Biosensors and Bioelectronics 25 (2010) 1143–1148

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Amperometric detection of benzoyl peroxide in pharmaceutical preparations using carbon paste electrodes with *peroxidases* naturally immobilized on coconut fibers☆

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ARTICLE INFO

Article history:
Received 11 August 2009
Received in revised form
16 September 2009
Accepted 30 September 2009
Available online 9 October 2009

Keywords:
Plant-tissue based biosensor
Amperometry
Peroxidase
Benzoyl peroxide

ABSTRACT

This paper describes the applications of a new carbon paste electrode containing fibers of coconut (*Cocus nucifera* L.) fruit, which are very rich in peroxidase enzymes naturally immobilized on its structure. The new sensor was applied for the amperometric quantification of benzoyl peroxide in facial creams and dermatological shampoos. The amperometric measurements were performed in 0.1 mol L^{-1} phosphate buffer (pH 5.2), at 0.0 V (*versus* Ag/AgCl). On these conditions, benzoyl peroxide was rapidly determined in the 5.0–55 μmol L^{-1}, with a detection limit of 2.5 μmol L^{-1} (s/n = 3), response time of 4.1 s (90% of the steady state) and sensitivity limit of 0.33 A mol L^{-1} cm^{-2}. The amperometric results are in good agreement with those obtained by spectrophotometric technique, used as a standard method.

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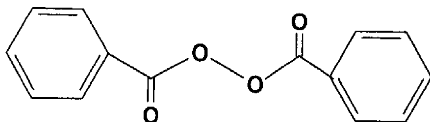
1. Introduction

Benzoyl peroxide (BZP) is widely employed as a polymerization initiator in the plastic and rubber industries, oxidizing agent in the food industry, bleaching agent for cheese, fats and oils fabrication or as an additive in flours. In pharmaceutical formulations, it acts as an antibacterial agent against *Propionibacterium acnes* in the skin, principally due its oxidative strength action. In the last case, BZP is absorbed into the skin, where is metabolized to benzoic acid and, subsequently, excreted as benzoate in the urine (Yeung et al., 1983).



Despite the large number of applications involving BZP, it was reported that this substance could assist the production of free radicals derivatives, promoting the growth and the development of tumors in mouse skin (Slaga et al., 1981; O'Connell et al., 1986). The metabolism of BZP in mouse keratinocytes occurs via homolytic cleavage of the peroxide bond, to produce benzoyloxyl free radicals, which can result in the production of the phenyl radicals and carbon dioxide, or abstraction of hydrogen atoms from biomolecules causing for instance DNA damage, lipid peroxidation and thiol oxidation (Kensler et al., 1988). A subsequent study suggested that BZP is activated to DNA-damaging intermediates via a copper-catalyzed cleavage of the peroxide bond (Swauger et al., 1991).

The reported methods for BZP determination include chromatographic techniques (Dehouck et al., 2003; Smith and Sherma, 1995; Chou and Locke, 1984; Gaddipati et al., 1983), and chemiluminescence techniques (Bowyer and Spurlin, 1987), which require complex separation and derivation steps involving the use of expensive equipments. The analytical methodology can be simplified by using electrochemical methods and carbon paste electrodes modified with (2,2′,6,2″-terpyridyl) copper (II) chloride complex [Cu(terpy)Cl]Cl.2H_2O] (Sotomayor et al., 2003) or with Os(byp)$_2$ClPyCH$_2$NHpoly(allylamine) polymer and *peroxidase* (Baldini et al., 2002), or alternatively Os(byp)$_2$ClPyCH$_2$NHpoly(allylamine) polymer and Fe-protoporphyrin IX (Turner et al., 1987).

The utilization of plant or mammalian tissues (Turner et al., 1987; Wijesuriya and Rechnitz, 1993; Lima et al., 1997; Lima et al., 1998; Lupetti et al., 2006; Fernandes et al., 2007) provides numerous advantages for development of biosensors such as, simultaneous presence of coenzymes and activators components, high enzymatic stability and activity, all at a more attractive price as compared with the commercial lyophilized enzymes.

☆ This work was part of the PhD of João Vitor Bueno Kozan, which was in progress in our lab. He died two days before his 27th birthday. We will ever remember his easy smile and his nice way to see the life.

