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Enzymatic biosensors based on ingá-cipó peroxidase immobilised on sepiolite for TBHQ quantification

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Sepiolite clay mineral was used as a support for the immobilisation of the peroxidase enzyme from ingá-cipó (*Inga edulis* Mart.) and was used with graphite powder, multi-walled carbon nanotubes (CNTs), mineral oil, and nafion 0.5% (v/v) in the development of a new biosensor for the determination of the antioxidant *tert*-butylhydroquinone (TBHQ) by square-wave voltammetry (SWV). For the optimisation and application of the biosensor, several parameters were investigated to determine the optimum experimental conditions using SWV. The best performance was obtained using a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0), 4.0×10^{-4} mol L^{-1} hydrogen peroxide, a frequency of 50 Hz, a pulse amplitude of 60 mV, and a scan increment of 6 mV. The biosensor showed good repeatability and reproducibility and remained stable for a period of 20 weeks. The analytical curve revealed a linear response range of 1.65 to 9.82 mg L^{-1} ($r = 0.994$) with detection and quantification limits of 0.41 and 1.25 mg L^{-1}. A recovery study of TBHQ in salad dressing samples yielded values from 99.6–104.8%. The proposed biosensor was successfully used for the determination of TBHQ in commercial salad dressing samples, giving a relative error of 5.4% in relation to the comparative method (chromatographic).

Introduction

Antioxidants are an important group of food additives that have the ability to prevent harmful changes (deterioration, discoloration, or rancidity). The oxidising nutrients block free radicals, converting them into stable products through redox reactions and, consequently, extend the shelf-life of foods. Generally, they are used in commercial mixtures in oils or fats, and mayonnaise is one of the foods that most contains these additives. Studies have revealed that the excess use of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ), in foodstuff may cause a loss of sustenance and produce toxic substances that are detrimental to human health. They are more extensively used than natural antioxidants due to some of their characteristics, such as chemical stability, availability and low cost. Based in this context, the quantification of synthetic antioxidants (*e.g.* TBHQ) in foods is very important for human health, food safety, and quality control in the food industries.[1–3]

TBHQ is a white or beige powder that is stable at high temperatures and moderately soluble in oil, with reduced solubility in water. It does not coordinate with copper or iron and, therefore, does not cause discoloration of foods. This additive is banned in the European Union and Japan, but it is legal in countries such as Brazil, the United States, and China, where the maximum allowed limit is 200 mg kg^{-1}.[4,5]

Some of the analytical methods reported in the literature for detection and quantification of TBHQ are gas chromatography,[6] FTIR spectroscopy,[4] micellar electrokinetics capillary chromatography,[7] spectrophotometry,[8] voltammetry,[9] and high performance liquid chromatography (HPLC) coupled with different detection systems.[5,10] Although HPLC methods are widely used, chromatographic techniques are laborious and time consuming since they involve several steps. Electroanalytical methods are advantageous because they have a low detection limit, fast response time, easy operation, and relatively low cost. Biosensors combined with voltammetric techniques are usually utilised in the development of electroanalytical methods for determination of antioxidant compounds.[11–13]

Graphite and an immiscible organic liquid in aqueous solution are used to construct a carbon paste electrode.[14] The internal modification capacity of the electrode material is one of the main advantages of using modified carbon paste when developing a sensor. These sensors offer versatility, low cost, wide range of working potential, low noise and the possibility of surface renewal, unlike what happens with solid electrodes in which the modification occurs only on the surface.[15,16] Because of these properties, the development of a carbon paste electrode with an immobilised enzyme (biosensor) is widely found in the literature.[17–19]

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A biosensor is an analytical device that consists of a biological material, often an enzyme, together with an appropriate transducer to produce an electronic signal capable of giving selective and/or quantitative analytical information about the analyte.[20] One of the important points in biosensor development is the establishment of fast electron transfer between the active site of the enzyme and the electrochemical transducer.

