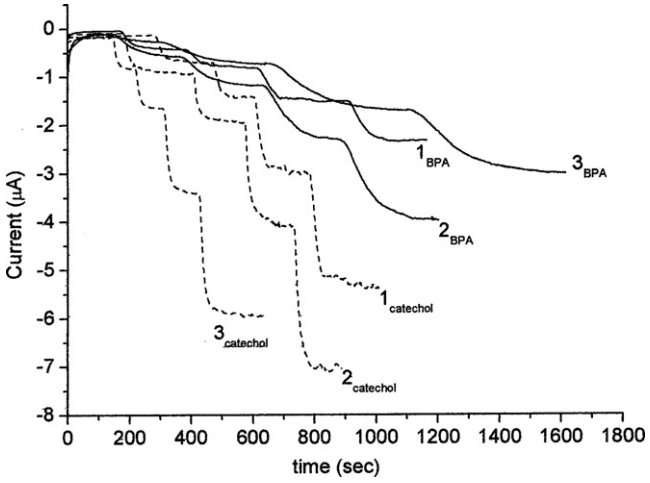

Fig. 3. Current responses to catechol (dotted lines) and to BPA (continuous lines) at thionine free electrode (curves 1), at 1 mM thionine electrode (curves 2) and at 10 mM thionine electrode (curves 3). Applied potential 200 mV vs. Ag/AgCl; 0.1 M sodium phosphate buffer plus KCl 0.1 M, pH 6.5.

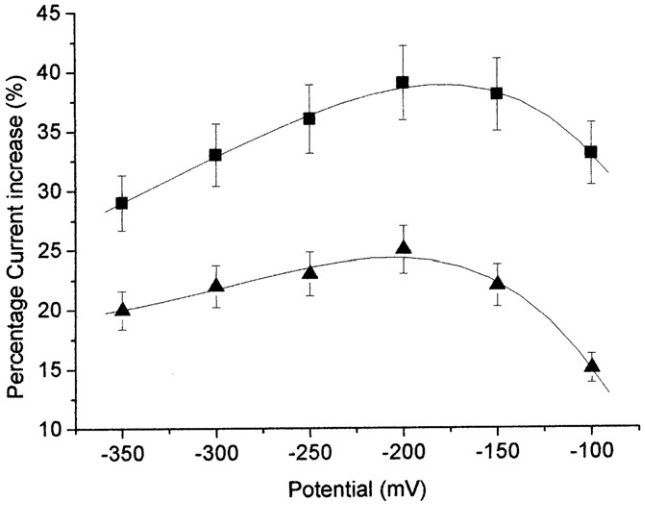

Fig. 4. Percentage current increase in function of the applied potential with catechol 10 μM (■) and with BPA 10 μM (▲) in 0.1 M sodium phosphate buffer plus KCl 0.1 M, pH 6.5.

thionine$_{red}$ at the working electrode, a quantity that decreases after the addition of BPA. These results confirm that thionine has electrocatalytic activity towards the reduction of the reaction product of BPA with tyrosinase.

3.2. Biosensor characterization

Having ascertained the electrocatalytic properties of thionine towards the reaction product of BPA with tyrosinase, we assembled the new biosensor by using graphite powder modified with thionine, mineral oil and tyrosinase.

First, the effect of different amount of thionine adsorbed onto the graphite was evaluated. In order to find the best operative conditions, three different working electrodes have been built, varying the thionine concentrations (0; 1 mM and 10 mM) and leaving constant the amount of graphite powder, mineral oil and tyrosinase. Fig. 3 shows the results obtained using the three electrodes subjected to a work potential of −200 mV when catechol was used as analyte. Once the background current was measured, four injections of standard amounts of catechol have been added: the first two equal to get a final concentration of 7.5 μM, for each injection, to assess the repeteability of the biosensor response; the third and the fourth ones to a final concentration of 30 μM and 52.5 μM respectively to assess the proportionality of the electrical signal to the analyte concentration. Repeteability and proportionality were clearly shown for each of the three electrodes (see Fig. 3). As expected, equal injections give signals equal within 3% and proportional injections give signals proportional within 4%. The results in Fig. 3 indicate that the electrode prepared with 1 mM thionine produces higher current values than those observed with the thionine-free electrode or those obtained with the electrode prepared with 10 mM thionine. The average response time was about 100 s.

Analogous results (see Fig. 3) have been obtained when BPA was used as analyte in the same concentration used before for catechol. In this case the average response time was about 200 s.

