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Development of a biosensor based on gilo peroxidase immobilized on chitosan chemically crosslinked with epichlorohydrin for determination of rutin

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Abstract

A new reagentless biosensor for the square-wave voltammetric determination of rutin in pharmaceutical formulations was developed by immobilization of gilo (*Solanum gilo*) crude extract in chitosan matrix. The gilo tissue acts as a source of peroxidase. The highest biosensor performance was obtained after immobilization of the peroxidase in chemically crosslinked chitosan with epichlorohydrin and glutaraldehyde that was incorporated in a carbon paste electrode. In the presence of hydrogen peroxide this enzyme catalyses the oxidation of rutin to quinone and the electrochemical reduction of the product was obtained at a fixed potential of +124 mV versus Ag/AgCl (3.0 M KCl). The performance and factors influencing the resulting biosensor were studied in detail. The bioelectrode exhibited a linear response for rutin concentrations from 3.4×10^{-7} to 7.2×10^{-6} M ($r = 0.9998$) and the recovery of rutin from the samples ranged from 96.2 to 102.4%. The detection and quantification limits were 2.0×10^{-8} and 6.3×10^{-8} M, respectively. The relative standard deviation was less than 1.0% for solutions containing 3.4×10^{-7} to 7.2×10^{-6} M rutin in 0.1 M phosphate buffer solution at pH 7.0 ($n = 10$). The lifetime of this biosensor was 8 months (at least 500 determinations).

Keywords: Epichlorohydrin; Rutin; Biosensor; Chitosan; Gilo (*Solanum gilo*)

1. Introduction

Flavonoids (or bioflavonoids) are benzo-γ-pyrone derivatives containing several hydroxyl groups attached to ring structures C_6–C_3–C_6. They can be subdivided into several groups: flavone (e.g., apigenin), flavonol (e.g., quercetin), flavanolo (e.g., taxifolin), isoflavone (e.g., genistein), flavanone (e.g., naringenin) and flavonol glycoside (e.g., rutin). They are widely distributed in plants and are known to possess strong antioxidant properties. The antioxidant activity can be due to both their radical-scavenging effect and to their metal-chelating properties, of which the former may dominate. Antioxidant flavonoids are naturally present in fruits, vegetables, tea and wine and have been found in vitro to inhibit oxidation of low-density protein [1,2].

Rutin, a natural flavone derivative, has been shown to act as a scavenger of various oxidizing species, i.e. superoxide anion (O_2^-), hydroxyl radical, and peroxyl radicals. As a result of these biological effects, its several pharmacological activities have been widely exploited including antibacterial, anti-inflammatory, antitumor, antiallergic, antiviral and antiprotozoal properties. Enzymatically decomposed rutin is generated by naringinase/hesperinase/rhamnosidase reactions followed by purification and its principal component is isoquercitrin, a compound naturally found in various plants and foods such as tea, wine and strawberries [3–5].

The quantification of rutin is of considerable interest. Many techniques including spectrophotometry [6–8], capillary electrophoresis [9,10] and electrochemical [11–14] measurement have been employed for the determination of this antioxidant. The spectrophotometric method has been widely used for individual and simultaneous determination of rutin and other flavonoids [6–8]. The procedure based on capillary electrophoresis with electrochemical detection was developed using a 300 μm diameter carbon disc electrode positioned opposite the outlet of the capillary [10]. The response was linear and ranged from 0.190×10^{-6} to 0.434×10^{-6} M. Electroanalytical methods and biosensors have proved to be effective for the study and determination of flavonoids in pharmaceuticals

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[11–14]. A biosensor was developed with Laccase *Coriolus Versicolor* as the biological reconnaissance element immobilized on derivatized polyethersulphone membranes [12]. A linearity ranging from 2.0 to 14.0×10^{-6} M and limit of detection of 1.0×10^{-6} M was obtained.

Peroxidase (EC 1.11.1.7) is widely distributed in nature and has been identified in plants, microorganisms and animals. In plants it participates in the lignification process and in the mechanism of defense in physically damaged or infected tissues. Most peroxidases are heme proteins and contain iron(III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. The high catalytic activity of this enzyme with a broad range of substrates is an attractive enzyme property for the construction and application of biosensors [15].

