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Biosensor based on laccase and an ionic liquid for determination of rosmarinic acid in plant extracts

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ABSTRACT

Novel biosensors based on laccase from *Aspergillus oryzae* and the ionic liquids (ILs) 1-*n*-butyl-3-methylimidazolium hexafluorophosphate ($BMIPF_6$) and 1-*n*-butyl-3-methylimidazolium tetrafluoroborate ($BMIBF_4$) were constructed for determination of rosmarinic acid by square-wave voltammetry. The laccase catalyzes the oxidation of rosmarinic acid to the corresponding *o*-quinone, which is electrochemically reduced back to rosmarinic acid at +0.2 V *vs.* Ag/AgCl. The biosensor based on $BMIPF_6$ showed a better performance than that based on $BMIBF_4$. The best performance was obtained with 50:20:15:15% (w/w/w/w) of the graphite powder:laccase:Nujol:$BMIPF_6$ composition in 0.1 mol L^{-1} acetate buffer solution (pH 5.0). The rosmarinic acid concentration was linear in the range of 9.99×10^{-7} to 6.54×10^{-5} mol L^{-1} ($r = 0.9996$) with a detection limit of 1.88×10^{-7} mol L^{-1}. The recovery study for rosmarinic acid in plant extract samples gave values from 96.1 to 105.0% and the concentrations determined were in agreement with those obtained using capillary electrophoresis at the 95% confidence level. The $BMIPF_6$-biosensor demonstrated long-term stability (300 days; 920 determinations) and reproducibility, with a relative standard deviation of 0.56%.

1. Introduction

Ionic liquids (ILs), in particular those derived from imidazolium cation posses unique association of physicochemical properties such as excellent ionic conductivity, high chemical and thermal stability, negligible vapor pressure and wide electrochemical windows [1–3]. These liquids have been widely used in biocatalysis, liquid–liquid extraction processes, materials science, catalysis and organic synthesis [1]. In particular, properties such as non-flammability, high ionic conductivity and electrochemical and thermal stability make ILs ideal electrolytes in electrochemical devices like in batteries, capacitors, fuel cells, photovoltaics, actuators and electrochemical sensors [4–15].

The first carbon paste electrode (CPE) was proposed by Adams in 1958 [16] and since then is widely used in the electroanalytical methods because it offers several important advantages, including controlled bulk modification, ease of construction, low cost, versatility, renewability and low background current [16–18]. It consists of electrically conducting graphite powder and a nonconducting organic liquid as the binder. Nujol and paraffin are the most used binder components of pastes [16–18]. Recently, the replacement of traditionally used binders by ILs has been shown to be an attractive and efficient alternative due to the high ionic conductivity and sensitivity of the resulting electrodes [4–15].

One of the most important enzymes in terms of applicability and versatility in industry is laccase. It is a multicopper oxidase, one of the extracellular glycoprotein enzymes expressed by white-rot fungi and other organisms that play a crucial role in the terrestrial carbon cycle by aiding the degradation of lignocellulosic materials such as wood. Laccase acts on phenolic substrates by catalyzing the oxidation of their phenolic hydroxyl groups to phenoxy radicals while molecular oxygen is reduced to water [19–21]. The general application of this enzyme has been reported [19–26]. However, biosensors based on the laccase and ionic liquids for the determination of phenolic compounds have not been described in the literature. There is only one report on the use of biosensor containing laccase from *Trametes vericolor* and ionic liquid for the inhibition of laccase activity by halide ions [27] and there are two other reports on the use of glucose oxidase and laccase from *Coriolos versicolor* immobilized at multiwalled carbon nanotubes-ionic liquid gel modified electrodes used as catalysts of anode and cathode of biofuel cells [28,29].

