Talanta 79 (2009) 222–228

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Biosensor for chlorogenic acid based on an ionic liquid containing iridium nanoparticles and polyphenol oxidase

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ARTICLE INFO

Article history:
Received 18 December 2008
Received in revised form 12 March 2009
Accepted 13 March 2009
Available online 26 March 2009

Keywords:
Biosensor
Ionic liquid
Iridium nanoparticle
Chlorogenic acid
Coffee

ABSTRACT

A biosensor based on the ionic liquid, 1-*n*-butyl-3-methylimidazolium hexafluorophosphate containing dispersed iridium nanoparticles (Ir-BMI.PF_6) and polyphenol oxidase was constructed. This enzyme was obtained from the sugar apple (*Annona squamosa*), immobilized in chitosan ionically crosslinked with oxalate. The biosensor was used for determination of chlorogenic acid by square wave voltammetry. The polyphenol oxidase catalyzes the oxidation of chlorogenic acid to the corresponding *o*-quinone, which is electrochemically reduced back to this substance at +0.25 V *vs.* Ag/AgCl. Under optimized operational conditions the chlorogenic acid concentration was linear in the range of 3.48×10^{-6} to 4.95×10^{-5} mol L^{-1} with a detection limit of 9.15×10^{-7} mol L^{-1}. The biosensor was applied in the determination of chlorogenic acid in organic and decaffeinated coffee and the results compared with those obtained using the capillary electrophoresis method. The recovery study for chlorogenic acid in these samples gave values of 93.2–105.7%.

1. Introduction

Ionic liquids (ILs) derived mainly from the association of the 1,3-dialkylimdiazolium cation with various non-coordinating anions are compounds with various interesting physico-chemical properties. Most of these fluids are non-volatile, do not have measurable vapor pressure, possess good electrochemical and thermal stability, high density, excellent ionic conductivity, and good solubility of inorganic and organic substances, and they are non-flammable [1–3]. In particular the hydrophobic 1-*n*-butyl-3-methylimidazolium hexafluorophosphate (BMI.PF_6) is one of the most popular and most used ILs [4]. The imidazolium-based ILs are used in several areas, such as electrolytes for battery, solvents for organic synthesis and catalysis, electrolytes for conducting polymer actuator systems and supports for the immobilization of enzymes [1–5].

Catalysis can be considered as one of the most popular applications of transition metals, especially noble metals, because of their high catalytic activity in many chemical reactions. The incorporation of metal (e.g., gold, iridium, platinum, palladium and silver) nanoparticles into carbon, and even in metal electrodes, has received considerable interest. The catalytic effect of these nanoparticles has been shown to increase the sensitivity of the electrode because electron transfer is better promoted [6–17]. The relation between transition metal nanoparticles and ILs offers considerable potential and has been used in the construction of sensors [8–13]. The high performance of biosensors based on metal nanoparticles has been reported in the literature [8–17], verifying the advantages of effective mass transport, stability, high effective surface area and control over the electrode microenviroment.

Enzymes are generally active in ILs [2]. The existence of hydrogen-bonded nanostructures with polar and non-polar regions in ILs may be responsible for the stabilization of the supported enzyme, which can sustain their functionality under strongly denaturing conditions. The application of ILs in the determination of the direct electrochemistry of enzymes has been investigated since they are considered appropriate media for supporting biocatalytic processes with high polarity, selectivity, fast rate and enzyme stability [1–3].

Immobilized enzymes are used mainly in biosensors for analytical applications because, compared with free enzymes, they generally have lower activity and a better Michaelis constant [18]. Immobilization of an enzyme under conditions close to those of its natural environment usually results in a more stable and efficient enzyme, due to interactions with the support. Chitosan has been widely used for the immobilization of enzymes and other biomolecules owing to its biocompatibility with this biomaterial [18,19]. As with other polymer gels, such as gelatin, xanthan and agarose, it is used as a matrix to immobilize enzymes for its application in biosensor development [19]. Chitosan is a polysaccharide

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doi:10.1016/j.talanta.2009.03.039

composed mainly of 1,4-linked 2-amino-2-deoxy-b-D-glucan and it has been widely crosslinked with many covalent or ionic crosslinking agents and modified physically to change its properties [18–22]. It is a polycationic polymer and, consequently, reactions with negatively charged components, either molecules or ions, can lead to the formation of a network through ionic bridges between the polymeric chains. Ionic interactions between the negative charges of the crosslinker and positively charged groups of chitosan are the main interactions inside the network [19,23].

Polyphenol oxidase is a copper-containing enzyme, extensively distributed in nature. This enzyme is responsible for the catalytic oxidation of *o*-diphenols to their corresponding *o*-quinones. These *o*-quinones take part in a series of chemical reactions and react non-enzymatically with a diversity of substances in the presence of oxygen to form pigments. Therefore, they are responsible for the quality deterioration of many fruits and vegetables during post-harvest processing. Moderately unstable, this enzyme is susceptible to inactivation at elevated temperatures and under extreme pH conditions [24,25].

