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Highly sensitive amperometric biosensor based on a biocompatible calcium phosphate cement

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ABSTRACT

Brushite is a biocompatible calcium phosphate mineral with properties of solid electrolyte. In this study we take advantage of this characteristic to develop an enzymatic amperometric biosensor based on brushite cement. The biosensor was prepared by immobilizing tyrosinase (PPO) on a brushite cement layer which was subsequently cross-linked with glutaraldehyde (GA) on the surface of a glassy carbon electrode. The system was optimized for the detection of phenolic compounds in both aqueous and non-aqueous solutions. Several variables involved in the enzyme immobilization method such as glutaraldehyde cross-linking time, PPO/brushite ratio and thickness of the brushite film were investigated. Furthermore, the effects of the pH, temperature and applied potential on the biosensor performance were also optimized. On the other hand, the biosensor analytical properties were studied in presence of different organic solvents: dioxane, acetonitrile and ethanol. In both, phosphate buffer solution (PBS) and acetonitrile/PBS solution, the biosensor exhibits a rapid response (12 s); a wide linear range (0.001–3 μM and 0.007–2 μM respectively); low detection limit (1 and 2 nM respectively); and high sensitivity (46.6 and 28.6 A M^{-1} cm^{-2} respectively). The performance of the biosensor in the analysis of phenols in real samples was successful.

1. Introduction

Calcium phosphate based materials (CaPs) have a wide range of applications in medicine (implants), agriculture (fertilizers), pharmacy (tabletting, toothpaste), chromatography (columns), and food industry (additives) (Bohner, 2000). Two different categories of CaPs should be distinguished: (i) low temperature CaPs obtained by precipitation from an aqueous solution at room temperature, and (ii) high-temperature CaPs obtained by thermal reaction. Calcium phosphate cements belong to the first category and are usually constituted by dicalcium phosphate dihydrate (DCPD), octacalcium phosphate and hydroxyapatite.

DCPD, also called brushite, ($CaHPO_4{\cdot}2H_2O$) is a biocompatible material present in human tissues and easy to synthesize. The crystal structure of brushite consists of compact sheets of parallel chains, in which calcium ions are coordinated by oxygen atoms of phosphate groups and water molecules. Two types of water molecules, adsorbed and lattice water, are present in brushite. Both types of molecules can be electrolyzed with the subsequent proton migration (Tortet et al., 1997). Therefore the conductive properties of brushite, together with its biocompatibility and protein adsorption capacity, suggest potential applications in the field of biosensors and bioelectronics. Accordingly, this study exploits these properties for the fabrication of brushite based amperometric biosensors.

Phenolic compounds are widely distributed in plant tissues and are extensively used in industry and agriculture. Hence, the development of accurate and versatile analytical methods for detecting these compounds (Rogers et al., 1999; Naczk and Shahidi, 2004; Kronholm et al., 2004) in hydrophilic (i.e. wine, fruits and water) and hydrophobic samples (i.e. oils and fats) implies an analytical challenge. Biosensors have been used for detecting phenol species in aqueous and non-aqueous media due to the high specificity, minimum sample pretreatment, and low cost and maintenance of these devices. Most of amperometric biosensors for phenolic compounds are based on the enzyme tyrosinase (monophenol polyphenoloxidase, E.C. 1.14.18.1), a copper-protein widespread in plants and animals that catalysis the hydroxylation of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones (Sánchez-Ferrer et al., 1995). Finally, the enzymatically generated *o*-quinone is detected by electrochemical reduction at the electrode.

A wide variety of enzyme immobilization strategies have been applied for development of biosensors in aqueous and organic solvents, including entrapment in different matrices (Stanca and

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Popescu, 2004; Hervás Pérez et al., 2006), covalent bonding (Rajesh and Kaneto, 2005) and adsorption on different materials (Morales et al., 2005; Mousty et al., 2007; Shan et al., 2003a,b).

In this work, a novel procedure for PPO immobilization by adsorption on brushite cement is reported. The immobilization system and the biosensor response were optimized using catechol solutions as substrates, since catechol has been probed to be the best substrate for PPO. Furthermore, the biosensor performance in the detection of phenolic compounds was investigated in aqueous and non-aqueous solutions, as well as in waste water samples.