Biosensors are constructed by integrating biological sensing systems (e.g., enzyme), with transducers. These receive a chemical signal produced by the interaction of the biological system with an analyte and transduce (e.g., potentiometric, amperometric, conductometric) it into a measurable response. Enzymes may be immobilized by a variety of methods, which may be broadly classified as physical, where there are weak interactions between support and enzyme, and chemical, where covalent bonds are formed with the enzyme. The properties of immobilized enzymes are governed by the properties of both the enzyme and the support material [16–18].

The enzyme peroxidase has been covalently bonded to a wide variety of water-insoluble supports [15]. Recently the chitosan biopolymer has attracted considerable attention and has been used as a support for this enzyme [19,20]. Chitosan is obtained by the alkaline deacetylation of chitin, one of the most abundant biopolymers in nature. The principal characteristics of chitosan that may affect its sorption properties are its degree of deacetylation, crystallinity and, to a lesser extent, molecular weight. The degree of deacetylation controls the fraction of free amine groups that will be available for interaction with metal ions and facilitates covalent binding of enzymes. The amine groups on chitosan are much more reactive that the acetamide groups on chitin [16,19–22].

The present work describes the construction, performance and application of a biosensor for the determination of rutin in pharmaceutical formulations based on chitosan/carbon paste electrode modified with gilo crude extract. The crude extract of gilo was used as an enzymatic source of peroxidase. This enzyme catalyses the oxidation of rutin to quinone in the presence of hydrogen peroxide. The chitosan biopolymer was chemically crosslinked with epichlorohydrin and glutaraldehyde. The influence of different experimental parameters was evaluated to optimize this biosensor.

2. Experimental

2.1. Chemicals and other supplies

All aqueous solutions were prepared using water purified with a Milli-Q system (Millipore, Bedford, MA, USA) and all reagents were of analytical grade. Guaiacol was purchased from Aldrich and a 5.0×10^{-2} M stock solution was prepared daily in 0.1 M phosphate buffer solution at pH 7.0. Hydrogen peroxide was purchased from Merck and a 0.1 M stock solution was prepared in the same phosphate buffer solution. Rutin was purchased from Sigma and a 1.1×10^{-4} M stock solution was dissolved in 10% (v/v) ethanol and prepared in 0.1 M phosphate buffer solution at pH 7.0. Reference solutions from 3.4×10^{-7} to 7.2×10^{-6} M were prepared from the stock solution by appropriate dilutions with the same buffer solution. Chitosan with a degree of deacetylation of approximately 80% was obtained from Sigma and used as the support for peroxidase. Epichlorohydrin and glutaraldeyde used for the crosslinking of chitosan were obtained from Sigma. The carbon paste was prepared using Acheson 38 graphite powder from Fisher and mineral oil from Aldrich. Crude extract of gilo was used as a source of peroxidase. Healthy gilo (*Solanum gilo*) was purchased from a local producer in Florianópolis/SC, washed, hand-peeled, chopped, and cooled in a refrigerator at 4 °C. Two Brazilian pharmaceutical formulations containing rutin (Nova Rutina and Rutin Manipulada) were acquired from a local drug store in Florianópolis (Santa Catarina, Brazil) and analyzed using the proposed biosensor.

2.2. Apparatus

Square-wave and cyclic voltammetry experiments were performed using an Autolab model PGSTAT12 (Eco Chemie, The Netherlands). All experiments were carried out using a conventional three-electrode system with the enzyme electrode made in our laboratory being used as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl (3.0 M KCl) electrode as the reference electrode. Electrochemical experiments were performed in an electrochemical cell holding 15.0 ml of the supporting electrolyte. A Hewlett-Packard (Boise, ID, USA) Model 8452A UV–vis spectrophotometer with a quartz cell (optical path of 1.00 cm) was used for peroxidase activity, total protein determinations and comparative method. A Hitachi Model Himac CR 20B2 centrifuge was used to obtain the gilo (*S. gilo*) crude extract.