Phenolic compounds are extensive secondary plant metabolites, derived from benzoic and cinnamic acids and they are known to play an important role in plant resistance and growth regulation. Moreover, they have been shown to have potential beneficial effects on human health and are considered to be an important contributor to diverse biological activities acting as anti-oxidant, anti-carcinogenic, and anti-inflammatory agents. Chlorogenic acid is a phenolic compound present in plants such as artichoke leaves, burdock root, dandelion root, echinacea root and in the popular coffee bean, the major source of this acid in the human diet [26–28].

Studies have revealed the particular effects of habitual coffee drinking on various aspects of health such as neurological conditions, metabolic disorders, psychoactive responses and liver function. Roasting degrades approximately 32–52% of the chlorogenic acid in coffee beans and it is easily hydrolyzed into caffeic acid and quinic acid, compounds responsible for the astringent and bitter flavors of the coffee aroma. Therefore, the determination of chlorogenic acid in coffee is an important aspect of the quality control of the final product, since its aroma characteristics determine the commercial value [27,28]. In view of this, it is very important to develop analytical methods of reduced cost but with good sensitivity and selectivity, as well as short response times. Several analytical methods have been used to determine chlorogenic acid (Table 1) including chemiluminescence, high performance liquid chromatography, capillary electrophoresis and electroanalytical methods [29–37].

In this study, we propose a novel biosensor for determination of chlorogenic acid in organic and decaffeinated coffee. Polyphenol oxidase from the sugar (or custard) apple (*Annona squamosa*) was immobilized in chitosan ionically crosslinked with oxalate. These biomaterials, along with the IL 1-*n*-butyl-3-methylimidazolium hexafluorophosphate containing dispersed iridium nanoparticles (Ir-BMI.PF_6), were used to build the biosensor. Furthermore, a novel biosensor for the determination of chlorogenic acid with simplicity, high sensitivity, fast response and good stability characteristics was developed.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and all solutions were prepared with water from a Millipore (Bedford, MA, USA) Milli-Q system (Model UV Plus Ultra-Low Organic Water). Chlorogenic acid, catechol, caffeine, caffeic acid, citric acid, fructose, glucose, glutamic acid, sucrose and tartaric acid were purchased from Aldrich. Chitosan (deacetylation degree of 85%), sodium oxalate and Tween 80 were obtained from Sigma. Graphite powder (Acherson #38) and Nujol were obtained from Fisher Scientific and Aldrich, respectively. The sugar (or custard) apples (*A. squamosa*) were purchased from a local market in Florianópolis (Santa Catarina, Brazil).

2.2. Synthesis of ionic liquids BMI.PF_6 and Ir-BMI.PF_6

The ionic liquid 1-*n*-butyl-3-methylimidazolium hexafluorophosphate (BMI.PF_6) was prepared according to a known procedure and dried under reduced pressure (1.0×10^{-3} bar) at 65 °C for 24 h. The purity (>99.4%) of this ionic liquid can be determined through its ^{1}H NMR spectrum using the intensity of the ^{13}C satellites of the imidazolium M-methyl group as an internal standard. NMR analysis was performed on a Varian Inova 300 spectrometer. Infrared spectra were obtained using a Bomem B-102 spectrometer [38].

The ionic liquid BMI.PF_6 containing dispersed iridium nanoparticles (Ir-BMI.PF_6) was prepared in a Fischer–Porter bottle containing an orange solution of the organometallic precursor iridium. Bis(1,5-cyclooctadiene) tetrafluoroborate {[Ir(cod)$_2$]BF_4} (5 mg, 0.01 mmol) was added to 1 mL of the BMI.PF_6 ionic liquid and the mixture was stirred at room temperature for 15 min. The system was then maintained for 2 h at 75 °C and 4 bar of H_2 pressure. After stirring for 2 h a black 'solution' was obtained containing the Ir-BMI.PF_6 (0.36% of Ir) [39] and a sample was analyzed by transmission electron microscopy (TEM).

2.3. Ionic liquid Ir-BMI.PF_6 characterization instrumentation

The morphology and the size distribution of the Ir-BMI.PF_6 were obtained through TEM analysis. The morphology and the electron diffraction (ED) of the obtained particles were investigated using a JEOL JEM – 2010 equipped with an energy dispersive X-ray spectroscopy (EDS) system and JEOL JEM – 1200 EXII electron microscope operating at accelerating voltages of 200 and 120 kV, respectively.

The sample for TEM analysis was prepared by dispersion of the iridium nanoparticles in the ionic liquid (Ir-BMI.PF_6) in acetone at room temperature. The suspension was collected on a carbon-coated copper grid. The histogram of the nanoparticle size

Table 1
Analytical methods for determination of chlorogenic acid.

