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Development of biosensor for phenolic compounds containing PPO in β-cyclodextrin modified support and iridium nanoparticles

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ABSTRACT

A biosensor based on the iridium nanoparticles dispersed in ionic liquid (IL) 1-butyl-3-methylimidazolium hexafluorophosphate (Ir-BMI·PF_6) and a celery (*Apium graveolens*) extract as a source of polyphenol oxidase (PPO) was constructed. A modified support based on β-cyclodextrin (β-CDEP) was used for enzyme immobilization. The behavior of phenolic compounds was investigated by square-wave voltammetry and rutin was selected by presenting the greatest signal. The best performance was obtained with a composition of 70:10:10:10% (w/w/w/w) of the graphite powder:β-CDEP:Nujol:Ir-BMI·PF_6 composition, a PPO concentration of 500 units mL^{-1}, in 0.1 M phosphate buffer solution (pH 6.0) with frequency, pulse amplitude and scan increment at 100 Hz, 60 mV, and 3.0 mV, respectively. Under optimized conditions, the cathodic currents increased linearly for the rutin concentration range of 1.3×10^{-7}–2.0×10^{-6} M with a detection limit of 7.9×10^{-8} M. This sensor demonstrated acceptable repeatability and reproducibility and the results for the rutin recovery ranged from 92.8 to 103.4%. A relative error of 0.7% was obtained in the rutin determination in simulated samples.

1. Introduction

The use of extracts or plant tissue with enzymes source presents several advantages compared with commercial and purified enzymes [1], mainly because they are so easy to obtain and the presence of co-factors for biological source material gives greater stability to enzyme [2]. The indispensable factor to development of biosensors is an immobilization of enzymes. Supports such as chitin, chitosan, montmorillonite and cyclodextrin have been widely used in the enzyme immobilization process to produce biosensors with better performance [3–6]. In particular, the cyclodextrins (CD) have a molecular structure similar to a truncated cone with six, seven or eight glucose units (α, β and γ-CD, respectively) linked by α-1,4 bonds, which present a receptive cavity where different chemical species can interact by forming inclusion complexes [7,8]. In addition, Franzoi et al. [6] have demonstrated that the application of β-CD modified with epichlorohydrin (EP) for laccase immobilization improves the biosensor performance, due to the formation of an effective polymeric network between β-CD and EP, providing a favorable environment for the enzyme.

Another relevant point in the development and construction of sensors is the use of modifiers, the main objective of which is to obtain devices with greater sensitivity, selectivity and stability. In this sense, ionic liquids (IL) have been extensively applied in biosensors. IL are composed entirely of ions and they possess fascinating properties, including low volatility, tunable viscosity and miscibility, and electrolytic conductivity, which make them unique and useful for many applications in chemical analysis [3,9–13].

An important application of IL, in particular imidazolium-based IL, is their use as suitable media for the generation and stabilization of metal nanoparticles [14–16]. Metal nanoparticles, especially noble metals, exhibit outstanding features including excellent catalytic ability, high surface area, chemical stability and biocompatibility, which have led to their extensive use in biosensors. Due to such properties these nanomaterials can provide a stable environment for the biomolecule immobilization while preserving its activity and they can also to facilitate electron transfer between the biological material and the electrode surface [17–19]. Several studies reported in the literature have demonstrated that the incorporation of metal nanoparticles dispersed in IL have generated high performance biosensors for phenolic compound determination [4–6,20,21].

Phenolic compounds comprising a wide range of substances of natural occurrence as vitamins, hormones, antioxidants, and also various industrial products such as detergents, drugs, *etc.* [22]. Two examples of phenolic substances are rutin, a drug with many pharmacological properties including anti-carcinogenic, cytoprotective, anti-platelet, anti-thrombic, vasoprotective, and cardioprotective activities [23,24] and fisetin, which is present in foods and beverages such as black tea and green tea (*Camellia sinensis*) and present

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great antioxidant activity [25] and a potential enzyme inhibitor in inflammatory processes [26]. Consumption of these compounds even at low concentrations affect the quality of human life, especially when inappropriately drug consumed, therefore the presence and amount of phenolic substances ingest in food, beverages and medications must be monitored constantly.