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[1] In memorian.

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doi:10.1016/j.bios.2009.09.044

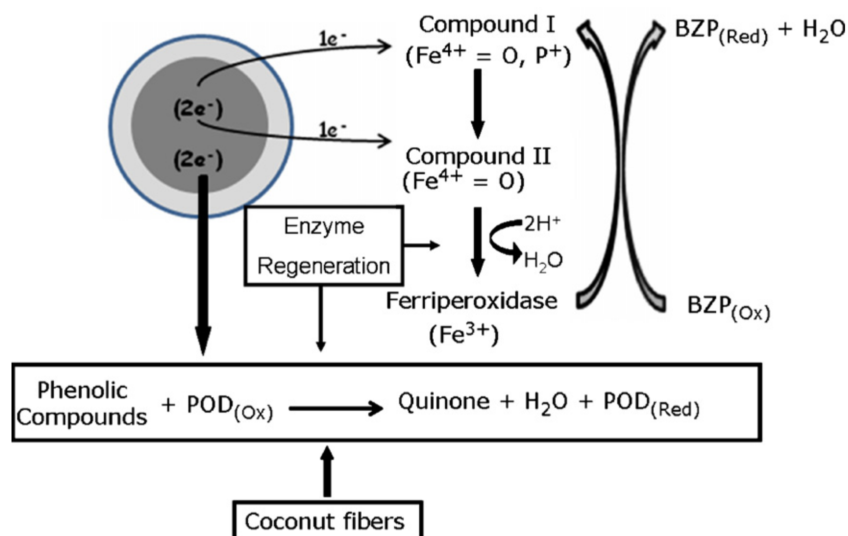
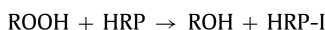


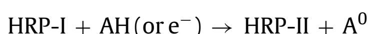
Fig. 1. Possible enzymatic and electrochemical coupled reactions at the biosensor surface during the BZP detection—schematic diagram.

Horseradish *peroxidase* (HRP) is known to catalyze the reduction of hydrogen peroxide and certain organic peroxides according to the following reaction (Maely, 1955; Everse et al., 1991):



$$\text{ROOH} + \text{HRP} \rightarrow \text{ROH} + \text{HRP-I}$$

Subsequent reduction of HRP-I to HRP occurs through two separated steps (Maely, 1955; Everse et al., 1991; Yamada and Yamazaki, 1974) generating two one-electron transfer processes, which can be found via mediated electron transport using AH molecule, or by direct heterogeneous electron transfer between the electrode and the hemin group of the HRP (Yao et al., 1984; Sanchez et al., 1990; Schubert et al., 1991; Garguilo et al., 1993; Ohara et al., 1993; Wang et al., 1993). In the last case, it is necessary the occurrence of an intimate contact between the enzyme and the conducting surface:



$$\text{HRP-I} + \text{AH}\,(\text{or } e^-) \rightarrow \text{HRP-II} + A^0$$

$$\text{HRP-II} + \text{AH}\,(\text{or } e^-) \rightarrow \text{HRP} + A^0$$

Generally, *peroxidase* reacts quickly with alkyl peroxides (Anni and Yonetani, 1992), however it seems that the enzyme requires the presence of free hydroperoxy moieties (–OOH).

In this paper naturally occurring POD, firmly immobilized in tissue derived from coconut fruits (Kozan et al., 2007), was used to develop carbon paste based biosensors which were employed to determine BZP in cosmetics (facial creams and dermatological shampoos) matrixes. The crude coconut tissue is rich in phenolic substrates (Lima et al., 1997), which are able to improve the rate of electron transfer between the electrode and the immobilized enzyme (Rosatto et al., 2002). The enzyme, immobilized on the electrode surface, reacts with peroxide to produce the enzyme oxidized form, which is further regenerated to the native form by direct heterogeneous electron transfer or via phenolic compounds mediation. In the last case phenolic derivatives, naturally present in the coconut fibbers, are electrochemically converted to the respective quinones on the biosensor surface, producing a reduction current which is proportional to the peroxide concentration in solution (Fig. 1).

BZP amperometric signal is attributed to the reduction of oxidized *peroxidase* admixed in a carbon paste together with the *peroxidase*, which is in direct contact with the graphite particles in the interface electrode/solution. In the catalytic cycle, BZP acts as an electron acceptor while phenol as an electron donor.