Methods	Accuracy	Linear range	Detection limit	Reference
HPLC	90%	0.8–3 mg L^{-1}	0.2 mg L^{-1}	[31]
HPLC	85.56–94.92%	0.16–2.5 μg mL^{-1} and 2.5–40.0 μg mL^{-1}	0.04 μg mL^{-1}	[32]
CZE	95%	1–20 μg mL^{-1}	0.25 mg L^{-1}	[33]
HPLC	96%	0.2–4 μg mL^{-1}	0.02 mg L^{-1}	
Chemiluminescence	94.0–105.2%	5.0×10^{-8} to 5.0×10^{-5} g mL^{-1}	5.7×10^{-9} g mL^{-1}	[34]
Biosensor	93.2–106.1%	5.0×10^{-6} to 1.45×10^{-4} mol L^{-1}	8.0×10^{-7} mol L^{-1}	[35]
Biosensor	96.5–102.6% and 91.3–115.5%	4.89×10^{-6} to 3.20×10^{-4} mol L^{-1} and 4.89×10^{-6} to 4.85×10^{-5} mol L^{-1}	8.02×10^{-7} and 8.52×10^{-7} mol L^{-1}	[36]
Biosensor	92–119%	1.0×10^{-6} to 5.0×10^{-5} mol L^{-1}	7.0×10^{-7} mol L^{-1}	[37]
Biosensor	93.2–105.7%	3.48×10^{-6} to 4.95×10^{-5} mol L^{-1}	9.15×10^{-7} mol L^{-1}	This work

distribution was obtained from measurement of around 300 particles, and was reproduced in different regions of the Cu grid, assuming spherical shape, found in an arbitrary chosen area of enlarged micrographs.

2.4. Instrumentation and electrodes

Electrochemical measurements were performed at room temperature using an Autolab PGSTAT12 potentiostat/galvanostat (Eco Chemie, The Netherlands) operating with data processing software (GPES, software version 4.9.006, Eco Chemie). The three-electrode system was composed of the biosensor as the working electrode, an Ag/AgCl electrode (3.0 mol L^{-1} KCl) as the reference and a platinum wire as the auxiliary electrode. A Hitachi centrifuge, model Himac CR 20B2, was used in the preparation of the sugar apple homogenate. A B572 (Micronal) spectrophotometer was used with an optical path of 1.0 cm (quartz cell) for the determination of polyphenol oxidase activity. A scanning electron microscope (SEM - Philips, model XL30 microscope) at an accelerating voltage of 15 kV was used in the analyzed of the carbon pastes modified with the Ir-BMI.PF_6 and PPO used for the construction of the biosensor. Electropherograms were obtained with an Agilent Technologies automated $HP^{3D}CE$ capillary electrophoresis apparatus (Palo Alto, CA, USA), equipped with a diode array detector.

2.5. Obtainment of the polyphenol oxidase and determination of the activity

The polyphenol oxidase (PPO) enzyme was obtained from the sugar apple (*A. squamosa*) tissue. Twenty-five grams of this plant material were homogenized in a mixer with 100 mL of phosphate buffer solution (0.1 mol L^{-1}; pH 7.0) for 1 min. The homogenate was rapidly filtered and centrifuged at 18,000 rpm for 2 min at 4 °C. The resulting supernatant was stored at 4 °C in a refrigerator and utilized as a source of polyphenol oxidase after determination of the activity. One unit of activity was defined as an increase in the amount of enzyme that causes a change in absorbance of 0.001 min^{-1} at 420 nm for *o*-quinone produced by the reaction between 2.8 mL of catechol solution (5.0×10^{-2} mol L^{-1}) in 0.1 mol L^{-1} phosphate buffer (pH 7.0) and 0.2 mL of the enzymatic extract.

2.6. Chitosan crosslinking, polyphenol oxidase immobilization and biosensor construction

The chitosan used as the support for immobilization of polyphenol oxidase was prepared according to the following procedure: chitosan (1.0%, m/v) was dissolved in an aqueous solution of acetic acid (3.0%, v/v) containing three drops of the surfactant Tween 80 (2.0%, v/v) at room temperature. Ionic crosslinked chitosan was formed spontaneously on adding 50.0 mL of sodium oxalate (5.0%, w/v) to the chitosan solution under stirring. The solid was separated by centrifugation (10 min; 3000 rpm), filtered, and washed twice with water and phosphate buffer solution (pH 6.0) to remove the excess of reagents. The crosslinked chitosan powder was collected, dried and stored in a desiccator at room temperature until use.

Aliquots of the homogenate containing 300–1500 unit of PPO mL^{-1} were added to 30.0 mg of the ionic crosslinked chitosan powder. These mixtures were dried at room temperature and investigated together, in different Ir-BMI.PF_6:Nujol proportions (0:100; 25:75; 50:50; 75:25; 100:0% (w/w)), for the building of the biosensors.