Due to these important properties, several analytical methods have been reported for the quantitative determination of phenolic compounds, including capillary electrophoresis [27], chemiluminescence [28], high-performance liquid chromatography [29] and spectrophotometry [30]. Electrochemical methods using sensors [31–34] and biosensors [5,35–37] have been applied for phenolic compounds determination as a viable alternative to the above-mentioned methods. These offer the advantages of low cost, high sensitivity, easy operation and the possibility for the construction of simple portable devices for fast screening purposes. Fernandes et al. [38] described a biosensor for chlorogenic acid using PPO immobilized in chitosan ionically crosslinked with oxalate and ionic liquid containing dispersed iridium nanoparticles. The biosensor presented a good stability mainly due the efficiency of the immobilization of the enzyme and the good response for this phenolic compound was associated to the synergic effect of the IL and the enzyme.

In this paper, a modified support based on β-CD and EP was used for PPO immobilization and iridium nanoparticles dispersed in an IL derived from 1-butyl-3-methylimidazolium hexafluorophosphate (BMI·PF_6) were applied as a binder in the biosensor fabrication. Herein, the combination of the β-CDEP and Ir-BMI·PF_6 improve the electrochemical performance of the biosensor. In addition, the biosensor and technical parameters were optimized and applied for rutin determination by square-wave voltammetry (SWV) as it showed the greatest response by comparing with other tested substrate.

2. Experimental

2.1. Chemicals and solutions

Reagents were of analytical grade and used as received. All solutions were prepared with ultrapure water (18.2 MΩ cm) obtained from a Milli-Q purification system. A phosphate buffer (0.1 M, pH 6.0) solution was used as the supporting electrolyte throughout the experiments. Caffeic acid, catechin, fisetin, quercetin and rutin were acquired from Sigma–Aldrich. A stock solution of phenolic compounds was prepared daily in 30% (v/v) ethanol and 0.1 M phosphate buffer solution at pH 6.0. The IL 1-butyl-3-methylimidazolium hexafluorophosphate (BMI·PF_6) and iridium nanoparticles dispersed in BMI·PF_6 (Ir-BMI·PF_6) were synthesized as previously described in the literature [39,40]. The β-cyclodextrin (β-CD) modified with epichlorohydrin (EP) was prepared following the steps described in the previous paper [6]. The carbon paste was prepared using graphite powder (Acheson 38, Fisher Scientific) and high purity Nujol purchased from Sigma–Aldrich.

2.2. Obtainment of the polyphenol oxidase and measurement of activity

Celery tissue (*Apium graveolens*) was used as a source of PPO enzyme and was obtained through the following procedure: 25 g of celery was homogenized for 30 s in 50 mL of 0.1 M phosphate buffer solution (pH 7.0), filtered and centrifuged (18,000 rpm; 2 min; 4 °C). The resulting supernatant (homogenate) was stored at 4 °C and used as the source of the enzyme to construct the biosensor after the determination of the activity. The activity of PPO was determined in triplicate by measuring the absorbance at 420 nm of *o*-quinone produced by the reaction between 2.8 mL of 0.05 M catechol solution in 0.1 M phosphate buffer (pH 7.0) and 0.2 mL of the enzymatic extract. One activity unit is defined as the quantity of enzyme that causes an increase of 0.001 absorbance per min under the conditions described above [38,41].

2.3. Polyphenol oxidase immobilization and biosensor construction

Aliquots of the celery homogenate containing 100–900 units mL^{-1} of PPO were added to the β-CDEP support. The resulting material was dried at room temperature and used for the developed of the biosensor.