2. Experimental

2.1. Chemicals

Tyrosinase from *Mushrooms* (1530 U mg^{-1}), catechol, phenol, *m*-cresol, *p*-cresol, 3-clorophenol, 4-clorophenol, β-tricalcium phosphate (β-TCP), monocalcium phosphate anhydrous (MCPA), pure brushite and glutaraldehyde solution 25% in water were purchased from Sigma–Aldrich (St Louis, MO, USA). Acetate/phosphate buffer solutions were prepared from sodium dihydrogen phosphate and sodium acetate (Panreac, Spain). Phosphate buffer solutions were prepared from sodium monohydrogen and dihydrogen phosphate (Panreac, Spain). All reagents were used as received and the water was Milli-Q quality (Millipore, Milford, MA, USA).

2.2. Adsorption materials

Two varieties of CaPs were used as materials for the adsorption of the enzyme; commercial pure brushite, and brushite cement. The brushite cement was obtained by precipitating at room temperature the brushite precursors in water. The cement powder was prepared by mixing β-TCP (1.43 g) with MCPA (0.80 g). This powder reacts with water according to the setting reaction given by

$$Ca_3(PO_4)_2 + Ca(H_2PO_4)_2 + 8H_2O \rightarrow 4CaHPO_4{\cdot}2H_2O$$

2.3. Biosensor preparation

Brushite colloidal suspensions (2 mg mL^{-1}) were prepared by dispersing the brushite cement precursors or pure brushite in water and stirring overnight. These suspensions were mixed with a PPO solution (2 mg mL^{-1}) (1:1, v/v). Subsequently, 25 μL of the aqueous mixture (25 μg of PPO and 25 μg of brushite) was spread on the surface of the glassy carbon electrode and the coating was left to dry at room temperature for 2 h. In order to induce protein cross-linking, the electrode was incubated in a glutaraldehyde saturated atmosphere for 15 min. Finally, the obtained PPO/brushite biomembrane was hydrated for 20 min in a 0.1 M phosphate buffer solution (pH 6.0). As control, a brushite free, glassy carbon electrode was prepared by drying 12.5 μL of a PPO solution (2 mg L^{-1}) on the electrode surface and exposing it to glutaraldehyde vapor for 15 min.

2.4. Apparatus and measurements

The morphology of the biosensors was studied by scanning electron microscopy (SEM). The aqueous mixtures were left to dry on standard SEM stabs. All samples were gold coated and micrographs were taken with a JSM6400 microscope (JEOL USA, Peabody, MA, USA) operating at 20 kV and at magnifications of 2000–5000.

The biosensor amperometric measurements were carried out in a Metrohm Polarecord potenciostat (Model E-506). The electrochemical measurements were performed in a three-electrode cell with a glassy-carbon working electrode, a Pt counter electrode and a SCE reference electrode. The working electrode surface was polished with an alumina slurry paste and was rinsed with ethanol and water.

All experimental measurements were made in 0.1 M PBS, except in the pH study carried out in 0.05 M acetate/0.05 M phosphate buffer. The pH of buffer solutions was adjusted using a Mettler Toledo MP230 pH meter. Biosensors were immersed into 10 mL solution and calibration curves were obtained. The linear region of the calibration plots was fixed according with the best correlation coefficient (R^2); sensitivity was expressed as the slope of these plots. The detection limit (DL) was defined as a signal-to-noise ratio of 3 and the response time was determined as the time required to reach the 95% of the current.

3. Results and discussion

3.1. Comparison between biosensors based on brushite cement and pure brushite

The use of brushite cement as an enzyme immobilizing system implies brushite crystal growth in the presence of PPO on the electrode surface. However, using pure brushite, crystals' formation *in situ* is no longer a parameter affecting the biosensor. An initial comparison between brushite cement and a pure brushite based biosensor was performed in order to evaluate if the effect of brushite crystal formation affect on the final biosensor performance.