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenylactic acid. It has a number of interesting biological activities

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such as inhibiting the HIV-1 virus and blood clots, it is also antihepatitis (protecting the liver), antibacterial, antiviral, and anti-inflammatory and it can act as an astringent. It is one of the most efficient natural antioxidants, hence its application in the food industry [30,31]. High performance liquid chromatography is a method commonly used for determination of this substance [32]. However, this generally necessitates lengthy analysis, expensive materials and preliminary steps to prepare the sample before injection. Capillary electrophoresis has been used since it offers considerable reductions in terms of sample preparation, analysis time and reagent consumption [33–36]. Recently, Santhiago et al. [37] have developed a biomimetic sensor based on a dinuclear Fe(III)Zn(II) mimetic complex for the active site of purple acid phosphatase for determination of rosmarinic acid. The rosmarinic acid concentration was linear in the range of 2.98×10^{-5} to 3.83×10^{-4} mol L^{-1} with a detection limit of 2.30×10^{-6} mol L^{-1}. The coupling of electroanalytical methods with the selectivity and sensitivity of enzymatic reactions is one the most attractive, elegant and promising techniques for the construction of biosensors. Rosmarinic acid is found in many plants and in leaf material of lemon balm (*Melissa officinalis*) it is the majority component. It is added as antioxidant in the cosmetic formulations; however, there are no standard methods for its quantification. So, it is important to develop methods with this main goal.

In this study, biosensors based on laccase from *Aspergillus oryzae* and the ionic liquids 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIPF_6$) and 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIBF_4$) were constructed and evaluated. The influence of different experimental parameters including the Nujol:$BMIPF_6$ ratio, enzyme percentage, and solution pH, along with the frequency, pulse amplitude and increment for the square-wave voltammetry, were investigated in order to optimize the performance of the biosensors employed in the determination of rosmarinic acid. The results obtained in the determination of this substance in plant extract samples using the selected biosensor compared favorably with those obtained using the capillary electrophoresis method.

2. Experimental

2.1. Reagents and solutions

Laccase from *A. oryzae* was purchased from Novozymes (Denilite® II BASE) in the form of microspheres. The ionic liquids 1-butyl-3-methylimidazolium hexafluorophosphate and 1-butyl-3-methylimidazolium tetrafluoroborate were synthesized as previously described in the literature [38,39]. Graphite powder (grade #38) and Nujol were supplied by Fisher Scientific and Aldrich, respectively. Adrenaline, caffeic acid, catechin, chlorogenic acid, ferulic acid, gallic acid, hesperetin, hesperidin, luteolin, *m*-coumaric acid, *p*-coumaric acid, rosmarinic acid, sinapic acid, syringic acid, tannic acid, rutin and vanillic acid were purchased from Sigma. Eriodictyol-7-O-glucoside was purchased from Extrasynthese. Acetate buffer (0.1 mol L^{-1}, pH 5.0) solution was used as the supporting electrolyte. For the capillary electrophoresis analysis, chlorogenic acid (internal standard) was prepared in methanol at 800 mg L^{-1}. Lemon balm (*Melissa officinalis L.*) plant extracts (A, A′: Melissa HG FS 157660705 and B, B′: Melissa 18745—100%), used for the manufacture of cosmetic products, were obtained from Farma Service Bioextract (São Paulo, SP, Brazil). All reagents were of analytical grade and used without further purification. Ultrapure water (18.2 MΩ) was obtained from a Milli-Q purification system and used throughout the experiments.

2.2. Instrumentation

Voltammetry measurements were carried out using an Autolab PGSTAT12 potentiostat/galvanostat (Eco Chemie, The Netherlands) connected to data processing software (GPES, software version 4.9.006, Eco Chemie). The three-electrode system was composed of a biosensor (geometric area 1.0 mm^2) based on laccase and ionic liquids ($BMIPF_6$ and $BMIBF_4$) as the working electrode, a platinum wire as the auxiliary electrode and an Ag/AgCl (3.0 mol L^{-1} KCl) as the reference electrode. Electropherograms were obtained with an Agilent Technologies automated $HP^{3D}CE$ capillary electrophoresis apparatus (Palo Alto, CA, USA), equipped with a diode array detector. The measurements were performed at 25 °C on an uncoated fused-silica capillary (48.5 cm × 50 μm I.D. × 375 μm O.D, 8.5 cm of effective length) obtained from Microtube (Araraquara, SP, Brazil). The acquisition software for data treatment was HP Chemstation®.

2.3. Construction of biosensors

The biosensors were prepared by hand-mixing laccase (40.0 mg; 20%, w/w) and graphite powder (100.0 mg; 50%, w/w) in a mortar for 20 min to ensure uniform dispersion. Nujol (30.0 mg; 15%, w/w) and $BMIPF_6$ or $BMIBF_4$ (30.0 mg; 15%, w/w) were added to this mixture which was then mixed for at least 20 min to produce the final paste. A portion of the resulting paste was packed firmly into the cavity of a syringe and the electrical contact was established via a copper wire. The biosensors were stored at room temperature when not in use. The carbon paste electrode without laccase was prepared following the same steps.