The biosensor was constructed according to the procedure described by Fernandes et al. [38]. Briefly, 20 mg (10% w/w) of β-CDEP containing immobilized PPO (500 units mL^{-1}) and graphite powder (140 mg; 70%, w/w) were mixed in a mortar for 20 min to ensure a uniform mixture. Subsequently, 20 mg of Nujol (10% w/w) and 20 mg of Ir-BMI·PF_6 (10% w/w) were added and mixed for at least 20 min to form a final paste. A portion of the resulting paste was packed firmly into the cavity of a syringe and a copper wire was inserted to obtain the external electrical contact.

2.4. Instrumentation and electrochemical measurements

Square wave voltammetry (SWV) measurements using the biosensor were performed on an Autolab PGSTAT12 potentiostat/galvanostat (Eco Chemie, Utrecht, The Netherlands) connected to data processing software (GPES, version 4.9.006, Eco Chemie). A conventional three-electrode system was used with the biosensor as the working electrode, a platinum wire as the counter electrode and Ag/AgCl (3.0 M KCl) as the reference electrode. Voltammetric measurements were carried out in an unstirred, phosphate buffer solution (pH 6.0) at 25.0 ± 0.5 °C and all potentials were measured and reported *vs.* Ag/AgCl. Firstly, 10 mL of the supporting electrolyte was transferred to a clean, dry cell and the required volume of phenolic compound was added by micropipette. After a stirring period of 60 s in order to homogenize the solution, square wave voltammograms were recorded, applying a sweep potential of between 0.5 and 0.0 V, at a frequency of 10–100 Hz, pulse amplitude of 10–100 mV and scan increment of 1.0–10.0 mV.

3. Results and discussion

3.1. Electroanalytical evaluation of phenolic compounds

PPO catalyzes the oxidation of a variety of phenolic compounds with the simultaneous reduction of the oxygen of the water molecule [5,38,42–48]. In order to investigate the response of the biosensor and determine the best substrate for the celery PPO, a comparative study between different compounds was carried out. The proposed biosensor responses to the selected substrates (caffeic acid, catechin, fisetin, quercetin and rutin) were obtained from solutions containing 2.8×10^{-3} M of the compound in 0.1 M phosphate buffer solution (pH 7.0). The results obtained are shown in Table 1. As can be observed, the proposed biosensor showed decreasing cathodic peak current (expressed in relative response) in the order: rutin > caffeic acid > catechin > fisetin > quercetin. Therefore, rutin was selected for subsequent experiments.

3.2. PPO immobilization and enzymatic reaction on the biosensor surface

A key factor in the construction of a biosensor is the need to achieve adequate and effective enzyme immobilization. The PPO enzyme has been successfully immobilized in a variety of supports, including chitosan, polypyrrole film and clay, using several techniques [5,38,45–48]. Franzoi et al. [6] demonstrated that the modified β-CDEP served as a very effective support to immobilize the laccase enzyme, preserving its activity and providing greater stability. In this study, this material was obtained with excellent properties and used as a support for PPO immobilization. Thus, the PPO enzyme is entrapped within the interstitial space of the β-CDEP and this system can result in considerable stabilization of the enzyme structure, hence increasing its activity. Basically, the formation of cross-linked system increases the interactions between the PPO enzyme and the support, creating a network of crosslinks. Crini et al. [49] proposed a possible structure for the β-CDEP polymer.

A broad range of polyphenols have been determined with the use of PPO biosensors [5,38,45–48]. PPO catalyzed the oxidation of rutin to quinone and this product was electrochemically reduced to rutin on the biosensor surface at a potential of +0.28 V *vs.* Ag/AgCl. Fig. 1 illustrates the immobilization procedure and the electrochemical reaction.