The analytical properties of both biosensors in catechol solutions (PBS; pH 6.0), at 20 °C and −0.1 V vs SCE are summarized in Table 1. These results confirm that enzyme adsorption on either pure brushite or brushite cement improves the analytical properties of the biosensor. Both biosensors had a similar maximum current response, stable amperometric response, and a fast response time (12 s). This implies (i) good substrate accessibility to the enzyme active site; (ii) good diffusion of the product to the electrode surface, and (iii) rapid electrode reaction. However, the sensitivity and detection limit of the biosensor based on brushite cement was 10 times higher than that of the biosensor based on pure brushite. This highlights the importance of brushite crystal formation in the presence of PPO for obtaining an optimal biosensor response.

SEM micrographs revealed that the PPO/brushite matrices are constituted by small particles scattered homogeneously over the electrode surface (Fig. 1). Interestingly, the size of the particles was smaller in the PPO/brushite cement biosensor than in the pure brushite one. This difference in the particle size probably

Table 1
Analytical properties of the biosensors based on PPO.

Biosensor	Sensitivity (A M^{-1} cm^{-2})	J_{max} (μA cm^{-2})	Linear range (M)	R^2 (n)	DL (nM)
PPO/pure brushite/GA	4.4	162.9	3.0×10^{-7} to 1.0×10^{-5}	0.9975 (19)	100
PPO/brushite cement/GA	42.6	145.7	3.0×10^{-9} to 3.0×10^{-6}	0.9989 (25)	3
PPO/GA	2.5	108.4	1.5×10^{-7} to 3.0×10^{-5}	0.9996 (24)	150

J_{max}: maximum current density; R: correlation coefficient of the linear range (n: number of points in the linear range); DL: detection limit.

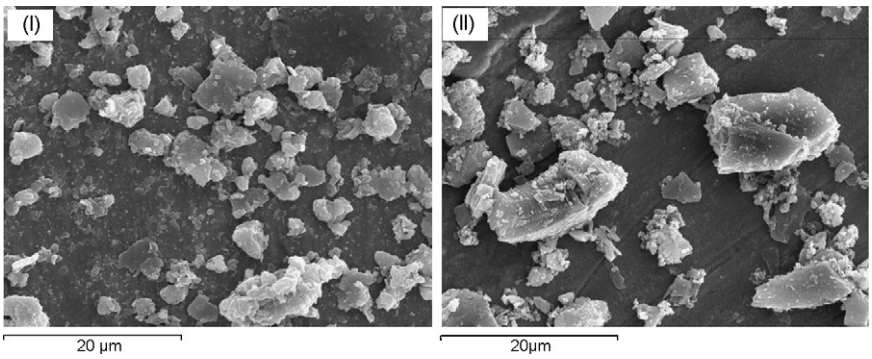

Fig. 1. SEM micrograph of (I) PPO/brushite cement (1:1, w:w) and (II) PPO/pure brushite (1:1, w:w).

involves a better substrate and product diffusion to the electrode surface, and would explain the better response obtained with the brushite cement based biosensor. According to these results, brushite cements were selected as immobilization matrix, and subsequent optimization of the biosensors was performed with this material. Hence, in the following experiments the term "brushite" was used to refer only to the cement.

3.2. Optimization of PPO/brushite based biosensor

The variables affecting the biosensor design were studied and once the optimal conditions for immobilization were adjusted, the analytical variables were optimized.

3.2.1. Biosensor composition

3.2.1.1. Enzyme/brushite ratio. According to previous work (Mousty et al., 2007) the optimal amount of PPO on the electrode surface should be around 25 μg. Higher or lower quantities cause a reduction in the biosensor performance. Thus, for the biosensor optimization we adjusted the amount of brushite, keeping the PPO weight constant. The amount of brushite crystals on the electrode surface has to be high enough to immobilize the enzyme, but without interfering with the substrate and product diffusion through the matrix.