The construction procedure for the biosensor involved hand-mixing 70.0 mg of graphite powder and 30.0 mg of support containing 1200 unit mL^{-1} of PPO for 20 min in a mortar. The Ir-BMI.PF_6 (60.0 mg) was then added to this mixture which was mixed for at least 20 min to guarantee uniform dispersion and produce the final paste. This uniform paste was tightly packed into the cavity of a syringe (1.0 mm internal diameter) and a copper wire was inserted to establish the external electrical contact. The carbon paste electrode without PPO was prepared following the same steps.

2.7. Electrochemical measurement and coffee sample preparation

Square wave and cyclic voltammetry measurements were carried out in 0.1 mol L^{-1} phosphate buffer (pH 6.0) and the required volume of chlorogenic acid or sample solutions with the following parameters: potential range between +0.40 and +0.10 V; frequency of 30.0 Hz; scan increment of 3.0 mV; pulse amplitude of 100.0 mV. All measurements were reported *vs.* Ag/AgCl (3.0 mol L^{-1} KCl) after a suitable initial stirring time of 60 s in order to homogenize the solution.

Two samples of organic coffee (A and B) and two of decaffeinated coffee (C and D) were acquired from a local supermarket in Flori-

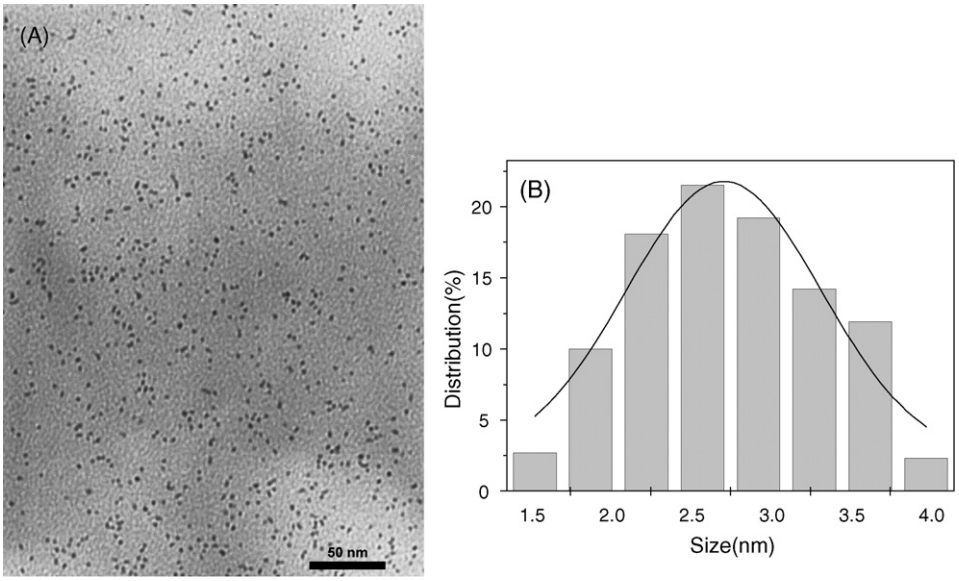

Fig. 1. (A) TEM micrograph of iridium nanoparticles in BMI.PF_6 and (B) histogram of the particle size distribution.

anópolis (Santa Catarina, Brazil). For the determinations, 1.5 g of soluble coffee (A and C) and 3.0 g instant coffee (B and D) were prepared in triplicate in 50 mL of hot water. For the electrochemical measurements using the proposed biosensor, 30 μl of these coffee samples were transferred to the cell containing 10 mL of phosphate buffer solution (0.1 mol L^{-1}; pH 6.0).

3. Results and discussion

3.1. Morphology and size of the iridium nanoparticles

Fig. 1A shows the morphology, investigated by transmission electron microscopy, of the iridium nanoparticles dispersed in the ionic liquid BMI.PF_6 and Fig. 1B gives the histogram of the particle size distribution. As can be seen, the mean diameter of the iridium particles was around 2.5 nm. The nanoparticle patterns are similar to those obtained by Fonseca [39] from the H_2 reduction of $[Ir(cod)Cl]_2$ in BMI.PF_6. Subsequently, this Ir-BMI.PF_6 was used as a binder in the construction of the biosensor containing polyphenol oxidase immobilized in chitosan ionically crosslinked with oxalate.

