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# Biosensor based on atemoya peroxidase immobilised on modified nanoclay for glyphosate biomonitoring

Grasielli C. Oliveira [a], Sally K. Moccelini [a,*], Marilza Castilho [a], Ailton J. Terezo [a], Juliana Possavatz [b], Márcia R.L. Magalhães [b], Eliana F.G.C. Dores [b]

[a] Departamento de Química, Grupo de Eletroquímica e Novos Materiais, Universidade Federal de Mato Grosso, 78060-900, Cuiabá, MT, Brazil
[b] Departamento de Química, Laboratório de Análise de Resíduos de Biocidas, Universidade Federal de Mato Grosso, 78060-900, Cuiabá, MT, Brazil

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ABSTRACT

A biosensor based on atemoya peroxidase immobilised on modified nanoclay was developed for the determination of glyphosate by the enzyme inhibition method. The inhibitor effect of the biocide results in a decrease in the current response of the hydroquinone that was used as a phenolic substrate to obtain the base signal. The biosensor was constructed using graphite powder, multiwalled carbon nanotubes, peroxidase immobilised on nanoclay and mineral oil. Square-wave voltammetry was utilised for the optimisation and application of the biosensor, and several parameters were investigated to determine the optimum experimental conditions. The best performance was obtained using a 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0), $1.9 \times 10^{-4}$ mol $L^{-1}$ hydrogen peroxide, a frequency of 30 Hz, a pulse amplitude of 50 mV and a scan increment of 4 mV. The glyphosate concentration response was linear between 0.10 and 4.55 mg $L^{-1}$ with a detection limit of 30 μg $L^{-1}$. The average recovery of glyphosate from spiked water samples ranged from 94.9 to 108.9%. The biosensor remained stable for a period of eight weeks.



## 1. Introduction

Several types of pesticides have been widely used in agriculture due to their high activity against fungi, insects and weeds. As a result, non-target organisms are inevitably exposed to these toxic compounds that can cause damages to human health and to ecosystems as a whole [1,2].

The state of Mato Grosso (Brazil), where headwaters of several rivers that form important aquatic ecosystems (Pantanal, Amazon and Araguaia) are located, is very rich in water resources. This state also has an important role in national agricultural production and, consequently, pesticides are used to maintain high levels of productivity and disease control. Therefore, it is likely that residues of these biocides remain in the soil and in surface and ground water, or are transported by atmospheric air, contaminating the water resources that constitute the main source of drinking water for the population [3,4].

Glyphosate [N-(phosphonometyl)glycine] is a post-emergent herbicide used worldwide that belongs to the chemical group of substituted glycines and is classified as non-selective and with systemic action. This pesticide presents a broad spectrum of action, which allows excellent control of weeds. Glyphosate acts as an inhibitor of the biosynthesis of essential aromatic amino acids, leading to various metabolic disorders, which include the suspension of protein synthesis and deregulation of the shikimate pathway, causing general metabolic disruption and death of the plant. Although considered to be of low toxicity to mammals, the effects of glyphosate on non-target organisms and its environmental impact are not yet fully understood. The high water solubility and extensive use of glyphosate indicate potential toxicological risk to aquatic environments that are exposed to this herbicide. Based on this context, it is necessary to develop versatile and inexpensive analytical methods to monitor glyphosate in aquatic environments [5,6].

Different methods have been described for glyphosate determination, including liquid chromatography [7] and gas chromatography [8] using various detectors. Other methods have been proposed, such as capillary electrophoresis [9], ion chromatography [5], spectrophotometry [10], the electrochemical method [11], NMR [12] and diffuse reflectance spectroscopy [13]. Biosensors have also been described for the detection and determination of glyphosate [14,15]. Redshaw et al. developed biosensors based on cells of *Escherichia coli* and *Salmonella typhimurium*. Both biosensors were sensitive for glyphosate and other harmful compounds and demonstrate that biosensor bioassays could be

* Corresponding author. Tel.: +55 65 3615 8769x238; fax: +55 65 3615 8764x244.
*E-mail address:* sallykm@gmail.com (S.K. Moccelini).