The calibration curves were performed at 20 °C and −0.1 V vs ECS in 10 mL of a 0.1 M PBS, pH 6.0. The enzyme/brushite ratio studied was ranged between 0.25 and 1.5, keeping always constant the amount of PPO in the mixture (25 μg) and modifying the amount of brushite to reach the appropriate ratio, 50 μg of the mixture was deposited on the electrode. Maximum current increased as a function of the PPO/brushite ratio up to the optimal ratio 1/1 (w/w) (I_{max} = 145.7 μA cm^{-2} μA/cm^2), and decreased beyond this point. The lower current density (14.50 μA/cm^2) obtained at low PPO/brushite ratio (0.25) pointed to a diffusional impairment caused by the high amount of brushite deposited on the electrode surface. Moreover, the increase of the response time from 12 to 20 s when the PPO/brushite ratio was decreased from 1 to 0.25 confirms this hypothesis. On the other hand, higher PPO/brushite ratio (1.5) caused a decrease in the current density (124.9 μA cm^{-2}) as a result of an insufficient amount of brushite crystals to immobilize the entire amount of enzyme. This hypothesis was confirmed by the presence of enzyme in the buffer solution used to clean the biosensor after its preparation. In consequence, the biosensors used in the following studies were prepared with a PPO/brushite ratio 1/1.

3.2.1.2. Amount of PPO/brushite mixture deposited on the electrode. The amount of PPO/brushite(1/1) was optimized by changing the amount of mixture deposited on the electrode from 30 to 100 μg. The maximum current density was proportional to the amount of PPO/brushite up to the optimal analytical conditions obtained with the biosensor prepared with 50 μg of PPO/brushite mixture (1:1). Beyond this point the biosensor (i.e. 100 μg) maximum current density decreased (83.3 μA cm^2), indicating an overloading of the electrode which makes a diffusional barrier to the substrate and the reaction products. The optimal configuration of 50 μg of PPO/brushite mixture (1/1) was thus chosen for the further experiments. The analytical properties of this device were the following: sensitivity 42.6 A M^{-1} cm^{-2}, maximum current density 145.7 μA cm^{-2}, linear range between 3.0×10^{-9} and 3.0×10^{-6} M (R^2 = 0.9989 for N = 25), and detection limit 3 nM. Beyond this point the biosensor maximum current density decreased, indicating an overloading of the electrode which makes a diffusional barrier to the substrate and the reaction products. The optimal configuration of 50 μg of PPO/brushite mixture was thus chosen for the further experiments.

3.2.1.3. Time of cross-linking with glutaraldehyde. Adsorption is a physical immobilization method that presents as disadvantage that the adsorbed enzyme could be lost. One way of preventing PPO lost consists of carrying out a chemical cross-linking with GA (Poyard et al., 1998). The effect of GA was tested by adjusting the exposure time of the biosensor to glutaraldehyde vapor, from 0 to 25 min, when 50 μg of a mixture of PPO/brushite (1/1) were deposited on the electrode. The sensitivity reached the maximum value after 15 min exposure, and no further changes in the calibration curve were observed beyond this point. Full retention of the enzyme after 15 min of cross-linking was verified by the absence of enzyme activity in the clean buffer solution. Under these conditions, the analytical properties were: sensitivity 43.57 A M^{-1} cm^{-2}, maximum current density 149 μA cm^{-2}, linearity 3.0×10^{-9} to 3.0×10^{-6} M, regression coefficient, r = 0.9997 (23 points) and detection limit 3 nM.

3.2.2. Effect of the applied potential, pH and temperature

Bearing in mind that catechol is monitoring by measuring the electrochemical reduction of *o*-quinone produced enzymatically, the influence of the applied potential (E_{app}) on the biosensor response was investigated in the range of 0.0 to −0.2 V vs SCE, using 0.02 μM catechol solution (0.1 M phosphate buffer pH 6.0 at 20 °C). The current generated by catechol was registered until the steady state was reached. It was found the maximum response at −0.1 V vs SCE and changes in any sense of the potential resulting in a decrease of the current, so, at −0.15 V the response was the 85% while at 0 V the response was only the 35% of the maximum. For $E_{app} < -0.1$ V, the current decreases due to oxygen depletion within the biocoating, with the subsequent decrease in the enzyme reaction rate (Shan et al., 2003a). Moreover, polymerization of the *o*-quinones produced in the enzymatic reaction may damage the electrode surface contributing to the current decrease.

Table 2
Amperometric performances of different biosensor based on PPO.