Carbon nanotubes (CNTs) have been shown to increase the kinetics of electron transfer reactions at low over potentials and, therefore, have been added to enzyme-based sensors. This increase is due to the special properties (high electrical conductivity, excellent chemical stability, mechanical strength, and high surface area) of CNTs, which yield biosensors with improved sensitivity and selectivity.[21,22] The utilisation of CNTs for electrode modification can be divided into two groups: modification of the bulk, such as carbon paste, and modification of the solid electrode surface, such as chemical vapor deposition (CVD)[23] or preparation of films.[24] Incorporated into carbon paste, CNTs provide their electrocatalytic properties for the sensors, improving signal transduction. Furthermore, the immobilised enzyme incorporation into carbon paste gives better protection from the external environment when compared to an enzyme immobilised on a surface of a biosensor, showing greater stability and durability of the biosensor.

In addition, when nafion is used in the design of biosensors, it enhances their stability because of its specific structure, which could form a conductive three dimensional interpenetrating network.[25] It combines the high hydrophobicity of the polytetrafluoroethylene backbone with the high hydrophilicity of the sulfonic acid groups that are alternatively arranged along the polymeric chain. The sulfonic acid groups that aggregate into clusters allow for the fast transport of ions and serve as a polymer electrolyte.[26] Nafion has been extensively used in electrochemistry to modify electrodes because of its antifouling capacity, chemical inertness, high permeability, mechanical strength, and thermal stability.[27]

Sepiolite is a natural hydrated magnesium silicate with a fibrous morphology that has the theoretical unit cell formula $Mg_8Si_{12}O_{30}(OH)_4 \cdot (H_2O)_4 \cdot 8H_2O$. Its crystalline structure consists of alternating blocks and channels that grow up in the fiber direction. The blocks are basically comprised of two layers of tetrahedral silica that sandwiches a central octahedral magnesium oxide–hydroxide layer.[28] Microchannels crossing the structure are filled with H_2O molecules that are zeolitic weakly bound and exchangeable cations.[29] The hollow needle-like structures with fine particle size results in a high surface area that combined with the presence of crystal channels provides excellent adsorptive and catalytic properties to sepiolite.[30] The catalytic activity of sepiolite is principally a function of its surface activity, especially of the silanol groups that are present on the surface, which can act as catalysts at reaction sites.[31] Since it is a good adsorbent, this silicate can be used as a solid support for enzyme immobilisation in order to stabilise and to provide for continuous use of the enzymes for bioanalytical applications.

Peroxidase enzymes are widely distributed in nature in plants, microorganisms, and animals and have important roles. Most peroxidases are heme proteins, containing iron(III) protoporphyrin IX as the prosthetic group, and catalyse the oxidation of a variety of substrates using peroxides. In their catalytic cycle, the native enzyme is oxidised by hydrogen peroxide to form an intermediate (compound I) and water, from the resulting reduction of peroxide. Then, compound I is reduced through the transfer of an electron from the substrate to form compound II, regenerating its ferric enzyme by a second electron transfer from one substrate molecule.[32] Peroxidases are widely used in the construction of biosensors for different applications.[33–35]

In this work, we describe a novel biosensor based on peroxidase from *Inga edulis* Mart. immobilised on sepiolite and incorporate into a carbon paste electrode (CPE) containing multiwalled CNTs and nafion for the determination of TBHQ. The electrochemical behaviour of TBHQ was investigated by square-wave voltammetry (SWV), and the bioelectrode was used for the determination of this antioxidant in samples of salad dressings. Finally, the results of the proposed bioelectroanalytical method were compared with those obtained using a HPLC method for TBHQ.

Experimental

Chemical, solutions, and samples

All reagents were of analytical grade and were used without further purification. All solutions were prepared with ultrapure water (18.2 MΩ cm) obtained from a Millipore (Bedford, USA) Milli-Q gradient purification system. Phosphate buffer solution (0.1 mol L^{-1}, pH 7.0) was used as the supporting electrolyte. Guaiacol, hydrogen peroxide, TBHQ, multi-walled CNTs (>90% carbon basis, O.D. × I.D. × *L* 10–15 nm × 2–6 nm × 0.1–10 μm), graphite powder, high purity mineral oil, and 5% nafion perfluorinated resin solution (Naf = nafion in the figure/table legends) were purchased from Sigma-Aldrich. The clay mineral sepiolite (SEP = sepiolite in the figure/table legends) was purchased from Tolsa. Acetonitrile (ACN) of HPLC grade and ethanol (J.T. Baker) were used in the preparation of the mobile phase for HPLC analyses and in the procedure extraction, respectively. The samples of salad dressing were purchased from a local store in Cuiabá.