3.2. Immobilization of polyphenol oxidase and enzymatic reaction

Chitosan, a natural biopolysaccharide, has attracted significant interest due to its well-documented properties, such as biocompatibility, low toxicity and biodegradability. It is widely used in the

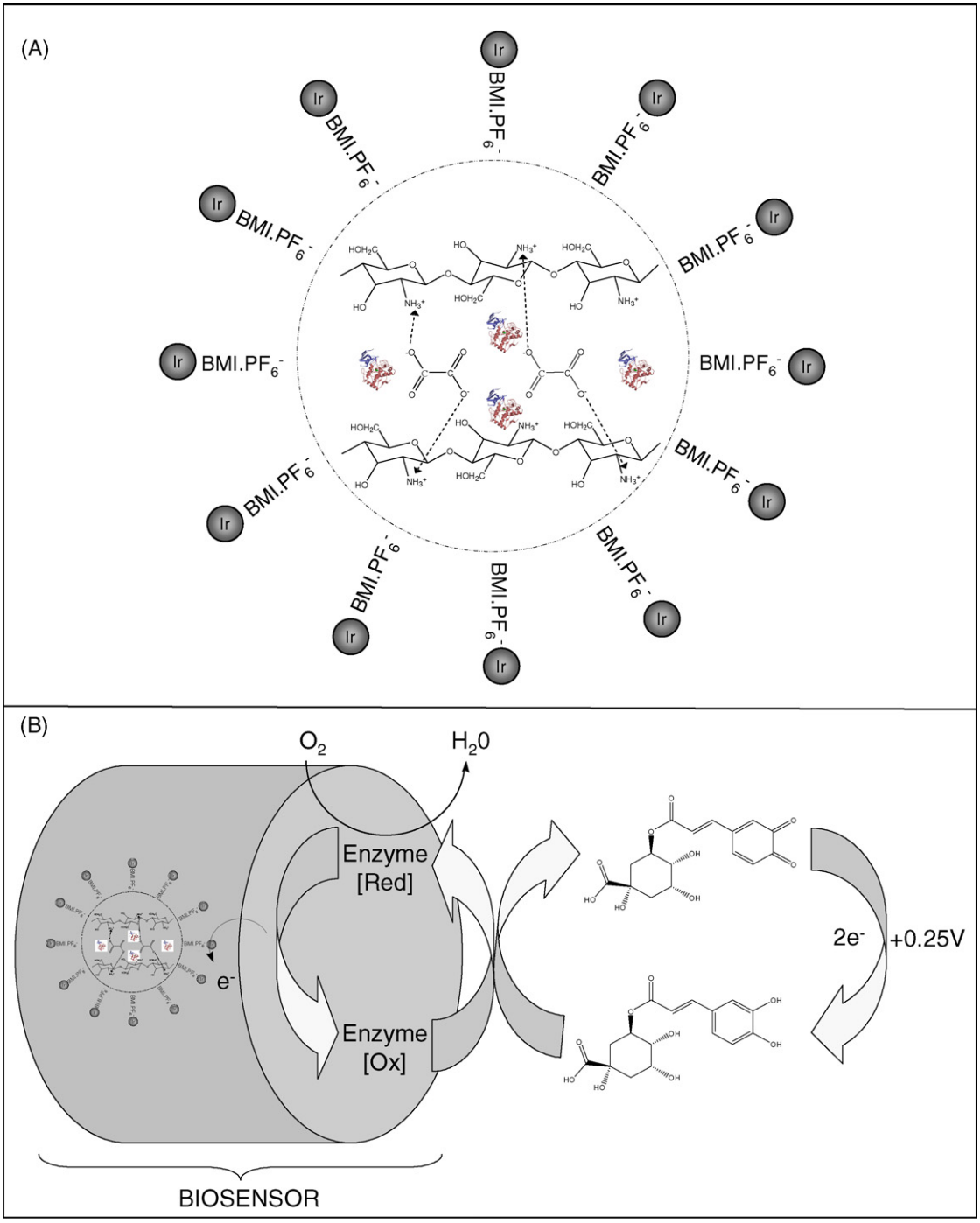

Fig. 2. Schematic representation of (A) immobilization of PPO on chitosan crosslinked with oxalate and (B) reaction involving chlorogenic acid on the surface of the biosensor.

pharmaceutical, food and biotechnological industries. Crosslinked chitosan is commonly used in controlled drug delivery [21,22,40]. Oxalate is an anion which interacts with chitosan via electrostatic forces to form ionic crosslinking. The positively charged amino groups of chitosan can react with the negatively charged oxalate via electrostatic attraction to form ionically crosslinked networks. Fig. 2A shows a schematic representation of the immobilization of the PPO from the sugar apple plant material in ionically crosslinked chitosan which leads to the proposed biosensor having a high stability and long lifetime.

Polyphenol oxidase catalyzes the oxidation of a variety of phenolic compounds, with the simultaneous reduction of the oxygen of the water molecule. Chlorogenic acid is oxidized in the presence of this enzyme to its respective *o*-quinone, which is electrochemically reduced back to chlorogenic acid on the biosensor surface at a potential of +0.25 V, as shown in Fig. 2B.