Inorganic materials	Sensitivity (A M^{-1} cm^{-2})	Linear range (M)	DL (nM)	Reference
Brushite	46.6	1.0×10^{-9} to 3.0×10^{-6}	1	Present work
Layered double hydroxydes	7.8	1.7×10^{-9} to 1.3×10^{-5}	1	Shan et al. (2003b)
$CaCO_3$ nanoparticles	6.8	6.0×10^{-9} to 2.0×10^{-5}	0.4	Shan et al. (2007)
Organoclay (*Cameroonian smectite*)	3.7	2.0×10^{-8} to 1.2×10^{-5}	9	Mbouguen et al. (2007)
Nafion silicate sol–gel	2.8	1.0×10^{-6} to 1.0×10^{-4}	350	Kim and Lee (2003)
ZnO sol–gel	2.4	1.5×10^{-7} to 4.0×10^{-5}	80	Liu et al. (2005)
Gold nanoparticles	1.5	5.0×10^{-7} to 5.0×10^{-5}	150	Carralero Sanz et al. (2005)

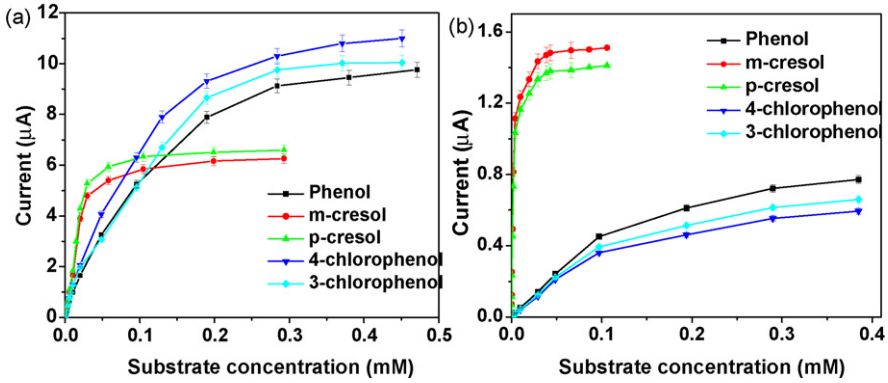

Fig. 2. Calibration curves for different monophenolic compounds in 0.1 M PBS, pH 6.0, and 20 °C: (a) $E = -0.1$ V vs SCE, (b) $E = -0.35$ V vs SCE.

The effect of pH and temperature was tested for catechol solutions (4 μM). The highest response was found at pH 6.0, which agrees with the optimum pH reported for the free enzyme (Brown et al., 1994). At pHs 5.0 and 7.0, the response was reduced to the 85%, and current decreased to the 40% at pHs 4.0 and 8.0. The biosensor retained the 85% of its maximum response between 20 and 35 °C although the enzyme denaturalization started at 25 °C. The Arrhenius plot is linear between 3 and 20 °C. The activation energy calculated from the slope was 44.2 kJ/mol, a value much higher than that of the PPO in solution (7 kJ/mol) (Shan et al., 2003a). It is worth noting that when PPO was immobilized in polyacrylamide microgels (Hervás Pérez et al., 2006) the Arrhenius plot showed a break around 10 °C (the temperature depends on the cross-linking content of the microgel) due to changes in the enzyme environment that affect the enzyme activity. This result seems to indicate that in the temperature range investigated the brushite matrix is scarcely affected by temperature. For the further experiments we chose 20 °C as working temperature to avoid the eventual inactivation of PPO.

3.3. Biosensor behavior under optimal conditions

Once the factors affecting the immobilization system and the analytical properties of the biosensor were optimized, a calibration curve at the optimal experimental conditions was obtained.

The brushite biosensor characteristics are summarized in Table 2. For comparison, Table 2 also shows the properties of biosensors based on PPO immobilized in other inorganic matrices previously reported. It must be emphasized the high sensitivity showed by the biosensor proposed in this work (around 6 times higher than any other previously reported).