Plant material

The fruits of ingá-cipó (*Inga edulis* Mart.) were collected from Cuiabá, Mato Grosso, Brazil in April 2013 and used as a source of peroxidase. The plant material was identified in the Herbarium of the Federal University of Mato Grosso (Cuiabá), where a voucher specimen (no. 40.738) was deposited.

Instrumentation

The determination of the peroxidase activity was carried out using a 50 Scan UV-Vis spectrophotometer (Varian, Australia) with an optical path of 1.0 cm (quartz cell). A Hettich centrifuge, model Rotanta 460 R, was used to determine the activity and preparation of the ingá-cipó extract. A vortex mixer, model Q220M (Quimis), an ultrasonic bath, model Ultra Cleaner 1400 (Unique), and an analytical balance, model AX200 (Shimadzu),

were used during the sample preparation. The pH measurements were performed on a Metrohn 827 pH meter.

The scanning electron microscopy (SEM) images of sepiolite and the enzymatic extract immobilised on sepiolite samples were obtained using a model Shimadzu SSX-550 Superscan microscope, with an accelerating voltage of 15 kV and 2000× magnification for all of the images.

Electrochemical measurements using SWV were performed on an Autolab PGSTAT302 potentiostat/galvanostat (Eco Chemie, The Netherlands) connected to data processing software (GPES, software version 4.9.006, Eco Chemie). All voltammetry experiments were carried out using a conventional three-electrode system with a biosensor as the working electrode, a platinum wire as the counter electrode, and Ag/AgCl (3.0 mol L^{-1} KCl) as the reference electrode.

Chromatographic analysis of TBHQ was carried out using a chromatographic system consisting of a Series 200 binary pump (Perkin Elmer, USA), UV-Vis detector (Varian, Australia), and Luna C18 column (250 mm × 4.6 mm × 5 μm; Phenomenex, USA).

Enzyme extract, activity, and total protein

Fresh pulp of ingá-cipó (25 g) was extracted with 100 mL phosphate buffer solution (0.1 mol L^{-1}, pH 7.0) for 1 min in a mixer. The extract was rapidly filtered and then centrifuged at 2000 rpm for 15 min at 4 °C. The resulting supernatant was maintained at 4 °C and utilised as a source of peroxidase in the construction of the biosensor.

The enzymatic activity was determined using a spectrophotometric method by measuring the absorbance at 470 nm of tetraguaiacol produced by the reaction between 0.05 mL of the enzymatic solution, 2.85 mL of the 0.05 mol L^{-1} guaiacol solution, and 0.1 mL of the 9.8 × 10^{-3} mol L^{-1} hydrogen peroxide solution in phosphate buffer solution (0.1 mol L^{-1}, pH 7.0). Control experiments were carried out under the same conditions, but in the absence of the enzyme. One unit of peroxidase was defined as the amount of enzyme sufficient to produce 0.001 units of absorbance per minute.[36]

The total protein concentration of the enzyme extract was determined by the Bradford method using serum albumin as the standard.[37] An analytical curve was constructed by adding Bradford reagent to solutions containing different concentrations of albumin (from 5.0 to 100.0 μg mL^{-1}), which absorbs at 595 nm. Absorbance was read from the enzymatic extract to determine the total protein. All measurements were performed in triplicate.

Biosensor preparation

The proposed biosensor was constructed by mixing 90.0 mg graphite powder, 10.0 mg multiwalled CNTs, and 20.0 mg of the sepiolite containing 9.3 units of peroxidase μg^{-1} of protein immobilised with a mortar and pestle. Then, 50.0 mg mineral oil and 80.0 mg 0.5% (v/v) nafion perfluorinated resin were added, and the mixture was homogenised for 20 min to produce the final paste. The obtained paste was compacted in a 1 mL plastic syringe with a 1.0 mm internal diameter and a copper wire was inserted to establish the external electrical contact.

Sample preparation

Samples of TBHQ-free salad dressings were fortified to a final concentration of 25, 100, and 200 mg L^{-1}, and the commercial salad dressings containing TBHQ were analysed. All samples were stored in a refrigerator prior to use. A similar procedure to the liquid–liquid extraction proposed by Medeiros *et al.*[38] was followed for the extraction of TBHQ from the salad dressing samples. A sample of about 5.0 g was dissolved in 5.0 mL ethanol in a large centrifuge tube. The mixture was shaken for 2 min using a vortex mixer, then placed in an ultrasonic bath for 5 min, and was then centrifuged at 2000 rpm for 15 min. The extraction procedure was repeated once and both supernatants were collected, and TBHQ was determined using the enzyme biosensor and chromatographic methods.