Comparison among curves in Fig. 3 indicates that: (1) the electrical currents in the presence of catechol are higher than those measured with BPA, catechol being a better substrate of tyrosinase enzyme as reported in literature (Mita et al., 2007; Andreescu and Sadik, 2004); (2) the electrodes modified with thionine give higher responses, demonstrating the electrocatalytic properties of thionine towards the reaction product of BPA with tyrosinase.

At this point further experiments were carried out to investigate whether the working potential of −200 mV was actually the best. To this aim, the increases in current for the electrode prepared with thionine (1 mM) in respect to the currents measured with the thionine-free electrode, also a function of the working potential, were measured. The percentage current increase (P.C.I. (%)) were calculated, at each working potential, by means of the equation:

$$\text{P.C.I.}\,(\%) = \frac{I_{\text{thion}} - I_{\text{thionfree}}}{I_{\text{thionfree}}} \times 100$$

where I_{thion} is the current with the electrode prepared with thionine (1 mM), $I_{\text{thionfree}}$ is the current obtained with the thionine free electrode. Fig. 4 (▲) shows the percentage current increases obtained in the case of catechol. From the same figure it is possible to see that the maximum increase (about 25%) occurs at −200 mV.

The same kind of experiment was repeated with BPA and the results are shown in Fig. 4 (■). Also in this case, the maximum percentage current increase, about 39%, occurs at a potential of −200 mV vs. Ag/AgCl electrode.

After ascertaining that the best working potential was −200 mV, we studied the response of both biosensors, the one prepared with 1 mM thionine and the thionine-free CPE, as a function of substrate concentration.

In Fig. 5 the responses of the two biosensors, when catechol was used as analyte, are displayed as a function of the analyte concentration. For both electrodes the detection limit is 0.15 μM, calculated according to the standard definition S/N = 3, where S is the current signal and N is the noise. The linear range is from 0.15 to 75 μM, while the sensitivity is 139.6 ± 1.1 nA/μM or 104.4 ± 0.5 nA/μM for 1 mM thionine or thionine-free electrode, respectively. It is worthy of mention that the sensitivity values obtained for our biosensors are higher than those reported in the literature for catechol: 114 nA/μM (Li et al., 2006), 70.2 nA/μM (Rajesh et al., 2004) or 26.23 nA/μM (Bingquan and Shaojun, 2000).

In Fig. 5 we have also reported as a function of the analyte concentration the responses of the two biosensors when BPA was used as analyte. For both the electrodes the detection limit is 0.15 μM and the linear range is from 0.15 to 45 μM, while the sensitivity is 85.4 ± 1.5 nA/μM or 51.1 ± 0.6 nA/μM for 1 mM thionine and thionine-free electrode, respectively. Also in the case of BPA, it is worthy of mention that the sensitivity values displayed by our biosensors are higher than those reported in the literature for BPA by Andreescu (Andreescu and Sadik, 2004) (S = 20.91 nA/μM) or by

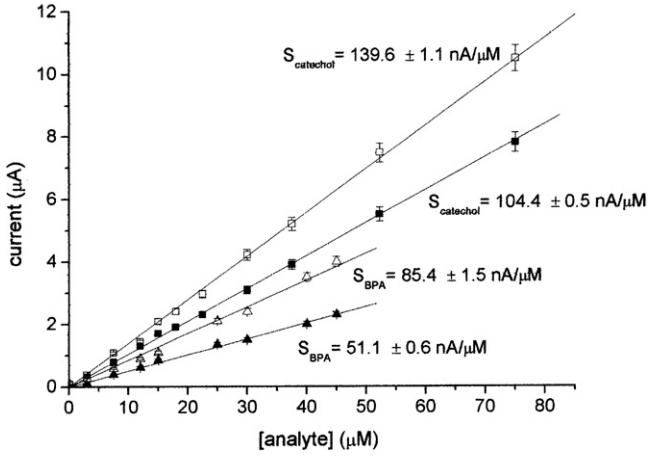

Fig. 5. Calibration curves obtained at thionine free electrode (full symbols) or at 1 mM thionine electrode (empty symbols) for catechol (■,□) and for BPA (▲,△). Applied potential 200 mV vs. Ag/AgCl; 0.1 M sodium phosphate buffer plus KCl 0.1 M, pH 6.5.

Dempsey (Dempsey et al., 2004) (S = 0.4 nA/μM), who also used thionine as electrochemical mediator or also than those reported in the literature for phenol compounds: 17.1 nA/μM (Rajesh et al., 2004) or 2.42 nA/μM (Bingquan and Shaojun, 2000).