2.4. Electrochemical and capillary electrophoresis measurements

Square-wave voltammetry and cyclic measurements were carried out in an unstirred, non-de-aerated acetate buffer solution (pH 5.0) at 25.0 ± 0.5 °C and all potentials were measured and reported *vs.* Ag/AgCl (3.0 mol L^{-1} KCl). In a typical run, 10 mL of the supporting electrolyte was transferred to a clean, dry cell and the required volume of the rosmarinic acid or sample of the plant extract was added by micropipette. After a stirring period of 60 s to homogenize the solution, a square wave or cyclic voltammograms were recorded.

Electropherograms were obtained using the injection pressure of 50 mbar (5.25 Pa) for 5 s and UV detection at 340 nm. The applied separation voltage was 30 kV, negative polarity. The background electrolyte (BGE) consisted of 10.0×10^{-3} mol L^{-1} (pH 9.3) of sodium tetraborate. At the start of each new working session, the capillary was conditioned at 25 °C and flushed with 1.0 mol L^{-1} sodium hydroxide for 10 min, followed by deionized water for 5 min and finally with the BGE for 10 min. Between runs with the same buffer, the capillary was rinsed for 0.5 min with BGE. At the end of the analysis the capillary was rinsed for 5 min with 1.0 mol L^{-1} sodium hydroxide and for 10 min with deionized water.

3. Results and discussion

3.1. Enzymatic reaction and voltammetric study of the biosensors

Reactions catalyzed by enzymes have long been used for the determination of different analytes. Numerous biosensors based on laccase have been described in the literature [24–27]. This is one of the blue copper oxidases which catalyze the four-electron reduction of molecular oxygen to water and the oxidation of a variety of aromatic compounds. In this study, laccase immobilized on the biosensor catalyzes the oxidation of rosmarinic acid present in

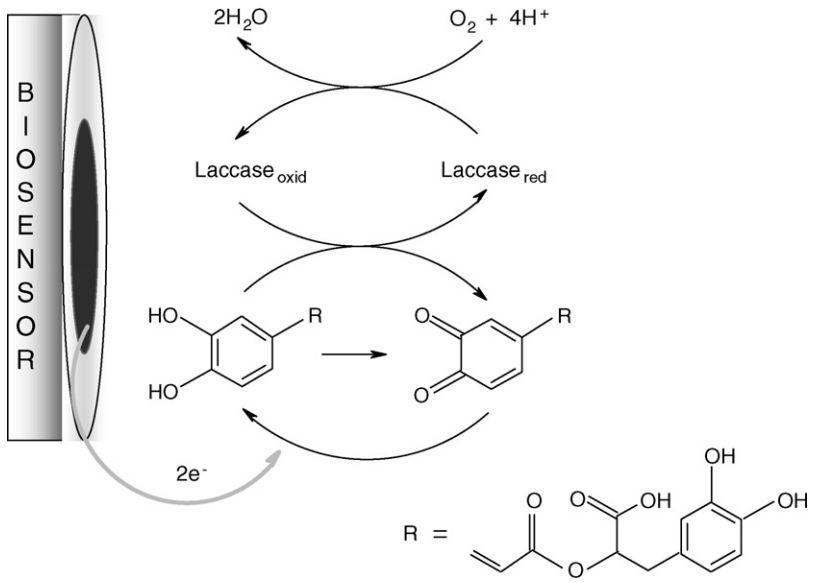

Fig. 1. Schematic representation of the reaction involving rosmarinic acid and laccase on the $BMIPF_6$-biosensor surface.

the solution to the corresponding *o*-quinone. This product is electrochemically reduced back to rosmarinic acid on the surface of the biosensor as shown in Fig. 1. The resulting reduction current is used as the analytical response for rosmarinic acid determination.