Voltammetric and chromatographic analyses

The SWVs were recorded by applying a sweep potential between +0.10 and −0.35 V and were performed under previously optimised conditions. A 1.0 mL aliquot of the sample extract was transferred to the electrochemical cell containing 8.0 mL of phosphate buffer solution (0.1 mol L^{-1}, pH 7.0) and 4.0 × 10^{-5} mol L^{-1} hydrogen peroxide, and then TBHQ was determined using the external standard method. All experiments were performed in triplicate at room temperature (25 °C ± 1 °C).

The chromatography was performed in the reversed-phase mode and under isocratic solvent conditions. Manual injections were performed using a Rheodyne injector valve with a 20 μL sample loop. The mobile phase was 1 : 1 (v/v) water–acetonitrile set at a flow rate of 1.0 mL min^{-1}. All the analyses were performed at ambient temperature (25 °C) and the UV detector was set at λ = 293 nm.[39] The concentration was determined by recording the peak height and comparing the results with the calibration curve.

Results and discussion

Peroxidase immobilisation and electrochemical response for TBHQ

Among the immobilisation techniques, the enzyme adsorption onto solid supports improves the enzyme stability against denaturation. In this study, the peroxidase enzyme was physically adsorbed onto surfaces of sepiolite, which exhibits properties, such as microfibrous morphology with a high superficial area and extended pore volume,[40] that make the material suitable for enzyme immobilisation. The surface morphologies of sepiolite and the peroxidase extract immobilised on sepiolite (Fig. 1) were observed by SEM. The sepiolite image (Fig. 1A) shows aggregates with a clear fibrous surface. However, when the enzymatic extract was adsorbed, the surface changed significantly (Fig. 1B), becoming more compact and homogeneous. According to de Fuentes *et al.*,[41] the adsorption mechanism for silicates must be produced through protonated lysines of the enzymes and the silanol residues of the sepiolite, which are able to promote cationic exchange. Furthermore, by having characteristics that are important during enzyme immobilisation, sepiolite provides a favourable microenvironment for

peroxidase, increasing its stability and enhancing their continuous use.

Sensors containing peroxidase were used to determine a wide range of phenolic compounds. In this biosensor, peroxidase catalysed the oxidation of the TBHQ substrate to quinone, and this product was electrochemically reduced to TBHQ on the biosensor surface at −0.15 V *versus* $E_{Ag/AgCl}$, as illustrated in Fig. 2. The resulting reduction current was proportional to the TBHQ concentration and was used to quantify the analyte.

The voltammetric behaviour of TBHQ was investigated in the potential range of 0.1 to −0.35 V *versus* $E_{Ag/AgCl}$ using SWV. Fig. 3 shows the voltammograms obtained using (a) CPE, (b) CPE–CNT, (c) CPE–CNT–SEP (d) CPE–CNT–SEP–nafion, and (e) CPE–CNT–SEP–nafion–peroxidase (biosensor) in a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) with 4.5×10^{-4} mol L^{-1} TBHQ and 4.0×10^{-5} mol L^{-1} H_2O_2. These five different sensors were constructed and compared to evaluate the contribution of each component of the biosensor to the electrochemical response of TBHQ. As shown in Fig. 3, the biosensor showed a higher response in the reduction wave of quinone to TBHQ and better sensitivity when compared with the other electrodes. These characteristics are attributed to the catalytic properties of peroxidase for phenolic compounds, which amplifies the TBHQ analytical signal and the excellent immobilisation of the enzyme. CNT incorporation into the carbon paste (b) intensified the TBHQ reduction current in contrast to CPE. This improvement is related to properties of the CNT, such as high electrical conductivity and highly electrochemically accessible surface area.[21,42] The response obtained with sensor (c), for which sepiolite was added, was better than that of the (a) and (b) electrodes. This was due to the catalytic properties of silicate, particularly of the silanol groups present on the surface.[31] The presence of the nafion (d) caused an increase in the analytical response for TBHQ, which may be related to the sulfonic acid groups, which allow for fast transport of ions and serve as a polymer electrolyte. Additionally, nafion can interact with the paste components (such as sepiolite, penetrating their structural channels) conferring greater stability to the sensor.[26,43] This experiment demonstrates the