3.3. Electrochemical response of PPO biosensors to rutin

The contribution of each component of the biosensor response was investigated by SWV in the potential range of +0.5 to 0.0 V *vs.*

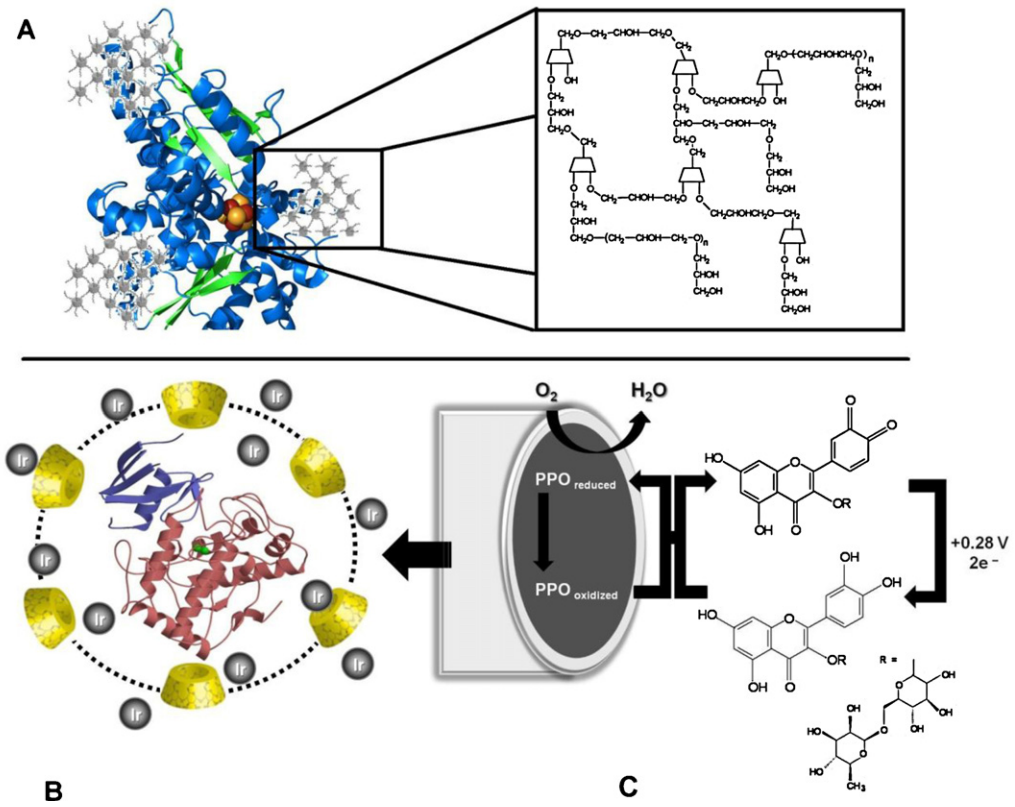


Fig. 1. (A) Immobilization procedure using β-CD modified support, (B) composition of the proposed biosensor and (C) PPO-catalyzed oxidation of rutin with its subsequent electrochemical reduction on the biosensor surface.

Ag/AgCl. Fig. 2 shows the voltammograms obtained using the (a) bare carbon paste electrode (CPE), (b) CPE + BMI·PF_6, (c) CPE + Ir-BMI·PF_6 and (d) biosensor + β-CDEP/Ir-BMI·PF_6 in 1.5×10^{-6} M rutin in 0.1 M phosphate buffer solution (pH 6.0), with optimized parameters. The cathodic current of *o*-quinone reduction to rutin was used as the analytical response (cathodic current values are shown in the inset of Fig. 2).

It can be observed that the use of the conductive binder (IL BMI·PF_6) led to an increase in the electrode response (b) and a large difference in the value of the cathodic current was observed when compared with the CPE (a). In addition, the incorporation of iridium nanoparticles dispersed in BMI·PF_6 for the construction of the electrochemical biosensor (c) enhanced the analytical performance with respect to other electrodes (a and b). The highest response was obtained using a combination of the PPO enzyme immobilized in an excellent support and Ir-BMI·PF_6 in the biosensor construction (d). This finding can be attributed to the combining of the biocompatibility of the material based on β-CDEP with the conductivity and enzymatic stabilization power of the Ir-BMI·PF_6. This behavior had been observed in other studies reported in the literature [4–6,20,21,38], since nanoparticles provide a suitable microenvironment for the enzyme, permitting direct electron transfer between the immobilized enzyme and the biosensor surface.