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a useful preliminary screening tool in forensic toxicology [14]. Songa's group [15] constructed a biosensor by electrostatically immobilising horseradish peroxidase onto the surface of a rotating gold disc electrode modified with poly(2,5-dimethoxyaniline)-poly(4-styrenesulfonic acid) nanoparticles. This biosensor was used for glyphosate analysis on spiked corn samples. Therefore, biosensors offer a promising alternative for the determination of glyphosate in environmental matrices. In view of this, it is important to develop analytical methods based on the biosensors with a reduced cost, acceptable sensitivity, a short response time, easy construction and easy operation.

A biosensor is usually defined as an analytical device in which a biological response is converted into a signal that can be quantified and processed [16]. Enzymatic biosensors can be used for the determination of pesticides through their inhibitory action on enzyme activity. The performance of an enzymatic biosensor depends on the success of the immobilisation of the biomolecule, which can be confined or bound in a particular support, and on maintaining its catalytic action. Among the materials described in the literature for enzyme immobilisation are the nanoclays (NCs). Owing to their physicochemical properties, such as swelling and thermal and mechanical stability, NCs can be effectively used as matrices for the immobilisation of enzymes [17]. A reduction in the dimensions of the enzyme carrier materials can generally improve the biocatalytic efficiency by promoting greater interaction with the enzyme and reducing diffusional limitations [18]. Biomolecules immobilised on different clay materials can be utilised in a wide variety of applications, including the construction of biosensors. Mbouguen et al. applied natural Cameroonian smectites grafted with either aminopropyl (AP) or trimethylpropylammonium (TMPA) groups to the immobilisation of glucose oxidase and TMPA-clays for polyphenol oxidase anchoring. These systems were evaluated as biosensing electrochemical devices for the detection of glucose and catechol analytes [19]. Shan et al. developed a biosensor for glucose by immobilising glucose oxidase into double-layered hydroxides [20]. In this study, we used a nanoclay based on montmorillonite modified with aminopropyltriethoxysilane and octadecylamine groups as a support for the immobilisation of the enzyme peroxidase. This material was utilised in the biosensor construction. This modification promotes an increase in the porosity and surface area of the support [21], which can extend the life of the enzyme and improve the stability of the biosensor.

The incorporation of enzyme immobilisation in the carbon paste for biosensor development is attractive due to its biocompatibility, renewable surface, low cost and ease of construction. These advantages can also be combined with the ability of carbon nanotubes (CNTs) to promote electron-transfer reactions. CNTs exhibit a high surface area, excellent electrical conductivity, chemical stability and mechanical strength, which make CNTs ideal for use in sensors [22]. In addition, they are useful because enzymes can be entrapped in the inner cavity [16].

The use of vegetables as an enzymatic source has been employed in the construction of biosensors to replace isolated enzymes. In vegetable tissues and crude extracts, the enzyme is maintained in its natural environment, thereby obtaining better stability and lower cost. Peroxidase, a heme-containing enzyme, belongs to the class of oxide-reductases, is widely found in nature and can be extracted from the cells of several plants. This enzyme utilises hydrogen peroxide to oxidise a variety of organic and inorganic compounds. High catalytic activity to a wide variety of substrates and thermoresistance are attractive properties for the development of biosensors [23,24].

In the present study, nanoclay modified with aminopropyltriethoxysilane and octadecylamine groups was employed as a support for the immobilisation of the peroxidase of atemoya in the construction of a biosensor, which was then applied in the determination of glyphosate in natural water. This biocide can be detected based on its inhibitory effect on peroxidase activity.

## 2. Experimental

### 2.1. Chemicals and solutions

The reagents were of analytical grade and employed without further purification. All solutions were prepared with ultrapure water (18.2 MΩ cm) obtained from a Millipore (Bedford, MA, USA) Milli-Q Gradient purification system. A phosphate buffer solution of 0.1 mol $L^{-1}$ (pH 7.0) was used as the supporting electrolyte throughout the experiments. The carbon paste was prepared using graphite powder. Multi-walled carbon nanotubes (Aldrich) and high purity mineral oil were purchased from Sigma-Aldrich. Nanoclay (montmorillonite clay base material containing 0.5–5 wt% aminopropyltriethoxysilane and 15–35 wt% octadecylamine), hydroquinone, hydrogen peroxide, guaiacol and glyphosate were obtained from Sigma. The atemoya (*Annona cherimolla* Mill × *Annona squamosa* L.) was purchased from a local market in Cuiabá (Mato Grosso, Brazil) and used as a source of peroxidase.