2.3. Obtention of the peroxidase, measurement of activity and total protein

The enzyme peroxidase was obtained from gilo tissue according to the following procedure: 25 g of the gilo pulp was homogenized in a liquefier with 100 ml of 0.1 M phosphate buffer solution (pH 7.0) for 1 min at 4 °C. The homogenate was rapidly filtered and centrifuged at 18,000 rpm for 5 min at 4 °C. The resulting supernatant was stored at this temperature in a refrigerator and used as the enzymatic source after the determination of the peroxidase activity and total protein [19,20,23–26].

Peroxidase activity was calculated as the increase in 0.001 unit of absorbance per min at 470 nm. The enzymatic activity of peroxidase present in the crude extract of gilo was determined in triplicate by measuring the absorbance at 470 nm of tetraguaicol produced by the reaction between 0.2 ml of supernatant solution, 2.7 ml of 0.05 M guaiacol solution and 0.1 ml of 10.0 mM hydrogen peroxide solution in 0.1 M phosphate buffer (pH 7.0) at 25 °C [19,20].

The total protein concentration of enzyme extract was determined in triplicate by the method of Folin using serum albumin as standard [27].

2.4. Chitosan crosslinking and immobilization of the peroxidase

The chitosan used as the solid support for peroxidase immobilization was chemically crosslinked with glutaraldehyde [19,20] and epichlorohydrin [28,29]. In order to prepare this an excess of 2.5% (v/v) glutaraldehyde solution made up in deionized water was transferred to a mass of 10.0 g chitosan powder, mechanically stirred for 30 min at room temperature and after this reaction time, the chitosan was washed with water to remove the excess of glutaraldehyde. Then, 30.0 ml of 3.7×10^{-4} M epichlorohydrin solution was added to the chitosan, heated at 50 °C for 1 h and 7.0 ml of 0.1 M sodium hydroxide solution was added and kept boiling for at least 1 h. The chitosan powder was washed with water, 0.1 M hydrochloric acid solution and 0.1 M sodium hydroxide solution, respectively, to remove the excess of epichlorohydrin. Finally, the chitosan powder was washed with water, phosphate buffer solution (pH 7.0) to remove the excess of crosslinking reagents and dried at room temperature.

The immobilization of the peroxidase was performed by adding aliquots from 30 to 400 μl of gilo crude extract solution containing 150–1950 units of peroxidase/mg protein to 0.100 g of the chemically crosslinked chitosan powder, and mixtures were gently stirred for 2 h at 25 °C. The chitosan containing immobilized enzyme was consecutively washed with 0.1 M phosphate buffer solution (pH 7.0) to remove unreacted enzyme, dried at room temperature and the solid obtained was used for construction of the biosensor.

2.5. Biosensor preparation and electrochemical measurement

The biosensor was prepared by mixing 0.100 g of chitosan containing 900 units of peroxidase/mg protein with 225 mg of graphite powder (75%, w/w) in a mortar for 20 min to ensure the uniform dispersion of the enzyme. Subsequently, 75 mg of Nujol (25%, w/w) was added and mixed for at least 20 min to produce the final paste. The resulting modified carbon paste was placed in a 1 ml plastic syringe and a silver wire was inserted to obtain the external electric contact [19,20]. The biosensor was stored in a refrigerator at 4 °C when not in use.

Square-wave and cyclic voltammetry measurements were performed in an unstirred and not de-aerated phosphate buffer solution (pH 7.0) containing 2.0×10^{-3} M hydrogen peroxide, at 25.0 ± 0.2 °C. The square-wave voltammetry measurements were performed by applying a sweep potential between +350 and −100 mV, at a pulse height from 5 to 100 mV and a frequency from 5 to 100 Hz after successive additions of rutin or sample solutions. All potentials were measured and reported versus Ag/AgCl (3.0 M KCl), after a suitable initial stirring time of 60 s in 0.1 M phosphate buffer solution (pH 7.0).