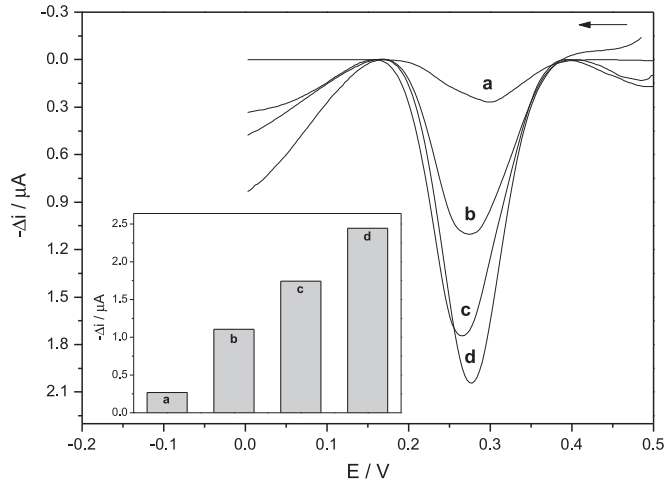


Fig. 2. Square-wave voltammograms obtained using (a) bare carbon paste electrode (CPE), (b) CPE + BMI·PF_6, (c) CPE + Ir-BMI·PF_6 and (d) biosensor + β-CDEP/Ir-BMI·PF_6 with 1.5×10^{-6} M rutin in 0.1 M phosphate buffer solution (pH 7.0) at frequency 100 Hz, pulse amplitude 60 mV and scan increment 3.0 mV. Inset: current values for each CPE and biosensor.

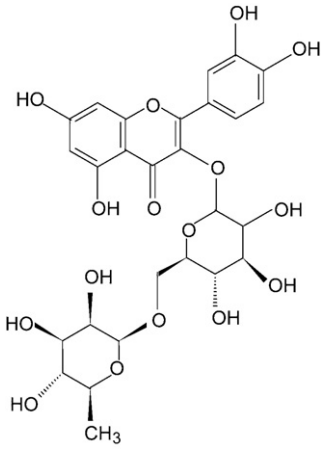
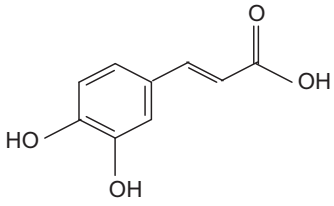
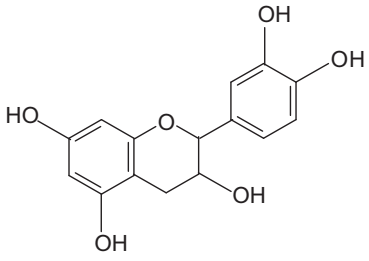
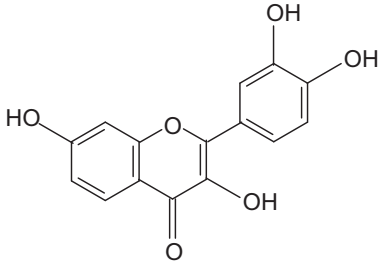
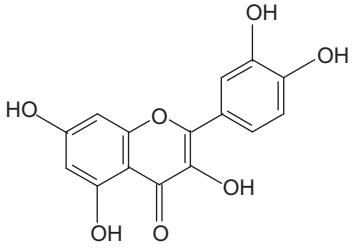
3.4. Optimization of the biosensor construction and analytical conditions

To optimize the performance of the biosensor in the determination of rutin some parameters, such as PPO concentration, supporting electrolyte and pH, along with the frequency, pulse amplitude and scan increment used in the SWV, were investigated.

The effect of PPO concentration on the biosensor response was studied in the range of 100–900 units mL^{-1}. The analytical signal (cathodic peak current) increased with the PPO concentration up to 500 units mL^{-1}, and thus this concentration was used for the construction of further biosensors.