### 2.2. Instrumentation

Square-wave voltammetry (SWV) experiments were performed in an electrochemical cell containing 5.0 mL of the supporting electrolyte at room temperature (25 °C) using an Autolab PGSTAT 12 potentiostat/galvanostat (Eco Chemie, The Netherlands) operating with data processing software (GPES, software version 4.9.006, Eco Chemie). All experiments were carried out using a conventional three-electrode system with the biosensor used as the working electrode, platinum as the auxiliary electrode and Ag/AgCl (3.0 mol $L^{-1}$ KCl) as the reference electrode. A Varian Cary® 50 UV–vis spectrophotometer with a quartz cell (optical path of 1.0 cm) was used for the determination of the peroxidase activity. A Hettich centrifuge, model Rotanta 460 R, was used in the preparation of the atemoya extract. The Fourier transform-infrared (FTIR) spectra of the nanoclay and the peroxidase immobilised into nanoclay matrix were obtained using Bomem FT-IR MB100 equipment with the samples in KBr pellets. Chromatographic separation of glyphosate was carried out using a Dionex® ICS-90 ion exchange chromatograph with a conductivity detector (model DS5), equipped with a Dionex® AS40 automatic sampler operating with Dionex® Chromeleon version 6.5 software.

### 2.3. Obtainment of the peroxidase and measurement of the activity

For the production of the raw extract, a portion of the atemoya (25 g) was homogenised in a mixer with 100 mL of 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0) for 1 min. The extract was filtered and centrifuged at 2000 rpm for 20 min at 4 °C, and the resulting supernatant solution was maintained at 4 °C and used as the enzyme source in the construction of the biosensor. The enzymatic activity of the peroxidase present in the atemoya extract was determined using a spectrophotometric method, in triplicate, by measuring the absorbance of the tetraguaiacol produced by guaiacol oxidation at 470 nm. Enzyme activity was measured in the solution containing 0.1 mol $L^{-1}$ phosphate buffer (pH 7.0), 0.2 mL of the enzymatic solution, 2.7 mL of the 0.05 mol $L^{-1}$ guaiacol solution and 0.1 mL of the 0.01 mol $L^{-1}$ hydrogen peroxide solution. Control experiments were carried out using the same procedure 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 min [25].

### 2.4. Biosensor preparation

The biosensor was constructed by the modification of a carbon paste electrode (CPE). An aliquot of the atemoya extract containing 500 units $mL^{-1}$ of the peroxidase was added to 20 mg of the nanoclay, and the mixture was dried at room temperature (25.0 °C) and used for the construction of the biosensor. This mixture was hand mixed with 94.5 mg of graphite powder and 10.5 mg of multiwalled carbon nanotubes in an agate mortar for 15 min to ensure a uniform mixture. Mineral oil (60.0 mg) was then added, and the mixture was homogenised for 20 min to produce the final paste. Finally, the modified paste was tightly packed into a plastic syringe (1.0 mm internal diameter) and a copper wire was inserted to establish the external electrical contact. When not in use, the biosensor was stored at room temperature.

### 2.5. Electroanalytical measurements

The square-wave voltammograms were recorded by applying a sweep potential between +0.2 and −0.5 V, using a conventional three-electrode system in which the biosensor was used as the working electrode, a platinum wire as the counter electrode and Ag/AgCl (3.0 mol $L^{-1}$ KCl) as the reference electrode. All measurements were performed in triplicate, at room temperature (25 ± 0.5 °C) and after a suitable time of 120 s (to homogenise the solution and obtain the maximum response).

### 2.6. Preparation and analysis of samples

The applicability of the biosensor was tested for the analysis of spiked water samples. The water samples were collected in the micro-basin of the Monjolo Stream, located in the town of Chapada dos Guimarães-MT, Brazil. Samples were collected in 4-L amber bottles and taken to the laboratory under ice. They were filtered through a cellulose acetate membrane (0.2 μm) and fortified with glyphosate. The fortification was performed in three levels (0.20, 1.00 and 1.75 mg $L^{-1}$).