2. Materials and methods

2.1. Apparatus

Amperometric experiments were performed using a μ-Autolab Type-III potentiostat (EcoChemie, Utrecht, Netherlands) controlled by a PC using the GPES 4.9.005β software. Amperometric experiments were done under stirring (450 rpm) in the presence of dissolved oxygen. All electrochemical experiments were carried out in a 5.0 mL glass cell thermostated at room temperature (23 ± 2 °C). The three electrodes system consisted of a biosensor (composed of mineral oil, carbon powder and coconut fiber tissue), Ag/AgCl, KCl 3.5 mol L^{-1} and platinum wire as working, reference and auxiliary electrodes, respectively.

A spectrophotometer (Fenton model 600 Plus) was utilized to perform the analysis of BZP using the well-established spectrophotometric method described in the reference (Bergmayer, 1974).

2.2. Reagents and solutions

All reagents were of analytical grade (purchased from Sigma or Merck) and utilized as received. Peroxidase enzymes, extracted from horseradish EC 1.11.1.7 (170 I.U. mg^{-1}) was supplied by Merck (São Paulo, SP, Brazil). All solutions were prepared with water purified from a Millipore (Bedford, MA, USA) Milli-Q ultra pure system (resistivity ≥ 18 MΩ cm). The supporting electrolyte used was a 0.1 mol L^{-1} phosphate buffer solution (PBS), pH 5.2. The standard solution of benzoyl peroxide (typically 1.0×10^{-2} mol L^{-1}) was prepared daily. The appropriate amount of BZP was dissolved in 10.0 mL of acetone/ethanol in the ratio of 1:4 (v/v). This mixture was homogenized by shaking in the vortex mini-shaker at 2200 rpm during 1.0 min and then, sonicated in an ultrasound bath. The same procedure was adopted to dissolve the samples of cream and dermatological shampoos. From these solutions, an adequate volume was transferred to another volumetric flask and the volume completed with phosphate buffer.

The carbon paste biosensor was prepared using graphite powder (Acheson 38, Fischer), mineral oil (Aldrich) and coconut tissue

Table 1
Amperometric and spectrophotometric determination of benzoyl peroxide (BZP) in cosmetic samples.

Sample	Presentation/% active substance	Nominal value[a] ($mol\,L^{-1}$)	Amperometry ± SD[c] ($mol\,L^{-1}$)	Spectrophotometry ± SD[c] ($mol\,L^{-1}$)	Δ^{d} (%)	Δ^{e} (%)	Δ^{f} (%)
1	Cream/5.0% BZP	0.206	0.200 ± 0.008	0.194 ± 0.004	(−) 2.9	(−) 5.8	(+) 3.0
2	Dermatological shampoo/2.5% BZP[b]	0.103	0.102 ± 0.003	0.0920 ± 0.001	(−) 1.0	(−)10	(+) 9.8
3	Dermatological shampoo/2.5% BZP[b]	0.103	0.0960 ± 0.006	–	(−) 6.8	–	–

(−) Values not obtained.
[a] Label value, presented by the manufacturer.
[b] Products of different producers.
[c] Average ± standard deviation for three determinations using the proposed biosensor and the spectrophotometric method, respectively.
[d] Relative deviation between the nominal and amperommetric values (%).
[e] Relative deviation between the nominal and spectrophotometric values (%).
[f] Relative deviation between amperometric and spectrophotometric methods (%).

fibers previously treated, as described below. The pharmaceutical formulations containing BZP were obtained from a local pharmacy.

2.3. Preparation of coconut tissue

Healthy green coconut fruits purchased from a local grocery store in São Paulo, Brazil, were used as the source of the biocatalytic material. The mesocarp tissue, located between the green thin skin and the woody shell (representing about 35% of the coconut weight) contains POD naturally immobilized and was used as an enzymatic source. This fibrous tissue (usually called "husk") was finely chopped with a stainless knife on a polycarbonate plate, washed in acetonitrile and dried in air, protected by a filter paper. After drying, the fibers were passed in sieves and the particles fraction in the range of 0.119 mm < particle < 0.250 mm were selected to construct the biosensors. The selected fibers were stored in closed glass flasks and maintained at room temperature.