The electrochemical behavior of rosmarinic acid using the bare CPEs and biosensors based on laccase and $BMIPF_6$ or $BMIBF_4$ as binders was investigated by square-wave voltammetry in the potential range of +0.5 to −0.5 V *vs.* Ag/AgCl. Fig. 2 shows the voltammograms obtained using (a′) $BMIBF_4$-CPE, (a) $BMIBF_4$-biosensor, (b′) $BMIPF_6$-CPE and (b) $BMIPF_6$-biosensor in a 6.10×10^{-5} mol L^{-1} rosmarinic acid solution in acetate buffer (0.1 mol L^{-1}, pH 5.0). As can be seen, for the $BMIPF_6$-biosensor, a higher response for the reduction wave of *o*-quinone to rosmarinic acid at a potential of +0.2 V was observed when compared with the $BMIBF_4$-biosensor at a potential of +0.1 V and CPEs containing ILs. A plausible explanation for the better performance of the $BMIPF_6$-biosensor compared to the $BMIBF_4$-biosensor is that the use of PF_6^- as the counter ion for the *N*,*N*-dialkyl substituted imidazolium cation causes the resulting structure to be hydrophobic. As a consequence, it has a greater stability, good electrochemical conductivity and a wide electrode potential window in aqueous solutions [8]. On the other hand, the BF_4^- forms a hydrophilic structure with the same cation and thus, for the construction of biosensors, it is unstable in aqueous solutions [1]. The miscibility in water of ILs based on anions such as BF_4^- is dependent on the structure of the cations. The miscibility decreases with an increase in the cation chain length [1–3]. Therefore, the $BMIPF_6$-biosensor was chosen for the development of this study.

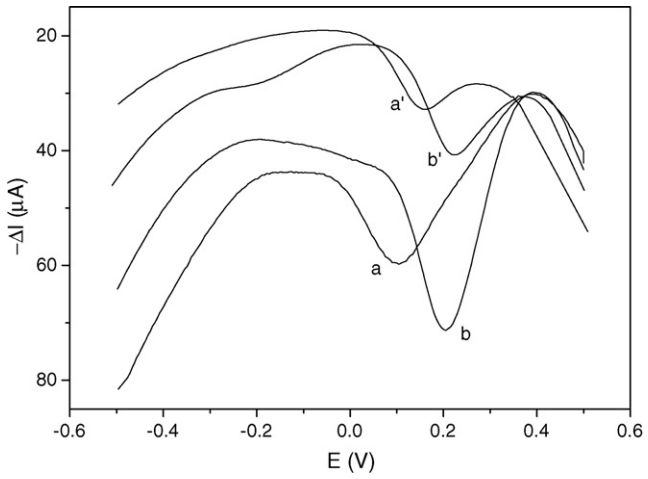

Fig. 2. Square-wave voltammograms obtained using (a′) $BMIBF_4$-CPE, (a) $BMIBF_4$-biosensor, (b′) $BMIPF_6$-CPE and (b) $BMIPF_6$-biosensor with 6.10×10^{-5} mol L^{-1} rosmarinic acid in 0.1 mol L^{-1} acetate buffer solution (pH 5.0); pulse amplitude 100 mV, frequency 30 Hz and scan increment 5.0 mV.

3.2. Optimization of the biosensor construction and experimental parameters for square-wave voltammetry

Parameters regarding the biosensor composition were investigated in order to build a biosensor with adequate performance. The Nujol:$BMIPF_6$ ratios (a) 100:0; (b) 75:25; (c) 50:50; (d) 25:75 and (e) 0:100% (w/w) were studied and the biosensor responses in the rosmarinic acid determination by (A) cyclic voltammetry and (B) square-wave voltammetry are compared in Fig. 3. All the biosensors had the same graphite powder:laccase ratio. As can been observed in the cyclic voltammograms (Fig. 3A), the background current increased with an increase in the $BMIPF_6$ content. It has been reported in the literature [9,13,15] that the addition of ILs to CPEs modifies the microstructure of the paste and that the charge transfer resistance decreases and charge transfer rate increases, due the higher conductivity of the ILs. The response of the proposed biosensor did not follow this behavior. It can be clearly observed that the background current (due principally to the capacitive current) increased considerably when $BMIPF_6$ represents over 50%. The same behavior was reported [8] for the oxidation of acetaminophen on carbon paste electrodes modified with different ILs. When the contribution of the capacitive current was suppressed (square-wave voltammograms showed in Fig. 3B; inset shows the current values for each biosensor composition), an increase in the current reduction peak was observed up to a Nujol:$BMIPF_6$ ratio of 50%. After this, the current decreased significantly. These results are in accordance with those obtained by cyclic voltammetry and confirm the efficiency of the $BMIPF_6$ as a binder when used in a suitable amount. Additionally, it is clearly observed that the sensitivity of