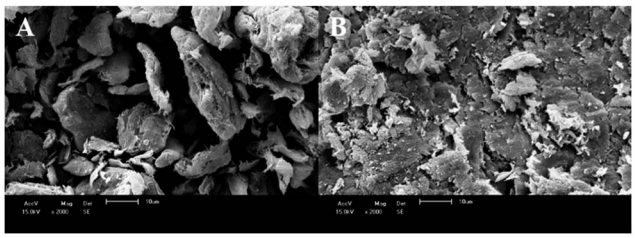

Fig. 1 SEM images of (A) sepiolite and (B) peroxidase immobilised on sepiolite (increase 2000×).

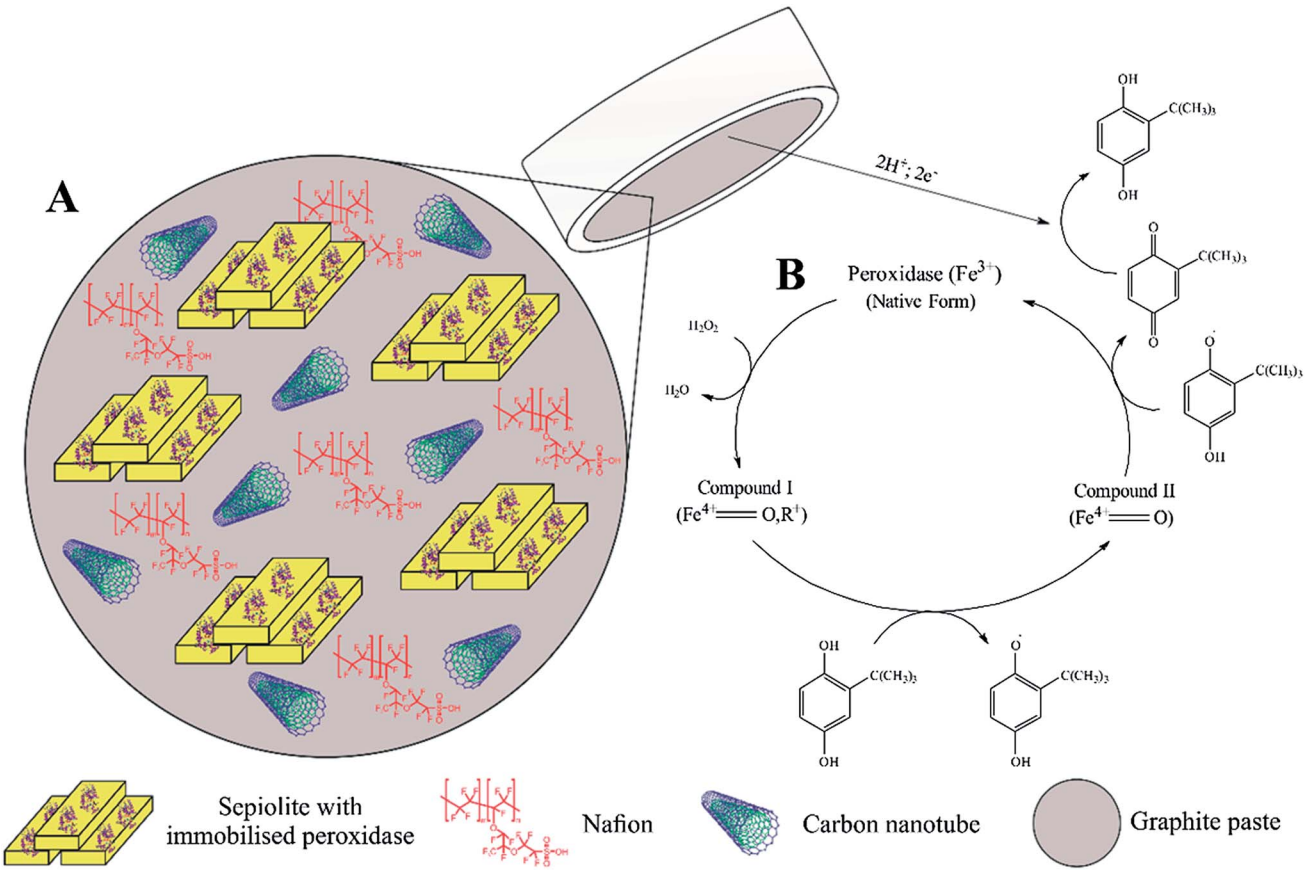

Fig. 2 Schematic representation (A) of carbon paste modified with CNTs, nafion and peroxidase immobilised on sepiolite. (B) TBHQ reaction with the peroxidase enzyme at the biosensor surface.