3.4. Phenolic compounds detection

The behavior of the proposed biosensor was tested using different monophenols: *m*-cresol, *p*-cresol, 3-chlorophenol and 4-chlorophenol. Calibration curves of the phenolic compounds were performed at different potentials (−0.1, −0.2, −0.3 and −0.35 V).

Fig. 2 illustrates the effect of applied potentials on the shape of the calibration curve, their shape suggests that PPO has a higher affinity for the methyl derivates, being the affinity for the chloride derivates very close to that of phenol. Concerning the saturation current, as probed Fig. 2, it appears strongly dependent on potential that could be related with the reduction of the corresponding quinone. To confirm it, a series of cyclic voltamperometry were carried out on a glassy carbon electrode covered by a dialysis membrane. The dialysis membrane was used to avoid the diffusion of the enzyme to the electrode and as so, its electrochemical reaction.

Each phenolic compound was added (0.5 M) to a PBS pH 6.0 in presence of PPO 0.2 mg/mL and a cyclic scan was registered from +1.0 to −1.0 V at 50 mV s^{-1}, all cyclic voltammograms presented a well-defined reduction peaks in the direct scan, changing the peak potential from −0.1 V (3-chlorophenol and 4-chlorophenol) to −0.26 V (*p*-cresol) and −0.27 V (*m*-cresol), while phenol showed an intermediate potential, −0.16 V. These results explain as the highest response of chlorophenols appears at −0.1 V (Fig. 2a) while cresols present more current intensity at −0.35 V (Fig. 2b), phenol shows a behavior very close to chlorophenols.

Table 3
Analytical properties of the biosensor in different organic solvent-buffer solution mixtures (98.5/1.5) (v/v).

Organic solvent	Sensitivity (A M^{-1} cm^{-2})	J_{max} (μA cm^{-2})	Linear range (M)	R^2 (n)	DL (μM)
Dioxane	0.02	1.0	5.0×10^{-6} to 5.0×10^{-5}	0.9967 (6)	5
Acetonitrile	3.30	66.6	4.0×10^{-8} to 7.0×10^{-6}	0.9982 (13)	0.04
Ethanol	0.43	6.9	5.0×10^{-7} to 9.0×10^{-6}	0.9989 (8)	0.5

J_{max}: maximum density current; R: correlation coefficient of the linear range (n: number of points in the linear range); DL: detection limit.

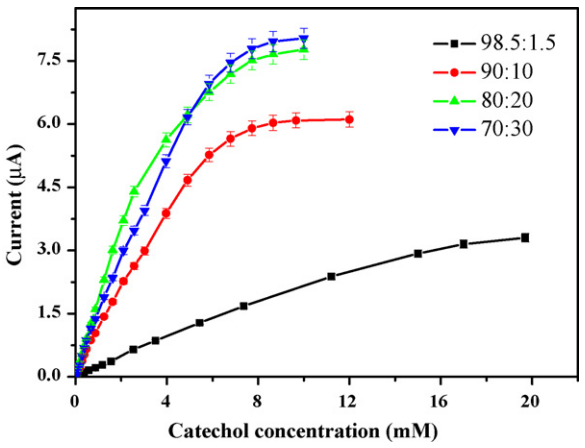

Fig. 3. Influence of the aqueous 0.1 M phosphate buffer percentage in acetonitrile:PBS mixtures (pH 6.0) on the biosensor response.

The apparent Michaelis Menten constant (K_M) of the different phenols derivatives have been estimated by Lineweaber Burk plot from the calibration curves obtained at a potential lightly more negative (~50 mV) that the cathodic peak. The apparent Michaelis Menten constant were found to be: K_M (4-chlorophenol) = 0.09 mM, K_M (3-chlorophenol) = 0.08 mM, measured at −0.1 V, K_M (phenol) = 0.05 mM, measured at −0.2 V and K_M (*p*-cresol) = 9.8×10^{-3} mM, K_M (*m*-cresol) = 6.6×10^{-3} mM, measured at −0.3 V. As expected, these result probe, that methyl substitution of the phenol increased the reaction rate when compared to phenol and substitution of halides slows down the reaction rate.