2.4. Construction of carbon paste electrodes

The CPE without the enzyme was prepared via the homogenization of graphite powder (67%) and mineral oil (33%) in a mortar for 10 min. The preparation of the biosensor involved mixing the graphite powder (62%, w/w) with coconut tissue fiber powder (5.0%, w/w). After homogenization of the resulting admixture, mineral oil (33%, w/w) was added, so that the resulting paste was thoroughly well mixed (Kozan et al., 2007).

The biosensor was built by inserting carbon paste containing coconut fibers into a glass tube (θ = 3.0 mm). To establish the electrical contact, a copper rod (θ = 2.9 mm) had been introduced from the opposite side in the glass tube and fixed near of its end, producing a cavity with ~2 mm depth, where the active material was packed. The electrode surface (0.07 cm^2) was smoothed using a weighing paper until obtain a smooth finishing before use.

To renew the electrode surface, a layer of carbon paste was removed from its extremity, a new active material was applied and the surface was smoothed again on weighing paper. This procedure was repeated for each new series of experiments. For comparison, carbon paste without coconut fibers was prepared and used at the same way.

2.5. Preparation of pharmaceutical samples

Gel and liquid pharmaceutical formulations containing BZP were purchased from a local pharmacy. A precisely known amount of each formulation was diluted (under stirring) in 7.0 mL of acetone/ethanol (1:4, v/v). After the dissolution, the volume was adjusted to 10 mL in a volumetric flask using the same mixture of solvents. No other treatment of the sample was required. To perform the quantification of BZP, a small volume of this solution was diluted in phosphate buffer and than the analysis was undertaken. Due the high viscosity of the facial cream, an adequate but known mass was weighed to prepare more dilute solutions, rather than employ volumes. The weighed samples were 500 times diluted and the dilutions were taken in account to calculate the final values presented in Table 1.

2.6. Spectrophotometric measurements

To build a spectrophotometric analytical curve, 100 mL of a solution containing the following reagents in 0.1 $mol\,L^{-1}$ phosphate buffer, pH 5.2 was prepared: 0.3 $g\,L^{-1}$ 4-aminoantipirine, 1.5 $g\,L^{-1}$ phenol, and 150 I.U. L^{-1} *peroxidase*. Aliquots of 5.0 mL of this solution were transferred to small flasks and, to each one, enough BZP to attain a final concentration ranging from 1.0×10^{-5} to 8.0×10^{-5} $mol\,L^{-1}$ was added, utilizing a stock solution containing 1.0×10^{-2} $mol\,L^{-1}$ BZP. All solutions were homogenized and incubated for 30 min (at 23 ± 2 °C) after which absorbance measurements were undertaken. An aliquot of the solution without BZP was employed as a control. 4-Aminoantipirine reacts with phenol and BZP in the presence of POD, to form a dye structure of quinoneimine, which adsorbs greatly at 500 nm. The absorbance measured is proportional of the quantity of dye produced, and consequently to the BZP concentration in the solution (Bergmayer, 1974). Analogous procedures were adopted for the spectrophotometric quantification of BZP in facial creams and dermatological shampoos.

2.7. Amperometric measurements

Amperometric measurements were carried out under stirring, in a cell containing 5.0 mL of PB solution, using a fixed potential at 0.0 V (*versus* Ag/AgCl, KCl 3.5 $mol\,L^{-1}$). After stabilization of the baseline signal (typically, in less than 3 min), successive additions of 1.0×10^{-2} $mol\,L^{-1}$ BZP standard solution aliquots were done. Experiments done with electrodes containing any coconut tissue resulted in the absence of current. When the electrodes containing coconut tissues were utilized, after each injection, the current increased rapidly, attaining a new steady state condition. Same procedures were employed for both the facial cream and dermatological shampoo analyses. For each sample, experiments were executed in triplicate.

Recovery tests were also undertaken. Approximately 0.1000 g of each sample was weighed and diluted in acetone/ethanol (in volumetric flasks of 10 mL). Each solution was well homogenized to ensure the complete dissolution of the samples (cream or dermatological shampoos). To perform the measurements, an adequate aliquot of sample was added to the electrochemical cell containing PBS, pH 5.2 and a final concentration of 10 or 20 $\mu mol\,L^{-1}$ were obtained (see Table 2).