The pH had a notable influence on the enzymes directly affecting enzymatic reactions. The effect of the pH of the 0.1 M phosphate buffer solution (in the range of 6.0–8.0) and acetate buffer solution (4.0–5.0) on the biosensor response to a 1.9×10^{-5} M rutin solution was studied. The best voltammetric response to rutin was

Table 1
Relative response of the proposed biosensor for the phenolic compounds investigated herein.

Phenolic compounds	Structure	Relative response (%)
Rutin		100
Caffeic acid		86.4
Catechin		55.4
Fisetin		25.8
Quercetin		19.9

obtained in phosphate buffer solution at pH 6.0. Therefore, this pH was selected and used for subsequent experiments.

The SWV parameters were optimized using the best response of the biosensor to 1.9×10^{-5} M rutin solutions. Values for frequency in the range of 10–100 Hz, pulse amplitude between 10 and 100 mV and scan increment from 1.0 to 10.0 mV were evaluated. The best resultant peak currents were obtained using values for frequency, pulse amplitude and scan increment of 100 Hz, 60 mV and 3.0 mV, respectively.

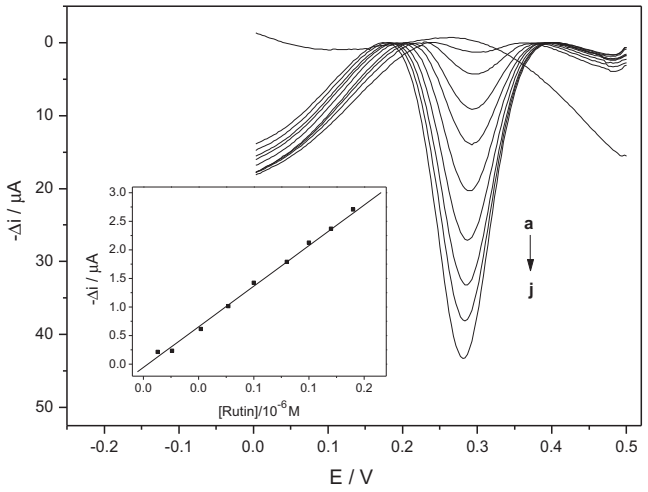


Fig. 3. Square-wave voltammograms obtained using the proposed biosensor based on (a) blank in phosphate buffer solution (0.1 M; pH 6.0) and rutin solutions at the following concentrations: (b) 1.3×10^{-7}; (c) 2.6×10^{-7}; (d) 5.2×10^{-7}; (e) 7.7×10^{-7}; (f) 1.0×10^{-6}; (g) 1.3×10^{-6}; (h) 1.5×10^{-6}; (i) 1.7×10^{-6} and (j) 2.0×10^{-6} M at frequency 100 Hz, pulse amplitude 60 mV and scan increment 3.0 mV. Inset: calibration curve for rutin.

3.5. Analytical performance of biosensor in rutin determination

Under the best working conditions established for rutin detection, typical analytical curves were obtained with the proposed biosensor by square wave voltammetry, in the potential range of +0.5–0.0 V *vs* Ag/AgCl. The electrochemical reduction to rutin was obtained at potentials of +0.28 V. Fig. 3 shows the voltammograms for rutin and the corresponding calibration curve, constructed from cathodic current peaks *vs* rutin concentration, can be seen in the inset. The corresponding linear range and regression equation obtained using this biosensor was:

1.3×10^{-7} to 2.0×10^{-6} M ($-\Delta i = 5.35 - 14.55 \times 106$ [rutin]; $r = 0.9995$)

where $-\Delta i$ is the cathodic current peak (μA), and [rutin] is the rutin concentration (M). The detection limit (three times the signal blank/slope) was calculated using the linear range and the value obtained was 7.9×10^{-8} M.