2.6. Preparation of pharmaceutical sample and application

The contents of 10 tablets were mixed well; from the powder an accurately weighed portion was taken, dissolved in 10% (v/v) ethanol and diluted to volume with phosphate buffer (pH 7.0, 0.1 M at 25 °C). When necessary, these solutions were sonicated for 5 min in an ultrasonic bath, filtered through a filter paper and made up to the mark with phosphate buffer.

All experiments were performed in a 15.0 ml thermostatically controlled glass cell at 25 ± 0.2 °C in 0.1 M phosphate buffer solution (pH 7.0). An accurate volume of 1.0 ml of rutin in pharmaceutical formulations containing 2.0×10^{-3} M hydrogen peroxide in 0.1 M phosphate buffer solution (pH 7.0) was kept and quantified after successive additions of reference rutin solution. After each addition, square-wave voltammograms were recorded by scanning the potential from +350 to −100 mV, at a pulse height of 30 mV and a frequency of 25 Hz.

A spectrophotometric method for the determination of rutin available in the Official Methods of Analysis [30] was used to compare the obtained analytical results with the proposed biosensor.

3. Results and discussion

3.1. Chemically crosslinked chitosan and peroxidase immobilization

The properties of both the enzyme and the support material govern the efficiency and properties of immobilized enzyme. Chitosan is known as an ideal support material for enzyme immobilization because of numerous characteristics including hydrophilicity, biocompatibility, biodegradability and antibacterial property. In addition, this biopolymer is susceptible to chemical modification due to the amine and hydroxyl groups that facilitate the covalent binding of enzyme [16,19,20].

Fig. 1 shows a suggestion for the immobilization of peroxidase on (a) chitosan (CTS) by crosslinking with (b) glutaraldehyde and (c) epichlorohydrin. The bifunctional glutaraldehyde reacts with the amine groups of chitosan to form Schiff bases, and with the addition of the epichlorohydrin, through the opening of the epoxide ring links are formed with carbon atoms and the chloride group is discharged. In a recent study realized by our group we reported that an excess of glutaraldehyde solution crosslinked 100% of the aldehyde groups of glutaraldehyde with the amino groups of chitosan [20]. The chemical crosslinking of chitosan with glutaraldehyde/epichlorohydrin and (d) peroxidase immobilization shows strong interactions, long-term stability, thermostability and high sensitivity compared with other recently constructed biosensors [19,20]. The high performance and strong interactions in this process of immobilization suggest that the enzyme peroxidase can be trapped within the interstitial space of the crosslinked substance and also covalently immobilized on this biopolymer (e).

Peroxidase is widely distributed in nature and we have developed several biosensors using crude extract and tissue of various vegetables [19,20,23–26]. The use of such biological materials is very attractive because of their high stability, high enzyme