Table 2
Standard addition method for amperommetric recovery of benzoyl peroxide (BZP) concentrations in cosmetic samples.

Pharmaceutical samples	Sample concentration (μmol L^{-1})	[BZP] sample + [BZP] added (μmol L^{-1})	[BZP] total Amperommetry (μmol L^{-1})	% Recovery obtained
1 (Cream)	20.0	40.0	39.0	97.5
2 (Dermatological shampoo)	10.0	30.0	28.8	96.0
3 (Dermatological shampoo)	10.0	30.0	28.1	93.7

3. Results and discussion

3.1. Optimization of experimental conditions

The initial problem was choosing a solvent (or solvent mixtures) in which BZP could be solved, since its solubility in phosphate buffer is very low. Several pure solvents and also mixture of solvents were evaluated. It was found that acetone + ethanol (in the proportion 1:4, v/v), was the best mixture to dissolve the analyte and also the pharmaceutical samples. Standard solutions of BZP as well as real samples were prepared in relatively high concentrations, so small volumes were necessary to prepare diluted solutions in phosphate buffer, reducing drastically the percentage of organic solvents to less than 0.5%. In these conditions no influence was observed on the analytical parameters.

Since that an optimum electrochemical reduction potential for the biocatalytically generated *peroxidase* had been obtained previously, using hydrogen peroxide as analyte guide (Kozan et al., 2007), identical protocols were used for BZP determination. Measurements at fixed potentials, ranging from −0.20 to +0.10 V (*versus* Ag/AgCl, KCl 3.5 mol L^{-1}), were undertaken and the best signals were obtained at 0.0 V (Fig. 2). This potential is different from that previously reported (−0.15 V) and the factors for this discrepancy include the molecular size of both analytes and the significant difference of solubility in water and in the mineral oil (which constitutes the carbon paste) of both analytes.

Amperometric current levels, obtained in the presence and absence of the oxygen were similar, probably because the fibrous mesocarp of the coconut fruit contains significant amounts of phenolic compounds, which can mediate the regeneration of the reduced form of the enzyme, with production of quinone derivatives, further reduced on the biosensor surface to establish the linear relation between reduction current and BZP concentrations in the measuring solutions. Direct reduction of the enzyme on the electrode surface cannot be discarded.

Quinone derivatives could be also formed by action of the *polyphenoloxidase* enzyme, simultaneously present in the vegetal tissue (Lima et al., 1997), or by action of the oxygen solved in the solution. The last two possibilities were not considered since the experimental reduction currents were proportional to the BZP concentrations and the presence of oxygen did not modify the current levels.

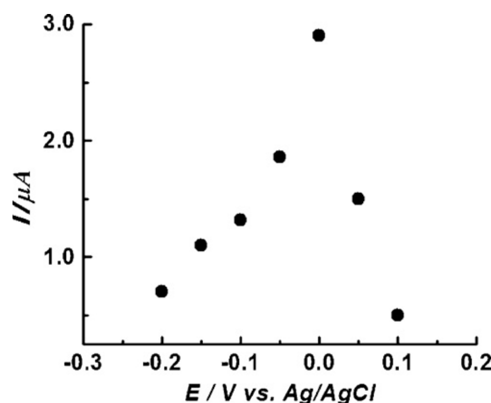


Fig. 2. Amperometric current recorded at the biosensor in 2.0×10^{-4} mol L^{-1} benzoyl peroxide in PBS, pH 5.2 as function of the applied potential. I (μA) correspond to the difference between the cathodic currents, recorded in the presence and absence of the analyte.

3.2. Long-term stability studies

Peroxidase–carbon paste electrodes prepared as described before exhibit very stable responses. The long-term stability was evaluated via repeating measurements over time. A series of 20 experiments (data not showed) utilizing 25 μmol L^{-1} BZP indicated very reproducible amperometric signals, attesting the long-term stability of the biosensor, an important aspect for prolonged operations. Such experiments were typically undertaken without the prior renewal of the electrode surface; the carbon paste biosensor was merely rinsed with pure water between each measurement series. The responses afforded a mean current value of 0.59 μA, with the signals changing in the interval of 0.58–0.61 μA with a relative standard deviation of 1.5% over the complete series.