3.5. Biosensor behavior in hydrophobic media

The performance of the biosensor was tested in organic solvents such as dioxane (lg*P* = −1.1), acetonitrile (lg*P* = −0.33), and ethanol (lg*P* = −0.24) to evaluate its possible use in hydrophobic environments. Highly apolar solvents such as hexane (lg*P* = 2.00) and chloroform (lg*P* = 3.50) could not be checked because of the overpotential generated in the electrode, probably due to the interaction between the apolar solvent and the inorganic matrix. As a result of this interaction, a vacuum film may develop between the solvent and the matrix, hindering the electric contact. To guarantee the solubility of the substrate in the solvent a small amount of water was added. Thus, the behavior of the biosensor was studied in organic solvent/phosphate buffer solutions (98.5/1.5, v/v).

The results listed in Table 3 show the good performance of the biosensor in both hydrophilic and hydrophobic media. It is worth noting that the best analytical properties were found in acetonitrile. Furthermore, the sensitivity registered in this solvent was much higher than the ones obtained with other recently reported biosensors based on poly (vinylimidazole) microparticles (sensitivity 1.1 mA M^{-1} cm^{-2}) (Sanchez-Paniagua et al., in press).

Fig. 3 shows the influence of the proportion of aqueous solution added to acetonitrile (v/v) on the analytical properties of the biosensor. The sensitivity and the current density increased with the aqueous phase content up to about 20%. The increase of the buffer solution in the mixture from 1.5 to 20% produces an increase of the sensitivity around 8 times (from 3.3 to 28.6 mA M^{-1} cm^{-2}) and a descend of the detection limit around 20 times (from 40 to 2 nM). At higher proportions a response nearly constant was observed, indicating that the amount of water needed for the enzymatic activity has been reached.

3.6. Precision and stability

The precision of the optimized biosensor was evaluated in terms of repeatability by performing 10 successive measurements at different substrate solutions. The catechol concentration of the solutions covered the whole linear range (3 nM, 0.5 μM, and 2 μM). The relative standard deviation (RSD) obtained for each concentration was 8.6, 2.6 and 1.6% respectively. Our results were compared with those obtained using the Horwitz equation (Horwitz, 1982).

RSD (%) = $2^{(1-0.5\log C)}$ and in all cases the RSD was lower than the Horwitz accepted values. In addition, 20 measurements at 0.5 μM catechol (intermediate concentration) were performed in two different days. No changes were observed in the average value or in the data dispersion. When comparing the *p*-value corresponding to the averages and the variances of the measurements carried out each day, (*p*-value = 0.8390 for averages and *p*-value = 0.8839 for variances) no statistically significant differences were observed.

The brushite biosensor retained 90% of its initial current after 6 days in aqueous solution and 3 days in acetonitrile/buffer solution. The current decreases gradually down to 40% after 9 days of storage in buffer solution and after 5 days in non-aqueous medium. On the other hand, the PPO/GA biosensor experienced a more accentuated loss of activity in PBS (~20% after 4 days, and ~60% after 7 days). This indicates that the PPO stability is improved upon adsorption on brushite cement.

3.7. Determination of phenolic compounds in water samples

Real waste water samples were analyzed with the optimized PPO/brushite biosensor to evaluate its potential applicability. Samples collected from an olive oil refinement facility dedicated to the production of olive-pumice oil were used for this purpose. The concentration of phenolic compounds was unknown. Water samples were taken at two different stages of the water purification process: after a primary treatment (physico-chemical treatment) and after a secondary treatment (biological treatment). Only with water from the primary treatment a response was obtained. Five replicates of the sample were analyzed and the average content of phenolic compounds, expressed as phenol concentration, was found to be 40 ± 1 μM. To carry out the recovery study, three aliquots were taken, and 40 μM of phenol was added. The resulting samples were analyzed, and the recoveries were always found within the range 92–95%.

4. Conclusions

In this work, a biocompatible tyrosinase biosensor based on brushite cement was proposed. This sensor had a fast response detecting phenolic compounds in both aqueous and non-aqueous media, and its sensitivity out performed previously reported systems by over a factor of 6. Moreover, the biosensor is effective in the analysis of real waste water samples collected from an olive oil refinement facility, demonstrating its analytical suitability for complex samples.

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