For the determination of glyphosate, the biosensor was initially immersed into a cell containing 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0), $1.89 \times 10^{-4}$ mol $L^{-1}$ hydroquinone reference solution and $1.9 \times 10^{-4}$ mol $L^{-1}$ hydrogen peroxide solution. The peak current was recorded as $I_0$ (base signal). The biosensor was incubated in standard solutions of the pesticide with different concentrations for 2 min and then transferred to the electrochemical cell containing 0.1 mol $L^{-1}$ phosphate buffer (pH 7.0), $1.89 \times 10^{-4}$ mol $L^{-1}$ hydroquinone and $1.9 \times 10^{-4}$ mol $L^{-1}$ hydrogen peroxide. The peak current was recorded as $I$ (after inhibition). The percentage of inhibition was calculated using different concentrations of glyphosate as follows [26]:

$$IR\% = I_0 - I/I_0 \times 100$$

where $I_0$ is the current value of hydroquinone before the inhibition, and $I$ is the peak current of hydroquinone after inhibition.

An ion chromatography method was used to compare the analytical results obtained from the proposed bioelectroanalytical method. The chromatographic system included an IonPac® AG14A guard column (4 × 50 mm) followed by an IonPac® AS14A analytical column (4 × 250 mm) and AMMS® III anionic micromembrane suppressor (4 mm) with a 50 μL sample loop injection. A $9.0 \times 10^{-3}$ mol $L^{-1}$ $Na_2CO_3$ solution at a flow rate of 1.0 mL $min^{-1}$ was used as the eluent, and a 0.025 mol $L^{-1}$ $H_2SO_4$ solution was used as the regenerant. The retention time of the analyte was 21.0 min.

## 3. Results and discussion

### 3.1. Immobilisation of peroxidase and voltammetric behaviour of hydroquinone

An essential problem for the development of biosensors is the immobilisation of the biological component while maintaining its biological activity and diffusional properties for the substrates. Due to their characteristic properties (hydrophilic, swelling and porosity), clays occupy an advantageous position as a support for the immobilisation of biological materials. The entrapment of enzymes in a clay matrix constitutes a fast, easy and economical method for the elaboration of biosensors [27]. In this work, the immobilisation procedure consisted of the physical adsorption of the enzymatic extract onto the modified nanoclay. Group modifiers (aminopropyltriethoxysilane and octadecylamine) likely occur in the inter-layer space of the montmorillonite, increasing the opening between the layers [28]. Perez-Santano and co-workers [21] reported that the intercalated clay shows a spongier aspect than the natural clay, and the inter-layer separation suggests a possible increase in the porosity and surface area. Therefore, the protein can be anchored on the external surfaces and the edges of the nanoclay or intercalated within the inter-layer space through hydrogen bonding, van der Waals, hydrophobic and electrostatic force interactions [29]. The enzyme backbone is positioned at the border of the nanoclay, whereas the side chains penetrate between the layers.

FTIR spectroscopy was employed to confirm the presence of the enzymatic extract in the immobilisation matrix. The FTIR spectra of the pure nanoclay and the enzyme–nanoclay are shown in Fig. 1. The amide bands are formed by the overlapping of several components that correspond to different elements of the secondary structure of the protein [17]. The bands between 1500 and 1650 $cm^{-1}$ correspond to the amide I and II vibrations of the amino acid bond of the protein chain. The addition of the enzyme on the nanoclay also caused an overlapping of bands between 3200 and 3430 $cm^{-1}$, which could be related to the amine and hydroxyl group vibrations.

The proposed sensing mechanism between peroxidase immobilised in nanoclay with hydroquinone as reducing substrate is