To evaluate the stability of the enzymes inside the carbon paste for longer periods, a larger amount of components (5 g) were admixed. This carbon paste was kept at room temperature and used during three months, to prepare new sensors. The signals have decreased only of 8% and 21% after one and three months, respectively, attesting the long-term stability of the enzymes naturally immobilized on the vegetal tissue.

3.3. Analytical characteristics of the biosensor

Fig. 3a shows a typical chronoamperometric curve for injections of BZP in phosphate buffer solution, under stirring conditions. The chronoamperometric response is observed to be fast, indicating that BZP reduction at the modified electrode is rapid and the steady state current is obtained almost instantaneously. The response time ($t_{90\%}$) was 4.1 s and the sensitivity, calculated for this series of measurements, from 5.0 to 55 μmol L^{-1} (I (μA) = $2.92 \times 10^{-2} + 2.35 \times 10^{-2}$ [BZP] μmol L^{-1}), was 0.33 A mol^{-1} L cm^{-2} with an estimated detection limit of 2.5 μmol L^{-1} (Fig. 3b). When the same experiment was carried using carbon paste electrodes without coconut fibers, any signal was recorded, attesting that the coconut fibers play a central role in this electrode process.

The high current density was attributed to the efficient transport of BZP to the electrode as consequence of the intense solution stirring associated to the fast enzymatic reaction.

Fig. 4 shows the reciprocal plot of $1/I_{ss}$ *versus* $1/C$. The apparent Michaelis constant, 0.13 mmol L^{-1}, calculated from the slope of the Lineweaver–Burk plot, is in good agreement with value reported in the literature (Kamin and Wilson, 1980), demonstrating that the *peroxidase* immobilized on the coconut fibers possesses a high enzymatic activity and high affinity for BZP. There is very little literature data concerning the kinetic behavior of peroxidase enzymes in the biotransformation of BZP. It is probably that the solubility of the organic peroxide (BZP) in the mineral oil (one of the components of the carbon paste electrode) plays an important role in the biosensor, since BZP specie is more effectively bio-transformed than hydrogen peroxide (Kozan et al., 2007).

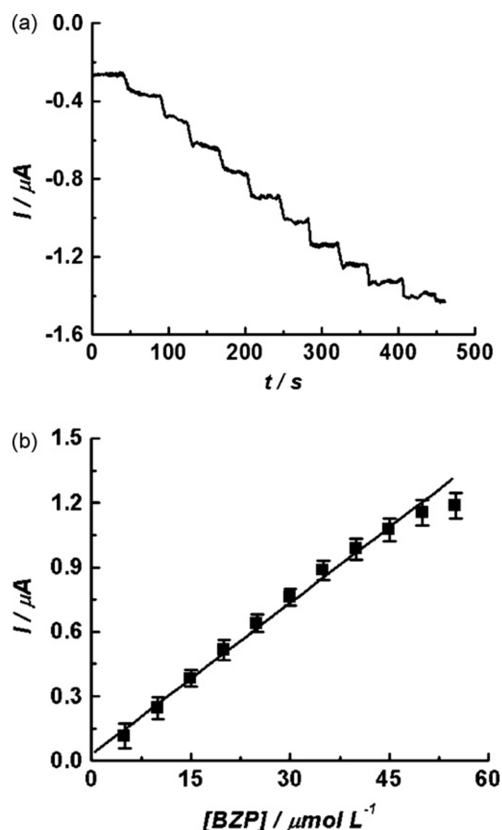


Fig. 3. (a) Amperometric current–time profile obtained during successive additions of 5.0 μmol L^{-1} benzoyl peroxide to the electrochemical cell containing 0.1 mol L^{-1} phosphate buffer solution. Experimental conditions: applied potential: 0.0 V *vs.* Ag/AgCl$_{(KCl\ 3.5\ mol\ L^{-1})}$ under constant stirring. (b) Analytical curve: [I (μA) = $2.9 \times 10^{-2} + 2.4 \times 10^{-2}$ [BZP] (μmol L^{-1})]. Data related to (a).