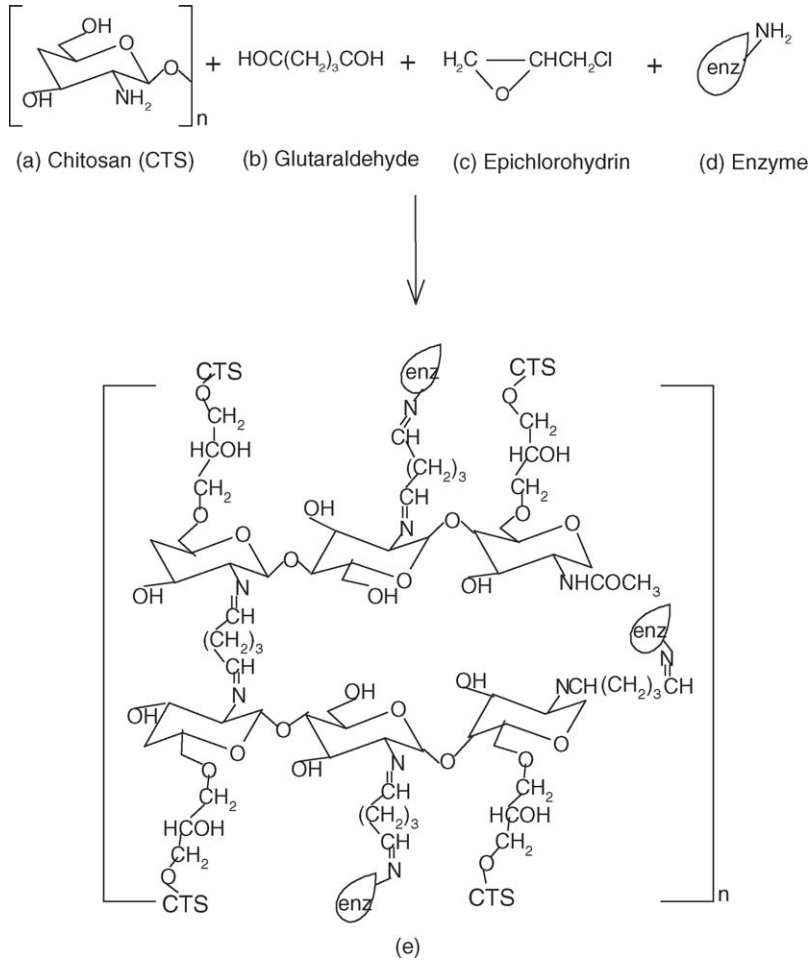

Fig. 1. Reaction between chitosan, epichlorohydrin, glutaraldehyde and peroxidase enzyme.

activity concentration, very low cost and fewer cofactor requirements in comparison with the pure enzymes. Fig. 2 shows a scheme of the enzymatic process among rutin, hydrogen peroxide and peroxidase of the crude extract incorporated into the chitosan and carbon paste electrode.

The electrochemical catalytic reduction of rutin by biosensor was examined by cyclic voltammetry. This enzyme in the presence of hydrogen peroxide catalyses the oxidation of rutin to the corresponding *o*-quinone from which electrochemical reduction back to rutin occurs at a potential of +124 mV as illustrated in Fig. 3. In this study, cyclic voltammetric measurement was performed by scanning the potential between +450 and −150 mV versus Ag/AgCl at a scan rate of 100 mV s^{-1} in an unstirred phosphate buffer solution (pH 7.0) containing (a) 2.0×10^{-3} M

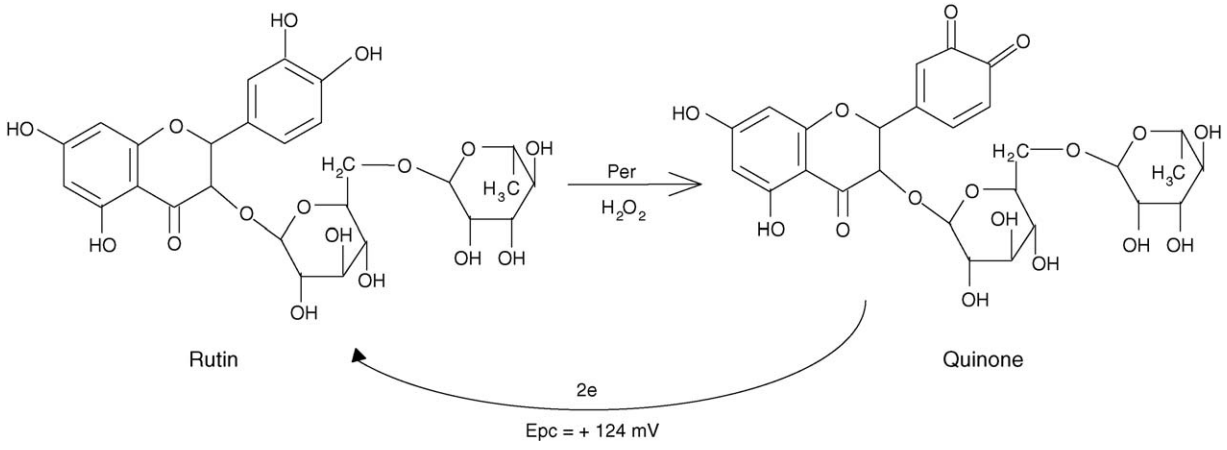

Fig. 2. Schematic representation of the enzymatic process among rutin, hydrogen peroxide and peroxidase.