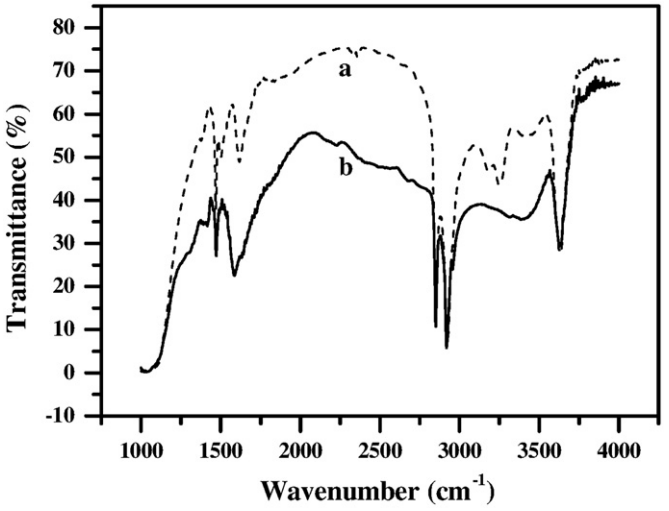


**Fig. 1.** Infrared spectra of (a) nanoclay; (b) peroxidase immobilised into nanoclay matrix.

summarised as follows. First, the peroxidase in the presence of hydrogen peroxide catalyses the oxidation of hydroquinone to *p*-benzoquinone. Afterwards, *p*-benzoquinone is electrochemically reduced at the biosensor surface at a potential of −0.1 V vs. Ag/AgCl (Fig. 2). The current obtained in the reduction of *p*-benzoquinone is quantitatively related to the hydroquinone concentration.

The square-wave voltammograms shown in Fig. 3 illustrate the efficiency of enzyme immobilisation on the nanoclay where (a) is the enzyme-free biosensor and (b) is the biosensor with immobilised enzyme in a $3.6 \times 10^{-4}$ mol $L^{-1}$ hydroquinone solution in 0.1 mol $L^{-1}$ phosphate buffer (pH 7.0) containing hydrogen peroxide. The response of the enzyme-free biosensor to hydroquinone was lower than that presented by the nanoclay biosensor. Based on this result, it is assumed that nanoclay offers

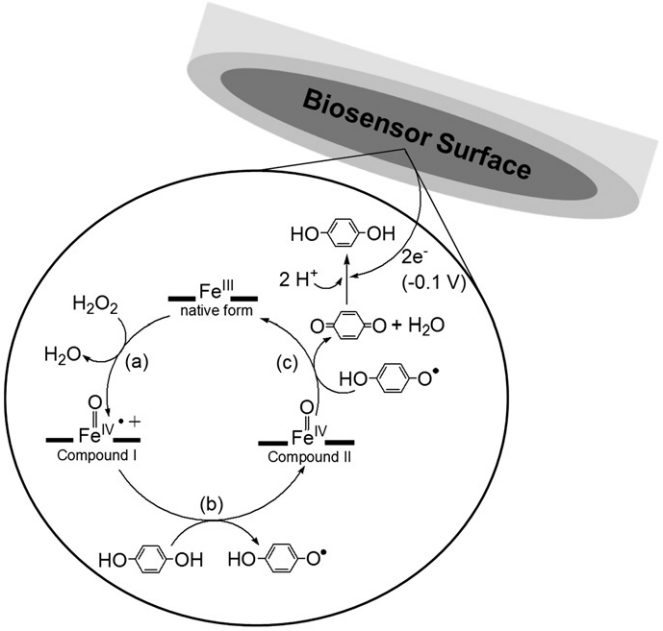


**Fig. 2.** Schematic representation of the proposed sensing mechanism between peroxidase immobilised in nanoclay with hydroquinone as reducing substrate.

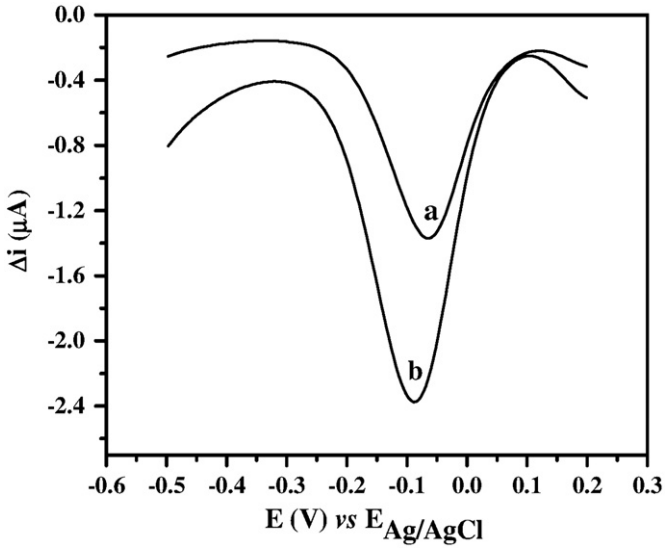


**Fig. 3.** Square-wave voltammograms obtained using (a) enzyme-free biosensor and (b) enzyme immobilised into nanoclay biosensor in $3.6 \times 10^{-4}$ mol $L^{-1}$ hydroquinone in 0.1 mol $L^{-1}$ phosphate buffer (pH 7.0).