3.3. Morphology of the plain CPE and biosensor

The morphological characteristics of the sensors were studied using scanning electron microscopy. Fig. 3A and B shows the plain carbon paste electrode (CPE) and the biosensor, respectively. Considerable differences in the surface of the Ir-BMI.PF_6-PPO and the plain carbon paste can be observed. The CPE is characterized by a surface formed of isolated and irregular carbon flakes of graphite and each layer could be clearly distinguished. On the other hand, the surface of the carbon paste modified with Ir-BMI.PF_6 and the immobilized PPO enzyme appeared to be smoother and more uniform, and no separated carbon layers could be observed due to the high viscosity of the ionic liquid, which was packed homogeneously into the empty spaces between the graphite powder particles. Because of the higher ionic conductivity, the IL is not limited to serving as a binder for graphite, but may also work as a charge-transfer bridge facilitating the electron transfer [41].

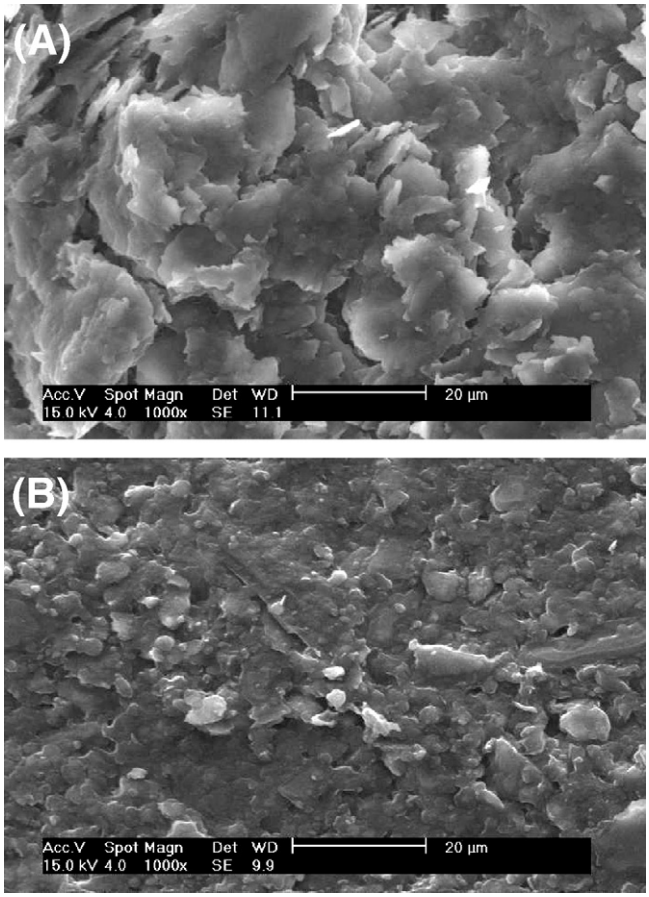

Fig. 3. Scanning electron micrograph of (A) plain CPE and (B) biosensor containing ionic liquid Ir-BMI.PF_6 and immobilized PPO.

3.4. Ir-BMI.PF_6–PPO contribution

To evaluate the contribution of the ionic liquid BMI.PF_6 containing iridium nanoparticles and PPO different sensors were constructed and compared. The electrochemical behavior of chlorogenic acid using these sensors was investigated by square wave voltammetry in the potential range of +0.5 to 0.0 V *vs.* Ag/AgCl. Fig. 4 shows the voltammograms obtained using the (a) plain CPE, (b) BMI.PF_6–CPE, (c) Ir-BMI.PF_6–CPE and (d) Ir-BMI.PF_6–PPO electrodes, in a 1.46×10^{-5} mol L^{-1} chlorogenic acid solution in phosphate buffer (0.1 mol L^{-1}; pH 6.0). As can be seen, a greater response to the chlorogenic acid solution was obtained using the biosensor containing Ir-BMI.PF_6 and immobilized PPO enzyme, when compared with the other sensors. Based on these results it is assumed that Ir-BMI.PF_6 offers a favorable microenvironment for PPO, which facilitates the electron transfer on the biosensor surface. This study confirms the catalytic contribution of the Ir-BMI.PF_6 and the efficiency of the immobilized PPO enzyme.

3.5. Optimization of the biosensor

The parameters of ionic liquid:Nujol ratio, pH and enzyme concentration, along with the frequency, pulse amplitude and scan increment used in the square wave voltammetry, were investigated to obtain the optimum response conditions for operation of the proposed biosensor.

The initial experiments were conducted to investigate the optimum ionic liquid content in the composition. Ratios of 100:0; 75:25; 50:50; 25:75 and 0:100% Nujol: IL were studied by square wave voltammetry. The analytical signals (resultant peak currents) for a 2.83×10^{-5} mol L^{-1} chlorogenic acid solution increased until only ionic liquid was present in biosensor. Since 100% of Ir-BMI.PF_6 was found to give the best sensitivity of the responses this was used for construction of the subsequent biosensors.