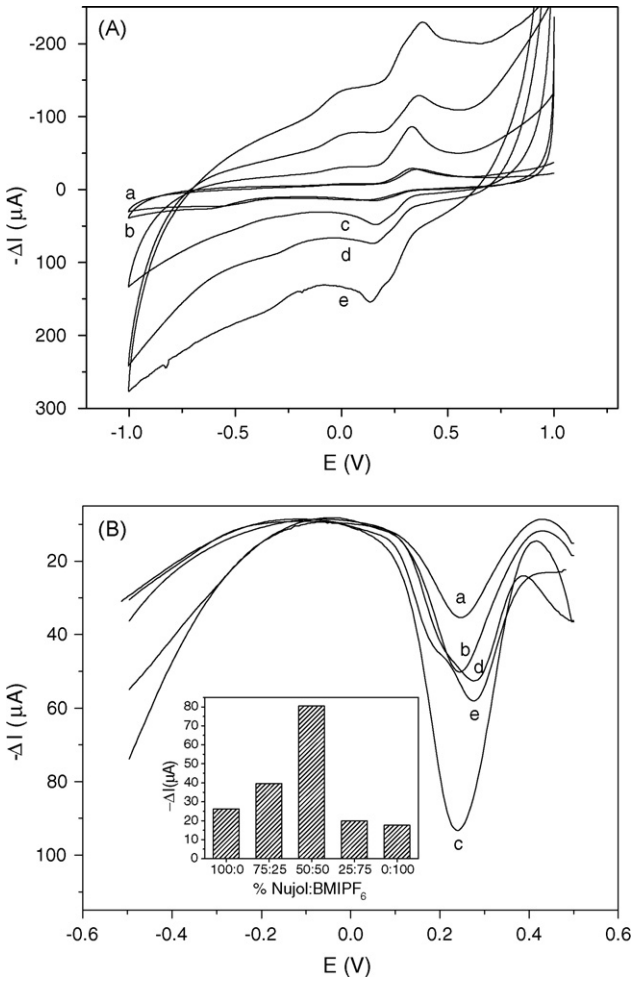

Fig. 3. (A) Cyclic and (B) square-wave voltammograms obtained using different Nujol:$BMIPF_6$ ratios in the carbon paste modified with laccase immersed in 6.10×10^{-5} rosmarinic acid solution and 0.1 mol L^{-1} acetate buffer solution (pH 5.0) at (A) 100 mV s^{-1} and (B) same conditions as in Fig. 2. Inset: current values for each biosensor composition.

the biosensor was greatly enhanced when the Nujol:$BMIPF_6$ ratio used was 50:50% (w/w) together with square-wave voltammetry. Therefore, this proportion was used for subsequent biosensor construction and square-wave voltammetry analysis for the determination of rosmarinic acid.

The effect of laccase in percentages of 10–30% (w/w) on the modified carbon paste electrode was investigated. The analytical signals (cathodic peak currents) increased with increases in the enzyme composition up to 20.0% (w/w). Consequently, this enzyme composition was selected for further studies and a composition of 50:20:15:15% (w/w/w/w) graphite powder:laccase:Nujol:$BMIPF_6$, respectively, was used in the construction of the $BMIPF_6$-biosensor.

After the optimization of the biosensor composition, other important experimental parameters were investigated, including solution pH (4.0–8.0) and square-wave voltammetry parameters (frequency of 10–100 Hz, pulse amplitude of 10–100 mV and scan increment of 1.0–5.0 mV) in order to obtain the best response for the proposed $BMIPF_6$-based biosensor. The response was analyzed on the cathodic peak current for a 6.10×10^{-5} mol L^{-1} rosmarinic acid solution. Table 1 summarizes the range over which each variable was investigated and the optimal value found. The best experimental conditions were selected for subsequent experiments.