The intraelectrode repeatability never exceeded the 2% (n = 5) while the interelectrode reproducibility was around 7%.

Concerning the stability, the carbon paste electrode was stable for about one month of working. The electrodes were discarded when their response to a catechol solution of 5 μM decreased 7% relative to the initial one.

Before concluding a significant experiment is described, in which the real functioning of our thionine-based biosensor is validated during a bioremediation process of an aqueous solution polluted by BPA. 10 mL of 1 mM BPA aqueous solution were allowed to react with a membrane on which laccase was immobilized. The immobilization method can be found in a previous paper (Mita et al., 2009). At zero time 200 μl were withdrawn from the reaction vessel for the subsequent measurements. 100 μL of those are diluted in 10 mL of buffer solution and put in contact with the biosensor, while 25 μL were processed in the HPLC. The procedure for HPLC assay is described in the same paper above indicated. Every 10 min the same operation was repeated until 100 min. In Fig. 6 the average results of five experiments are reported. Data in figure show: (i) the time decrease of the current intensity measured by the biosensor during the bioremediation process (continuous line and right scale); (ii) the time dependence of BPA concentration (left scale) as measured by HPLC (○) and as obtained by the calibration curve reported in Fig. 5 (●). It is interesting to note how: (i) a reduction of 29% in the current value corresponds to a reduction of 25% in the concentration value; and (ii) the BPA concentration values measured by HPLC are very similar to those obtained from the calibration curve. The conclusion is that the functioning of our thionine-tyrosinase based biosensor appears excellent.

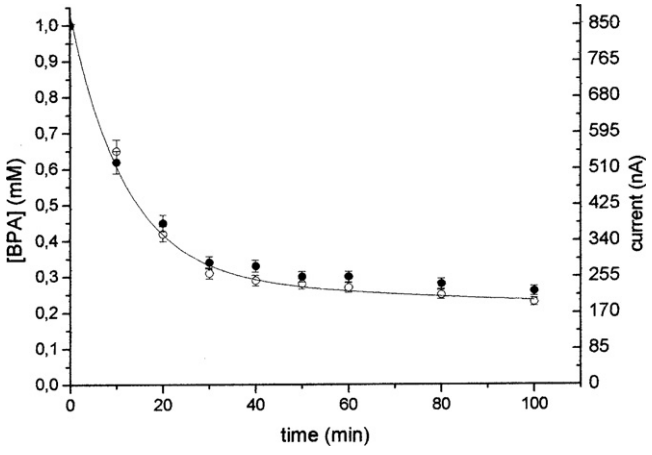

Fig. 6. Time dependence of biosensor response (continuous line, right scale) and validation of the response through the analysis of BPA concentrations (left scale) by means of HPLC (○) and the calibration curve (●) of Fig. 5.

4. Conclusions

This paper reports the simple and effective preparation of a carbon paste biosensor for catechol and BPA determination. It was prepared by using thionine as mediator and the enzyme tyrosinase as bioactive element. The thionine adsorption onto the graphite powder turned out to be a simple way to obtain a stable thionine-modified CPE. In addition, the use of thionine as monomer in the tyrosinase carbon paste biosensor seems to improve the analytical performances with respect to those exhibited by the glassy carbon paste modified with electropolymerised thionine and tyrosinase (Dempsey et al., 2004). In the latter case, in fact, lower sensitivity (0.4 nA/μM) and higher detection limit (23 μM) were observed for BPA. This behaviour can probably be ascribed to the better solubility of BPA in a hydrophobic system such as the one with oil present in the CPE. The oil, in fact, seems to play a significant role as reported in our previous paper (Mita et al., 2007). In that paper we have found with reference to the BPA graphite carbon powder biosensor the following characteristics: linear range from 0.1 to 15 μM; S = 68 ± 4 nA/μM; and detection limit = 0.1 μM. In the present work, thanks to thionine, we have further improved our BPA graphite carbon powder biosensor having found: linear range from 0.15 to 45 μM; S = 85.4 ± 1.5 nA/μM; and detection limit = 0.15 μM. In this way we have demonstrated that the combination of graphite powder, oil and thionine as mediator can be a useful system for BPA and catechol detection when tyrosinase is used as bioactive element.

Moreover, it is possible to improve the performance of our biosensor, for example, by using an enzyme with higher specific activity. Studies in this direction are being carried out in our laboratories.

Acknowledgments

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