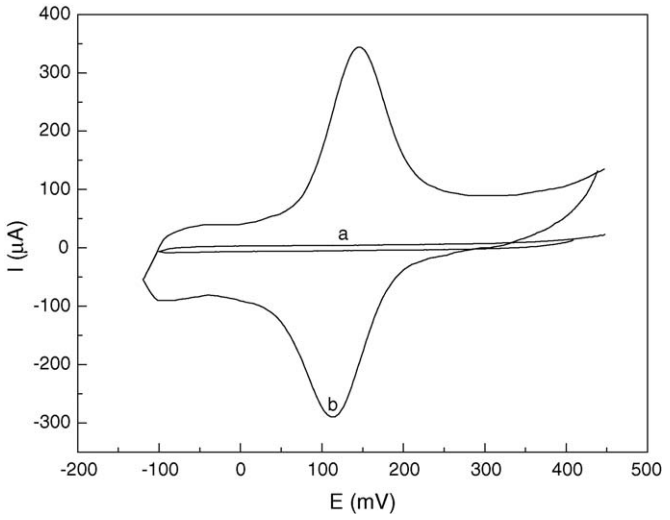

Fig. 3. Cyclic voltammograms obtained using a proposed biosensor in 0.1 phosphate buffer solutions (pH 7.0) for: (a) 2.0×10^{-3} M hydrogen peroxide and (b) 3.9×10^{-6} M rutin and 2.0×10^{-3} M hydrogen peroxide solution. Scan rate of 100 mV s^{-1}.

hydrogen peroxide and (b) 3.9×10^{-6} M rutin and 2.0×10^{-3} M hydrogen peroxide solution.

3.2. Optimization of the biosensor

To optimize the biosensor, various experimental parameters were investigated. In previous studies realized by our group [19,20,23–26] the best carbon paste composition was found using 75:25% (w/w) graphite powder:Nujol. Therefore, this carbon paste composition was used in the present investigation in the construction of the biosensor using the chemically crosslinked chitosan and peroxidase enzyme.

The effect of varying the enzyme concentration from 150 to 1950 units peroxidase/mg protein in the 75:25% (w/w) carbon paste on the biosensor response was studied. The analytical signals (cathodic peak currents) for 3.9×10^{-6} M rutin solution and 2.0×10^{-3} M hydrogen peroxide solution increased with the enzyme concentration up to 900 units peroxidase/mg protein (3.0 units peroxidase/mg carbon paste). This was then used for the construction of further biosensors.

The effect of the pH of the buffer solution on the biosensor response was investigated between 5.0 and 9.0 in 3.9×10^{-6} M rutin in the presence of 2.0×10^{-3} M hydrogen peroxide solution. The current response increased from pH 5.0 to 7.0, and achieved a maximum value between 6.0 and 7.0, before decreasing from 8.0 to 9.0, which was in agreement with results reported for the optimum enzymatic activity of the peroxidase [15,19,20]. Therefore, a pH of 7.0 was used in further experiments.

The effect of varying the frequency from 5 to 100 Hz on the biosensor response to 3.9×10^{-6} M rutin and 2.0×10^{-3} M hydrogen peroxide solutions was studied. The highest analytical signal was obtained at 25 Hz. After that, the pulse height was studied ranging from 5 to 100 mV. The highest analytical signal was obtained at 30 mV for 3.9×10^{-6} M rutin solution.

Table 1
Optimization of biosensor parameters

Biosensor parameter	Range studied	Optimal value
Enzyme concentration[a]	150–1950	900
Enzyme/carbon paste[b]	0.50–6.5	3.0
pH	5.0–9.0	7.0
Frequency (Hz)	5–100	25
Pulse height (mV)	5–100	30
E_{pc} (mV)	+350 to −100	+124

[a] Units peroxidase/mg protein.
[b] Units peroxidase/mg carbon paste.

Thus, these experimental conditions were selected for further experiments.

Table 1 summarizes the range over which each variable was investigated and the optimum value found in the optimization of this biosensor.