3.4. Real samples analyses

The developed biosensor was exploited for the quantification of BZP in cosmetic formulations (gel and liquid) products by using both, a direct and standard addition procedure. To assess the amperometric results with another analytical methodology, spectrophotometric determinations of BZP were simultaneously undertaken for these samples.

Table 1 shows the results obtained for the BZP determination in real samples using the amperometric and spectrophotometric methods. The results obtained by using both methods were slightly smaller than the nominal values; however the amperometric results were closer to the nominal values specified by the manufacturer as compared with spectrophotometric method, since that the last one is more affected by matrix interferences. The third sample remains turbid even after the dilution and as a consequence the spectrophotometric determination was unenviable.

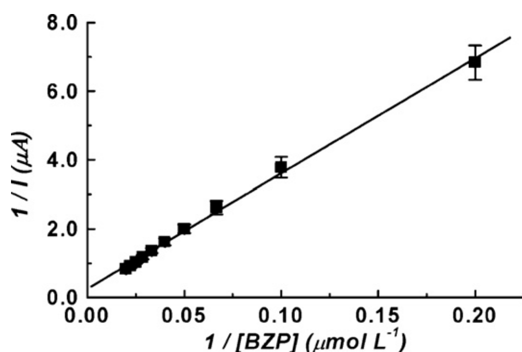


Fig. 4. Lineweaver–Burk plot corresponding to the electrocatalytic peak currents recorded in BZP solutions. Data obtained from the linear region [I (μA^{-1}) = $2.9 \times 10^{-2} + 2.4 \times 10^{-2}$ [BZP^{-1}] (μmol^{-1} L)] of the analytical curve.

Table 1 also shows a good agreement between the amperometric results and the labeled values with typical deviations in the order of 2.9% and 1.0%. In contrast, the spectrophotometric analysis showed deviations in the order of 3.0% and 9.8%. For the third sample, the most complex matrix, the deviation between the amperometric and the nominal results was 6.8%.

The lower BZP concentrations obtained in comparison with the values indicated by the manufacturer were attributed to some decomposition process, occurred in course during the shelf life of the product, or even as a result of low quality control issues. To the best of our knowledge, there are no certified solutions of this kind of sample.

To obtain additional information about the performance of the amperometric method recovery tests were undertaken using the same pharmaceutical preparations. The recovery results were similar to those obtained by the direct analysis, suggesting that the differences correspond to experimental errors and not to matrix effect (Table 2).

The results obtained with the new sensor are in good agreement with the values indicated in the cosmetic samples labels. The differences observed were attributed to many factors, between them the complexity of the samples investigated herein, some uncertainty in manufacturer specification and the inherent errors present in all analytical methods. For one of the dermatological shampoos (Table 1, sample number 3) it was impossible to determine the content of BZP utilizing spectrophotometry, whereas the amperometric method was able to quantify the content of the organic peroxide within acceptable error margins.

4. Conclusions

Carbon paste electrodes containing *peroxidases* naturally immobilized on coconut fibers are reliable sensors for the detection of benzoyl peroxide (BZP) in pharmaceutical preparations. Amperometric current levels, obtained in the presence and absence of the oxygen were similar, probably because the fibrous mesocarp of the coconut fruit contains significant amounts of phenolic compounds, which mediates the regeneration of the reduced form of the enzyme, with production of quinone derivatives, further reduced on the biosensor surface to establish the linear relation between reduction current and BZP concentrations in the measuring solutions. The fact of the vegetable tissue darks in the presence of atmospheric O_2 is an indicative of the simultaneous presence of catechols and *poliphenol oxidase* enzyme into the tissue and, therefore the mediated reaction via catechols is a well-built possibility.

The biosensor preparation is easy and reproducible and presents low cost. Additionally, short response time and low detection limit were obtained. The biocatalytic reaction readily enables rapid detection of BZP in cosmetic formulations within a linear range of the 5.0–55 μmol L^{-1} at a very low potential (0.0 V), minimizing interference problem.

Acknowledgements

The authors JVBK (*in memorian*) and RPS acknowledge the fellowships from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), respectively. AWOL, LA and SHPS thanks to CNPq grants and FAPESP. Many thank to Prof. Jay D. Wadhawan for the revision and suggestions for this manuscript. This work was supported with grants from IM^{2}C, CTPetro and SAMUTI projects.

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