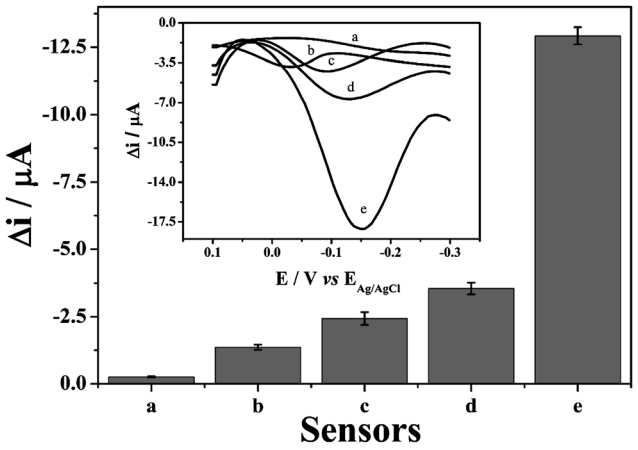

Fig. 3 Relative responses for the different electrodes and biosensors. The inset shows SWVs obtained using (a) CPE, (b) CPE–CNT, (c) CPE–CNT–SEP, (d) CPE–CNT–SEP–Naf, and (e) CPE–CNT–SEP–Naf–enzyme (biosensor) in phosphate buffer solution (0.1 mol L^{-1}, pH 7.0) containing 4.0×10^{-5} mol L^{-1} H_2O_2 and 4.5×10^{-4} mol L^{-1} TBHQ at a frequency 50 Hz, a pulse amplitude of 60 mV, and a scan increment of 6 mV ($\bar{x} \pm s$, $n = 3$).

significant contribution of each of these materials to improving the response of the analytical biosensor.

Optimisation of the experimental conditions

Various experimental conditions were carefully investigated in the optimisation of the proposed method, such as the enzyme units, pH of the electrolyte support, hydrogen peroxide concentration, frequency, pulse amplitude, and scan increment, to obtain the best experimental working conditions. These studies were performed using 4.5×10^{-4} mol L^{-1} TBHQ and 4.0×10^{-5} mol L^{-1} H_2O_2 in 0.1 mol L^{-1} phosphate buffer solution (pH 7.0).

The total protein concentration in the enzymatic ingá-cipó extract, determined by the Bradford method, was 78.1 μg of protein mL^{-1}. The effect of peroxidase concentration on the biosensor response was evaluated from 3.1–12.4 units of peroxidase μg^{-1} of protein. The best analytical signal was achieved at 9.3 units μg^{-1} of protein, and this concentration was used for further optimisation studies.

A study to select the best supporting electrolyte pH for the proposed bioanalytical method was carried out using 0.1 mol L^{-1} phosphate buffer solutions at different pH values (6.0, 6.5, 7.0, and 7.5). The highest voltammetric responses for TBHQ were obtained at pH 7.0, which were used in the following studies. The presence of hydrogen peroxide is essential for the biosensor response because the peroxidase requires hydrogen peroxide to catalyse the reaction. The influence of hydrogen peroxide concentration (from 1.0×10^{-5} to 1.0×10^{-4} mol L^{-1}) on the proposed biosensor response was investigated and the optimum concentration was found to be 4.0×10^{-5} mol L^{-1}. Therefore, this concentration was selected for subsequent studies. The SWV parameters and ranges investigated were: frequency (10–100 Hz), pulse amplitude (10–100 mV), and scan increment (0.5–10 mV). The best resultant peak currents were obtained using a frequency of 50 Hz, a pulse amplitude of 60 mV, and a scan increment of 6 mV. Therefore, these experimental conditions were applied in subsequent experiments.