The solution pH is important to the electrochemical reaction and was investigated by detection of chlorogenic acid in phosphate or acetate buffer solution (0.1 mol L^{-1}) ranging from 5.0 to 9.0. The

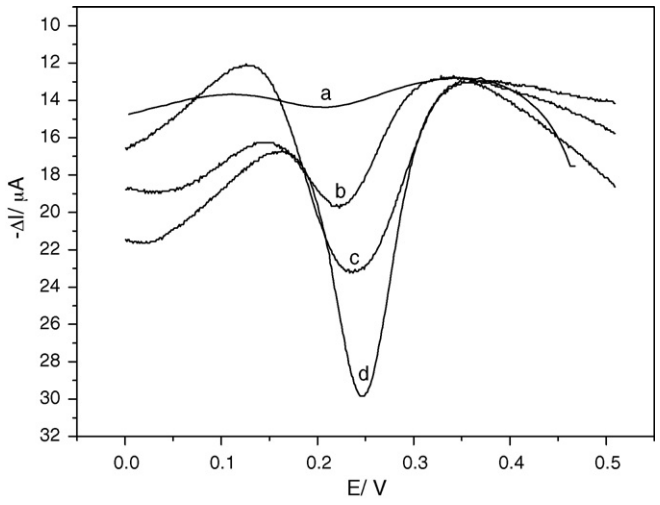

Fig. 4. Square wave voltammograms obtained using (a) plain CPE, (b) BMI.PF_6–CPE, (c) Ir-BMI.PF_6–CPE and (d) Ir-BMI.PF_6–PPO in a 1.46×10^{-5} mol L^{-1} chlorogenic acid solution in phosphate buffer (0.1 mol L^{-1}; pH 6.0) at pulse amplitude 100 mV, frequency 30 Hz and scan increment 1.0 mV.

Table 2
Optimization of experimental variables.

Parameters investigated	Range studied	Optimal value
IL composition (% w/w)	0–100	100
PPO (unit mL^{-1})	300–1500	1200
pH	5.0–9.0	6.0
Frequency (Hz)	10–100	30
Pulse amplitude (mV)	10–100	100
Scan increment (mV)	1.0–10.0	1.0

resulting peak-shaped pH profile showed a maximum sensitivity response at pH 6.0, the peak current of chlorogenic acid decreasing at higher values. Thus, pH 6.0 was adopted for further studies.

The amount of PPO enzyme in the paste preparation was optimized from 300 to 1500 unit mL^{-1} and the biosensor response increased until 1200 unit mL^{-1} and decreased with higher values of PPO in the paste. Based on this result, pastes containing 1200 unit mL^{-1} of PPO were prepared to perform further experiments.

Finally, the parameters of square wave voltammetry were optimized for the best performance of the biosensor under the same conditions cited above. Frequency (10–100 Hz), pulse amplitude (10–100 mV) and scan increment (1.0–10.0 mV) were evaluated. Overall, the optimized parameters can be summarized as follows: frequency 30 Hz; amplitude 100 mV and scan increment 1.0 mV. Table 2 summarizes the relevant analytical information and gives the best value found in the optimization of the biosensors.

3.6. Reproducibility, repeatability and stability of the biosensor

The reproducibility was investigated using three biosensors constructed independently, and indicated an acceptable reproducibility with a relative standard deviation of 1.81% for the response to 2.83×10^{-5} mol L^{-1} chlorogenic acid solution (pH 6.0). The repeatability of the biosensor performance also was studied by successive measurements of seven replicates of chlorogenic acid and the relative standard deviation was 4.30%. This indicates that the biosensor has good reproducibility due to the high sensitivity and efficiency of the immobilization of the PPO enzyme on the electrode.

The stability of the biosensor was investigated by measuring the electrochemical response (cathodic peak currents) to a 2.83×10^{-5} mol L^{-1} chlorogenic acid solution (pH 6.0) over a 150-day period without surface renewal. When the biosensor was stored at room temperature and measurements taken every 1–8 days, no notable change was observed.

3.7. Analytical performance of biosensor

Fig. 5 displays the square wave voltammetry response and the analytical curve of the cathodic peak current *vs.* chlorogenic acid concentration using the proposed biosensor under the selected conditions mentioned above. It was found that the resultant peak current was linear for chlorogenic acid concentrations in the range of 3.48×10^{-6} to 4.95×10^{-5} mol L^{-1}, with a detection limit (S/N = 3) of 9.15×10^{-7} mol L^{-1}. The linear regression equation was expressed as $-\Delta I$ (μA) = 0.4879 + 5.1621 × 10^7 [chlorogenic acid] with a correlation coefficient of $r = 0.9996$.

The good analytical performance and stability of the proposed biosensor can be attributed to the excellent immobilization of polyphenol oxidase from the sugar apple (*A. squamosa*) in chitosan ionically crosslinked with oxalate and the high conductivity of the ionic liquid containing dispersed Ir nanoparticles. The contribution of the iridium nanoparticles was compared with reports in the literature [16,17] and other biosensors based on gold, palladium and platinum nanoparticles [8–15].