Analytical features observed for rutin determination and those reported previously in the literature using modified carbon paste electrodes are shown in Table 2. The linear range and detection limit of the developed biosensor are comparable with or better than those described in the literature [5,35–37] considering the use of non-purified enzymes for the biosensor construction, with low cost and easy to obtain. The satisfactory analytical performance of the proposed biosensor can be attributed to the good stability of PPO in crude extract and the effective immobilization on the modified support, the ionic liquid and metal nanoparticles has synergic contributions to PPO stabilization and catalytic contribution facilitate the electron transfer, improving the biosensor sensitivity as reported by Fernandes et al. [38].

3.6. Repeatability and reproducibility of the biosensor

The study of the biosensor repeatability was evaluated by investigating its performance for five parallel determinations of

Table 2
Analytical response of different carbon paste sensors for determination of rutin.

Sensor composition	Linear range (M)	Detection limit (M)	References
CPE + Lac + PPO/MMT + Ir-BMI·BF_4	$9.2 \times 10-8$–$3.1 \times 10-6$	3.0×10^{-8}	[5]
CPE + PVP	$3.9 \times 10-7$–$1.3 \times 10-5$	1.5×10^{-7}	[35]
CPE + Lac + BMI·Tf_2N	$4.8 \times 10-6$–$4.6 \times 10-5$	4.5×10^{-7}	[36]
CPE + Lac/Chi-TPP	$6.0 \times 10-7$–$3.9 \times 10-6$	6.2×10^{-8}	[37]
CPE + PPO/β-CDEP + Ir-BMI·PF_6	$1.3 \times 10-7$–$2.0 \times 10-6$	7.9×10^{-8}	(This study)

CPE, carbon paste electrode; PVP, poly(vinylpyrrolidone); Lac, Laccase; MMT, montmorillonite; Chi-TPP, microspheres of chitosan crosslinked with tripolyphosphate.

Table 3
Recovery results for rutin in simulated sample using the proposed biosensor.

Rutin (3.0×10^{-5} M)		Recovery (%)
Added[a]	Found[a]	
2.37	2.20	92.8
4.70	4.86	103.4
7.00	6.95	99.3

[a] $n = 3$.

2.4×10^{-5} M rutin solution in phosphate buffer solution (0.1 M; pH 6.0). The relative standard deviation (RSD) was calculated as 9.0%.

Another important parameter that needs to be considered is the reproducibility of the biosensor which was investigated by detecting 7.0×10^{-5} M rutin. Three biosensors were constructed using the same procedure and were independently used for the detection of rutin under the optimized conditions described previously. The RSD was 8.1%, demonstrating that the biosensor has a good reproducibility.

3.7. Recovery tests and rutin analysis in simulated sample

The standard addition method was applied in the rutin recovery experiments. As can be seen in Table 3, rutin recoveries of 92.8–103.4% from simulated samples (3.0×10^{-5} M; $n = 3$) were obtained using the biosensor. These results are considered satisfactory and indicate the absence of matrix effects in these determinations. Rutin solutions of 2.4×10^{-7}, 4.7×10^{-7} and 7.0×10^{-7} M were added to the sample and the cathodic peak current was registered for the simulated sample.

To investigate the possible practical application of the novel biosensor, the determination was performed in a simulated sample (2.4×10^{-7} M) in triplicate. The relative error was only 0.7%, verifying that there is no significant difference between the measurements.

4. Conclusions

The main feature of this study was to propose a biosensor using plant extract for PPO source immobilized on a modified support together with iridium nanoparticles dispersed in BMI·PF_6 showing a good linear range and a low detection limit for rutin. The modified β-CDEP was biocompatible and able to immobilize PPO, leading to highly efficient, robust and stable biocatalysts. Another factor which contributed to the good performance of the biosensor is related to the conductivity of BMI·PF_6 and the advantages of the iridium nanoparticles in the electron transfer, enhancing the sensitivity of the biosensor. Moreover, the reported biosensor combines construction simplicity, fast response, good repeatability and reproducibility and an acceptable level of accuracy for rutin determination in a simulated sample.

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