Analytical characteristics of the biosensor

After establishing optimal experimental conditions, a calibration curve for TBHQ was constructed using SWV and the proposed biosensor. In the voltammograms obtained in this experiment, the reduction potential of TBHQ at the biosensor surface was observed at about −0.15 V *versus* Ag/AgCl (Fig. 4A). The analytical curve, which was constructed from a plot of the resulting current peaks *versus* TBHQ concentrations (Fig. 4B), showed a linear response between 1.65 and 9.82 mg L^{-1} TBHQ. This can be expressed according to the following regression equation: $-\Delta i = 1.34\ (\pm 0.02) + 0.11\ (\pm 0.004)$ [TBHQ], with a correlation coefficient of 0.994, where Δi is the resultant peak current (μA) and [TBHQ] is the TBHQ concentration (mg L^{-1}). The detection limit (LOD) and quantification limit (LOQ) were calculated using the standard deviation of the lower level concentration (s) and the slope of analytical curve (S) given by $\mathrm{LOD} = 3.3s/S$ and $\mathrm{LOQ} = 10s/S$. The LOD and LOQ were 0.41 and 1.25 mg L^{-1}, respectively.

In order to determine the precision of the method, the repeatability and reproducibility were verified by measuring the resulting current response, in triplicate and using SWV. For eight successive measurements using the same biosensor, the repeatability study produced a relative standard deviation (RSD)

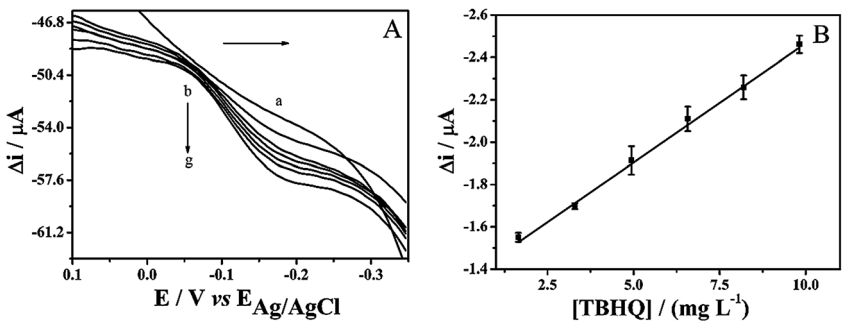

Fig. 4 (A) SWVs obtained using the biosensor proposed in (a) phosphate buffer solution (0.1 mol L^{-1}, pH 7.0) containing 4.0×10^{-5} mol L^{-1} hydrogen peroxide and TBHQ standard solutions in the following concentrations: (b) 1.65, (c) 3.30, (d) 4.94, (e) 6.57, (f) 8.20, and (g) 9.82 mg L^{-1} at a frequency of 50 Hz, a pulse amplitude of 60 mV, and a scan increment of 6 mV. (B) Calibration curve for TBHQ ($\bar{x} \pm s$, $n = 3$).

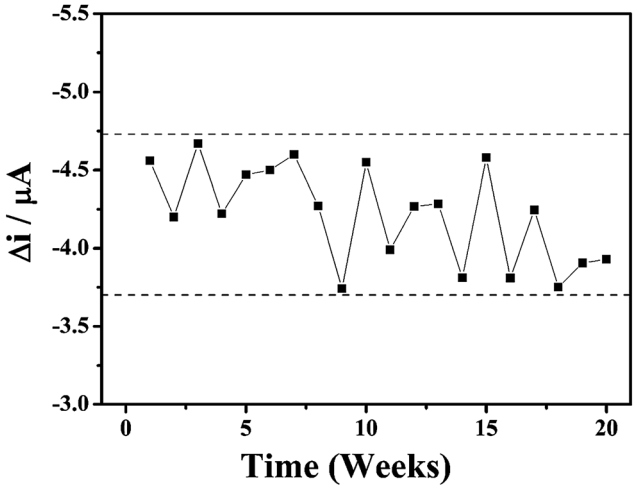

Fig. 5 Study of stability of biosensors by SWV in phosphate buffer solution (0.1 mol L^{-1}, pH 7.0) containing 4.0×10^{-5} mol L^{-1} H_2O_2 and 9.76×10^{-5} mol L^{-1} TBHQ. Confidence level of 99%.

of 6.2%, showing an acceptable precision between measurements. The reproducibility test was performed with five biosensors that were independently prepared and subjected to the same procedure. The test showed a good result, with a RSD of 3.4% under the optimised conditions described previously.