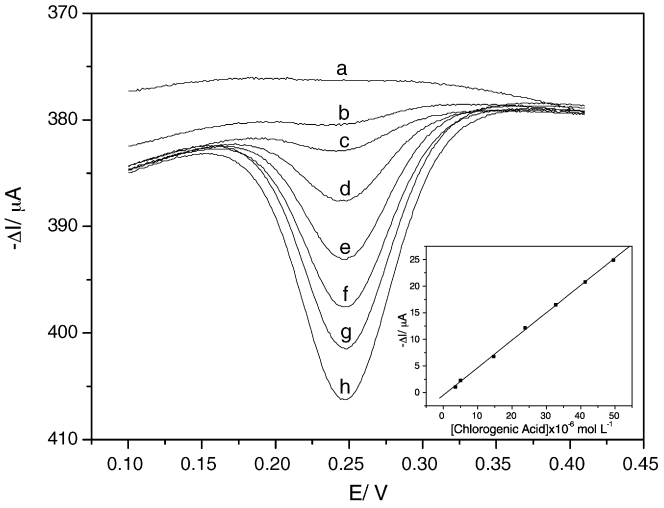

Fig. 5. Square wave voltammograms obtained using the proposed Ir-BMI.PF_6–PPO biosensor in (a) 0.1 mol L^{-1} phosphate buffer solution (pH 6.0) and chlorogenic acid solutions at the following concentrations: (b) 3.48×10^{-6} mol L^{-1}, (c) 4.95×10^{-6} mol L^{-1}, (d) 1.46×10^{-5} mol L^{-1}, (e) 2.38×10^{-5} mol L^{-1}, (f) 3.27×10^{-5} mol L^{-1}, (g) 4.13×10^{-5} mol L^{-1} and (h) 4.95×10^{-5} mol L^{-1} at pulse amplitude 100 mV, frequency 30 Hz and scan increment 1.0 mV. Inset: the analytical curve of chlorogenic acid.

The analytical features of the biosensor in terms of chlorogenic acid determination, and those for other analytical methods, are given in Table 1. The accuracy, stability (not shown in Table 1), linear range and limit of detection of the proposed biosensor were compared with others reported previously in the literature [31–37].

3.8. Recovery, interference and analytical application

Recovery experiments were carried out in order to evaluate the interference from matrix effects of coffee samples. This study was performed by adding known amounts of standard solutions to the samples (1.80, 8.65 and 15.0 mg L^{-1}) followed by analysis using the proposed biosensor. Satisfactory values between 93.2 and 105.7% for chlorogenic acid were obtained for the recovery, thus indicating that there is no interference from the sample matrix in the proposed method and also that the proposed method is suitable for use in these samples.

The effect of possible interferents, such as caffeine, caffeic acid, citric acid, fructose, glucose, glutamic acid, sucrose and tartaric acid, on the determination the chlorogenic acid in coffee was also investigated. The ratio of the concentration of chlorogenic acid to that of each substance was fixed at 1:1. Only caffeic acid caused a positive interference (12.0%) when present at the same concentration as the analyte. The observed interference was not critical, however, since caffeic acid is present at lower concentration levels than those investigated. The data were in agreement with those obtained employing other biosensors [35]. None of the other substances interfered in the performance of the proposed biosensors.

This biosensor was used to determine chlorogenic acid in different organic and decaffeinated samples using the standard addition method. Table 3 presents the chlorogenic acid concentrations determined in coffee samples employing the proposed biosensor and the capillary electrophoresis method. The results determined by the biosensor were in satisfactory agreement with those given by the capillary electrophoresis method, indicating that it is viable to apply the proposed biosensor to determine chlorogenic acid in coffee samples.

Table 3
Chlorogenic acid determination (g L^{-1}) in organic (A and B) and decaffeinated (C and D) coffee samples.

Sample	Chlorogenic acid (g L^{-1})		Relative error (Re%)
	Capillary electrophoresis	Biosensor[a]	
A	0.71 ± 0.01	0.71 ± 0.02	0.0
B	0.60 ± 0.02	0.58 ± 0.02	−3.33
C	0.97 ± 0.02	0.99 ± 0.01	+2.06
D	1.01 ± 0.02	0.96 ± 0.01	−4.95

Re = biosensor *vs.* capillary electrophoresis.
[a] $n = 4$; confidence level of 95%.

4. Conclusions

In this paper, the construction of a biosensor containing Ir-BMI.PF_6 and PPO immobilized in crosslinked chitosan was described. This biosensor offers advantages such as good sensitivity and linearity with a low detection limit and ease of preparation and renewal compared with the conventional carbon paste electrode. Immobilization of the enzyme in biocompatible chitosan together with Ir-BMI.PF_6 applied in the determination of chlorogenic acid in organic and decaffeinated coffee samples was showed to be a simple and efficient analytical method.

Acknowledgments

The authors are grateful to CNPq (Processes 472169/2004-1 and 472541/2006-4), MCT/CNPq/PADCT and also for the scholarship granted by CNPq and CAPES to SKM and SCF, respectively, and for their financial support of this work.

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