3.3. Repeatability, reproducibility and stability

The repeatability of the current response for the proposed biosensor in solutions containing 3.4×10^{-7} to 7.2×10^{-6} M rutin and 2.0×10^{-3} M hydrogen peroxide solution was investigated. The relative standard deviation (R.S.D.) was less than 1.0% for 10 successive assays.

The biosensor-to-biosensor reproducibility was determined from the response in solutions containing 3.4×10^{-7}, 1.1×10^{-6}, 2.2×10^{-6}, 3.3×10^{-6}, 4.3×10^{-6}, 5.2×10^{-6}, 6.3×10^{-6} and 7.2×10^{-6} M rutin and 2.0×10^{-3} M hydrogen peroxide solution at five different enzyme electrodes. These yielded a 2.2% R.S.D. in the analytical curve slopes. The good reproducibility may be due to the high sensitivity and efficiency of the immobilization of the peroxidase on the chitosan biopolymer.

Long-term stability is considered to be one of the key factors in biosensor performance. The stability of the biosensor was tested by repeated measurements without surface renewal over a 300-day period. When the biosensor was stored at 4 °C and measured every 2–4 days, no obvious change was found in the response to 3.4×10^{-7} to 7.2×10^{-6} M rutin and 2.0×10^{-3} M hydrogen peroxide solution. The good long-term stability could be attributed to the fact that there were strong interactions between the peroxidase-gilo crude extract, which could be firmly immobilized on the crosslinked chitosan and gave the biosensor better stability and performance.

3.4. Selectivity and recovery study

The selectivity of the proposed biosensor was assessed by studying the effect of common excipients used in pharmaceutical preparations on the determination of rutin. The substances usually found in pharmaceuticals such as sodium chloride, starch, poly(ethylene glycol), magnesium stearate, lactose, and sucrose were investigated. The ratios of the concentration of rutin to those of the excipient substances were fixed at 0.1, 1.0 and 10.0. None of these substances interfered with the proposed procedure.

Table 2
Results of recoveries of rutin standard solution in pharmaceuticals using the proposed biosensor

Sample	Rutin (mg l^{-1})		Recovery (%)
	Added	Found	
A	0.63	0.64 ± 0.03	101.6
	1.25	1.28 ± 0.04	102.4
	1.86	1.79 ± 0.02	96.2
B	0.63	0.61 ± 0.03	96.8
	1.25	1.22 ± 0.05	97.6
	1.86	1.80 ± 0.03	96.8

A recovery study was performed using two pharmaceutical formulations samples with three different standard concentrations: 0.63, 1.25 and 1.86 mg l^{-1} of rutin and the results obtained were compared with those added. The results obtained in the addition-recovery experiments are summarized in Table 2 and show that average recovery varied from 96.2 to 102.4%, demonstrating the accuracy of the developed proposed biosensor.

3.5. Analytical performance and application to commercial pharmaceutical

The proposed biosensor characteristics were thoroughly studied and the results are summarized in Table 1. Precision and accuracy were also evaluated to guarantee the reliability of this biosensor. Results obtained from calibration plots (Fig. 4) showed an excellent correlation coefficient in the linear analytical curve for rutin concentrations from 3.4×10^{-7} to 7.2×10^{-6} M ($I_{pc} = 30.73 + 4.869 \times 10^{7}$ [rutin]; $r = 0.9998$), where I_{pc} is the cathodic peak current in nA and [rutin] is the rutin concentration in M with a detection limit (three times the signal blank/slope) of 2.0×10^{-8} M rutin and quantification limit (10 times the signal blank/slope) of 6.3×10^{-8} M rutin.

Table 3
Determination of rutin in pharmaceutical formulations using the official method [30] and biosensor proposed

Sample	Rutin (mg)			Relative error (R.E.) (%)	
	Label	Official method	Biosensor	R.E.$_1$	R.E.$_2$
A	20.0	20.6 ± 0.06	19.7 ± 0.08	−4.4	−1.5
B	20.0	19.8 ± 0.08	20.1 ± 0.04	+1.5	+0.5

$n = 6$; confidence level of 95%. R.E.$_1$ = biosensor vs. official method value; R.E.$_2$ = biosensor vs. label value.