The biosensor stability was evaluated in a TBHQ solution over a period of 20 weeks (Fig. 5). The stability was verified and the measurements for each week remained within the limits of statistical control, showing normal random fluctuations. The biosensor was dry-stored and kept at room temperature (25 °C). The good performance of this biosensor was probably due to the success of the immobilisation of ingá-cipó peroxidase in the sepiolite, rather than the adsorption method, which induced fewer modifications on the active site conformation of the enzymes, thus preserving their activity.[41]

Recovery study and analytical applications

In order to determine the accuracy of the method presented here, the analytical recovery of TBHQ added to salad dressing samples was performed. Salad dressing samples were fortified with three different concentrations of TBHQ: 25, 100, and 200 mg L^{-1}. After the samples were then subjected to extraction with ethanol, a 1.0 mL aliquot was transferred to the voltammetric cell and the TBHQ was determined. In this study, TBHQ was also quantified by chromatographic analysis (adapted from Goulart *et al.*[39]) and compared with the results obtained by the proposed bioanalytical method. The results of the recovery rates for both methods were compared with the concentrations of TBHQ added to the samples. Table 1 shows TBHQ recovery values of 99.6–104.8% for the proposed biosensor and 97.3–105.2% for the HPLC method. The average recoveries demonstrate the satisfactory accuracy of the biosensor.

Commercial samples of salad dressings were subjected to extraction and analysed using the proposed and comparative methods. The results obtained using the biosensor and HPLC are summarised in Table 2.

Table 1 Recovery of TBHQ from salad dressing samples using the biosensor and HPLC methods

TBHQ (mg L^{-1})				
	Found		Recovery[b] (%)	
Fortified	Biosensor[a]	HPLC[a]	Biosensor	HPLC
25.0	26.2 ± 1.4	24.5 ± 0.9	104.8	98.0
100.0	99.6 ± 4.4	105.2 ± 2.7	99.6	105.2
200.0	204.9 ± 6.8	195.3 ± 6.5	102.4	97.3

[a] Mean ± standard deviation; $n = 3$. [b] Recovery = (mean found value/added value) × 100%.

Table 2 Results of the determination of TBHQ in salad dressing samples using the proposed biosensor and HPLC methods

	[TBHQ][a] (mg L^{-1})	RE[b] (%)	t_{cal}	t_{tab}[c]
Biosensor	50.9 ± 1.9	5.4	0.65	2.78
HPLC	48.3 ± 2.6			

[a] Mean ± standard deviation; $n = 3$. [b] RE (relative error) = biosensor *versus* comparative method. [c] Confidence level of 95%.

A relative error of 5.4% was obtained using the biosensor when compared to the comparative method. According to the Student's *t*-test, at a 95% confidence level, there are no significant differences between the mean concentrations obtained using the biosensor and the HPLC method in the commercial samples. We concluded that the proposed biosensor is suitable for the determination of TBHQ in salad dressings and can be used as an alternative method for TBHQ determination.

According to the Brazilian National Health Surveillance Agency (ANVISA), through Resolution (RDC) no. 4 of January 15, 2007, the maximum allowed value of TBHQ in sauces and condiments is 200 mg L^{-1}. The proposed biosensor was sufficiently sensitive for analysis of the quality control of foods, with LOD and LOQ values below those allowed by ANVISA.

Conclusions

The satisfactory analytical performance of the proposed biosensor can be attributed to the efficiency of the peroxidase immobilisation on the sepiolite and to the components included in the carbon paste (CNTs and nafion) facilitating electron transfer on the biosensor surface, which led to high biocatalyst efficiencies. The results of this study show that the proposed method offered good precision and accuracy for the determination of TBHQ in salad dressings and is also appropriate for the quantification of TBHQ in food samples. Furthermore, the biosensor construction was simple and relatively inexpensive, and the sensor displays long-term stability, and good repeatability and reproducibility for TBHQ quantification.

The design of the proposed biosensor offers a favorable microenvironment to maintain the bioactivity of the enzyme, increasing its sensitivity. Therefore, this design provides a promising base for the development of other biosensors.

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