Table 1
Optimization of the experimental conditions

Parameter investigated	Range studied	Optimal value
Nujol:$BMIPF_6$ (% w/w)	100:0–0:100	50:50
Laccase (% w/w)	10–30	20
pH	4.0–8.0	5.0
Frequency (Hz)	10–100	30.0
Pulse amplitude (mV)	10–100	100
Scan increment (mV)	0.5–5.0	5.0

3.3. Reproducibility and stability of the biosensor

To investigate the precision of the determination, the same biosensor was used for six parallel determinations of 6.10×10^{-5} mol L^{-1} rosmarinic acid solution in acetate buffer solution (0.1 mol L^{-1}; pH 5.0). The relative standard deviation was calculated as 0.94%. Four biosensors were constructed using the same procedure and were independently used for the determination of rosmarinic acid under the conditions described above. All the biosensors showed an acceptable reproducibility with a relative standard deviation of 0.56%, indicating that the results obtained with the proposed biosensor have a good reproducibility.

The lifetime and stability of the biosensor are very important features, and thus these parameters were also investigated. The biosensor was used for consecutive measurements without surface renewal over a 300-day period (over 920 samples were determined for each quantity of carbon paste used in the syringe). The response was recorded (cathodic peak currents) in a 6.10×10^{-5} mol L^{-1} rosmarinic acid (pH 5.0) solution. When the biosensor was stored at room temperature and measurements taken every 1–2 days in solutions of the same composition, no notable changes were observed in its response, indicating good stability and long lifetime.

3.4. Square-wave voltammograms and analytical curve

Under the selected conditions mentioned above, the analytical curve obtained was linear from 9.99×10^{-7} to 6.54×10^{-5} mol L^{-1} of rosmarinic acid and the regression equation found was $Ipc = -1.1210 + 0.5630 \times 10^6$ [rosmarinic acid] (Ipc: μA; [rosmarinic acid]: mol L^{-1}; $r = 0.9996$). The detection limit (three times the blank signal/slope) was calculated using this calibration curve and was found to be 1.88×10^{-7} mol L^{-1}. Fig. 4 shows these voltammograms and the corresponding analytical curve can be seen in the inset. The experiments were carried out in triplicate and the results shown in the inset correspond to the mean of the measurements.

3.5. Selectivity and interference study

Phenolic compounds, such as adrenaline, caffeic acid, catechin, chlorogenic acid, ferulic acid, gallic acid, luteolin, *p*-coumaric acid, rosmarinic acid, syringic acid, sinapic acid, tannic acid, rutin and vanillic acid were investigated, in order to determine the selectivity of the proposed biosensor. The sensor was more sensitive to rosmarinic acid (100%), adrenaline (63.0%), caffeic acid (25.0%), chlorogenic acid (22.0%), catechin (10.0%) and rutin (9.0%). No response was observed for the other phenolic compounds studied.

The effects of other substances on the determination of rosmarinic acid in lemon balm plant extracts, such as caffeic acid, eriodictyol-7-O-glucoside, hesperetin, hesperidin, *m*-coumaric acid, naringenin and naringin, were investigated using square-wave voltammetry. The ratios of the concentration of rosmarinic acid to those of the possible interferents were fixed at 1.0 and 5.0. Of these substances, only the caffeic acid and eriodictyol-