In order to evaluate the applicability of the proposed biosensor, (A) Nova Rutina and (B) Rutin Manipulada in its pharmaceutical formulations were analyzed. The results of the analysis using the biosensor were compared with those obtained using the standard spectrophotometric method, AOAC Method [30] and label values (Table 3). A *t*-test (95% confidence level) performed on these data showed that there was no significant difference between the results obtained with the two methods and with the label values.

Several techniques have been proposed for determination of rutin [6–14]. Some of these methods involve interferences, use of complex procedures with several sample manipulation, require long analysis time and use of expensive reagents. The spectrophotometric method proposed by Official Methods of Analysis [30] for the determination of this antioxidant is based on the measurement at 352.5 nm. Nevertheless, the excipients frequently found in some pharmaceutical formulations have strong absorption in the UV region. In addition, the biosensor proposed in this work using of gilo as biocatalyst offers several advantages. The gilo is rich in peroxidase and the enzyme from this

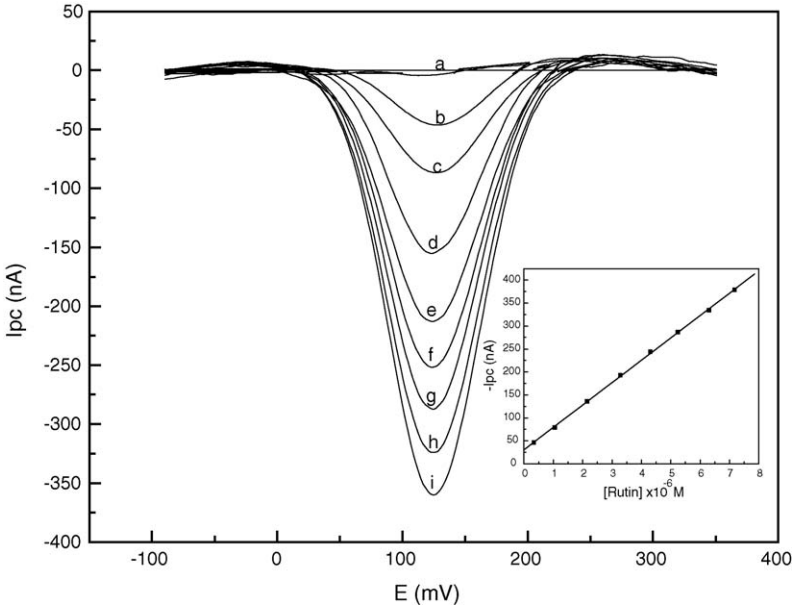

Fig. 4. Square-wave voltammograms obtained using the biosensor for (a) blank in phosphate buffer solution and rutin solutions at the following concentrations: (b) 3.4×10^{-7} M; (c) 1.1×10^{-6} M; (d) 2.2×10^{-6} M; (e) 3.3×10^{-6} M; (f) 4.3×10^{-6} M; (g) 5.2×10^{-6} M; (h) 6.3×10^{-6} M; (i) 7.2×10^{-6} M, at 30 mV pulse height and frequency of 25 Hz. Inset: the calibration curve of the biosensor.

vegetal is not yet commercially available, having high stability, low cost and fewer cofactor requirements. This enzyme electrode, combine the enzyme selectivity with the high sensitivity, stability and rapid response time, compared with the procedures described in the literature for determination the rutin [6–14].

4. Conclusions

In this paper we have introduced a novel procedure to construct a biosensor. The peroxidase obtained from the gilo tissue was successfully immobilized on the chitosan biopolymer and the performance was determined. The strong interactions between enzyme and crosslinked biopolymer can be firmly immobilized on the biosensor. The bioelectrode offers favourable analytical features for use as a biosensor including high sensitivity, good reproducibility, long-term stability, low detection limit and rapid response. This approach provides a simple and low cost procedure to develop a new class of electrochemical biosensor for rutin.

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