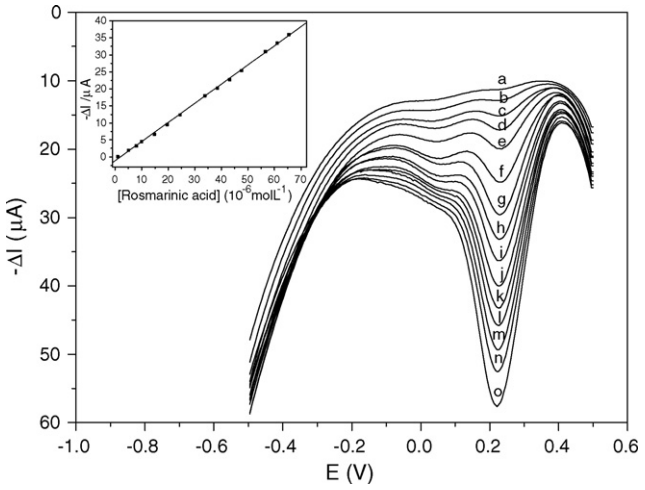

Fig. 4. Square-wave voltammograms obtained using the proposed biosensor for (a) blank in acetate buffer solution (0.1 mol L^{-1}; pH 5.0) and rosmarinic acid solutions at the following concentrations: (b) 9.99×10^{-7} mol L^{-1}; (c) 4.98×10^{-6} mol L^{-1}; (d) 7.94×10^{-6} mol L^{-1}; (e) 9.90×10^{-6} mol L^{-1}; (f) 1.48×10^{-5} mol L^{-1}; (g) 1.96×10^{-5} mol L^{-1}; (h) 2.44×10^{-5} mol L^{-1}; (i) 3.38×10^{-5} mol L^{-1}; (j) 3.85×10^{-5} mol L^{-1}; (k) 4.31×10^{-5} mol L^{-1}; (l) 4.76×10^{-5} mol L^{-1}; (m) 5.66×10^{-5} mol L^{-1}; (n) 6.10×10^{-5} mol L^{-1}; (o) 6.54×10^{-5} mol L^{-1}; other conditions are the same as in Fig. 2. Inset: the analytical curve of rosmarinic acid.

7-O-glucoside showed electrochemical activity on the proposed biosensor. Nevertheless, the concentrations of these compounds in the plant extracts were very low and did not interfere in the determination of rosmarinic acid. These data were agreement with those reported recently by Dastmalchi et al. [40] using high performance liquid chromatography and a biomimetic sensor [37].

3.6. Recovery study and analytical application

Recoveries of 96.1–105.0% for rosmarinic acid present in the plant extract samples ($n = 3$) were obtained using the biosensor. In this study 3.57, 10.48, 17.15 and 23.56 mg L^{-1} of rosmarinic acid solutions were successively added to each sample and the cathodic current peak analyzed. The recovery results obtained were satisfactory, as shown in Table 2, indicating an absence of matrix effects in the rosmarinic acid determinations using the proposed biosensor. Also, the results obtained with the $BMIPF_6$-based biosensor were compared with those obtained using the capillary electrophoresis method. The results for the two determination methods are shown in Table 3. A good agreement was obtained at the 95% confidence level, within an acceptable range of error, indicating that the biosensor is suitable for the determination of the antioxidant rosmarinic acid in plant extracts.

Table 2
Recoveries of rosmarinic acid solution in plant extracts using the biosensor proposed

Rosmarinic acid (mg L^{-1})			Recovery (%)
Sample	Added[a]	Found	
A	3.57	3.66 ± 0.02	102.5
	10.48	10.78 ± 0.01	102.9
	17.15	17.31 ± 0.02	100.9
	23.56	22.63 ± 0.03	96.1
B	3.57	3.61 ± 0.02	101.1
	10.48	11.01 ± 0.02	105.0
	17.15	17.68 ± 0.03	103.0
	23.56	22.97 ± 0.01	97.5

[a] $n = 3$.

Table 3
Determination of rosmarinic acid in plant extracts using the $BMIPF_6$-biosensor and capillary electrophoresis

Sample	Rosmarinic acid (mg L^{-1})		Relative error (%)[a]
	Capillary electrophoresis[b]	$BMIPF_6$-biosensor[b]	
A	229.5 ± 0.1	230.7 ± 0.2	+0.5
A′	229.5 ± 0.1	229.9 ± 0.1	+0.2
B	230.2 ± 0.1	215.9 ± 0.2	−6.2
B′	230.2 ± 0.1	212.2 ± 0.1	−7.8

[a] $BMIPF_6$-biosensor *vs.* capillary electrophoresis.
[b] $n = 3$; confidence level of 95%.

4. Conclusions

In this study, we present novel biosensors based on laccase from *A. oryzae* and the ionic liquids 1-butyl-3-methylimidazolium hexafluorophosphate or 1-butyl-3-methylimidazolium hexafluoroborate used as the binder. The IL-based biosensors were used for the determination of rosmarinic acid in plant extracts. The $BMIPF_6$-based biosensor showed more favorable analytical features such as high sensitivity, linear calibration range, low detection limit, rapid response, good reproducibility and long-term stability. In addition, the low cost, renewability, simplicity and fast construction of the biosensor, using square-wave voltammetry as the analysis method, make it superior to other techniques for rosmarinic acid determination. The results indicated that the antioxidant was successfully determined and the results obtained were satisfactory when compared with the capillary electrophoresis method.

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