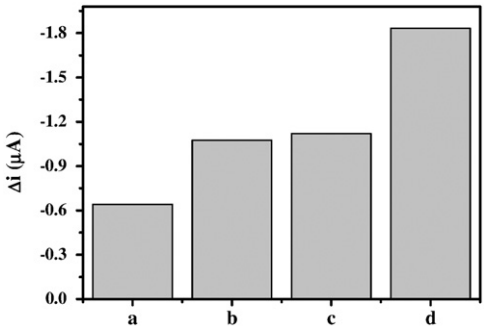


**Fig. 4.** Cathodic peak current values for (a) CPE, (b) CPE–CNT, (c) CPE–CNT–NC and (d) CPE–CNT–NC–enzyme (biosensor) in $3.6 \times 10^{-4}$ mol $L^{-1}$ hydroquinone in 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0).

a favourable microenvironment for peroxidase, facilitating the electron transfer on the biosensor surface and confirming the efficiency of the immobilisation. The electrochemical behaviour of hydroquinone was investigated in the potential range of +0.2 to −0.5 V vs. Ag/AgCl.

To evaluate the contribution of each component of the biosensor, different sensors were constructed and compared. The electrochemical behaviour of hydroquinone was also investigated using SWV with $3.6 \times 10^{-4}$ mol $L^{-1}$ of hydroquinone in 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0) containing hydrogen peroxide, in the potential range between +0.2 and −0.5 V vs. Ag/AgCl. The cathodic peak current values for (a) a CPE, (b) a CPE containing CNTs, (c) a CPE containing CNT–NC and (d) the CPE–CNT–NC–enzyme (biosensor) are shown in Fig. 4. The incorporation of CNTs into the carbon paste provided an increase in the current value of *p*-benzoquinone reduction to hydroquinone in relation to the CPE sensor. The greater response is attributed to the highly electrochemically accessible surface area of the carbon nanotubes combined with their high electronic conductivity [30]. In the CPE–CNT–NC, no significant difference in the response was obtained. However, a small contribution in the current response was observed. This may be due to the presence of metal ions in the nanoclay. An improvement in the catalytic current was observed in the biosensor. This behaviour is attributed to the catalytic properties of the immobilised enzyme, which amplified the analytical signal of the hydroquinone, resulting in a more sensitive sensor.

### 3.2. Optimisation of the biosensor

In the optimisation studies of the biosensor, some of the experimental parameters such as the enzyme units, pH of the electrolyte support, hydrogen peroxide concentration, frequency, pulse amplitude and scan increment were investigated to obtain the best experimental working conditions. The effect of the peroxidase concentration in the paste preparation was evaluated from 100 to 500 unit $mL^{-1}$ in nanoclay. The best analytic signal was attained at 500 unit $mL^{-1}$. This result was then used in the subsequent studies. To determine the influence of the pH of the electrolyte support on the biosensor response, a range of 6.0–8.0 was studied. The highest voltammetric responses for hydroquinone were obtained with 0.1 mol $L^{-1}$ phosphate buffer solution at pH 7.0. Consequently, this pH value was used in further studies. Peroxidase requires hydrogen peroxide to catalyse the reaction, so this parameter was also explored. The effect on the biosensor response due to varying the hydrogen peroxide concentration from $9.3 \times 10^{-5}$ to $6.9 \times 10^{-4}$ mol $L^{-1}$ in 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0) was investigated. The analytical response increased with a rise in the concentration of the hydrogen

peroxide up to $1.9 \times 10^{-4}$ mol $L^{-1}$, and this concentration was selected for use in this study. The following ranges were studied for the optimisation of the instrumental parameters of the square wave voltammetry: the frequency (10–100 Hz), pulse amplitude (10–100 mV) and scan increment (0.5–12 mV). This study was conducted in $3.6 \times 10^{-4}$ mol $L^{-1}$ of hydroquinone in 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0), containing $1.9 \times 10^{-4}$ mol $L^{-1}$ hydrogen peroxide. The best analytical signals were obtained for the biosensor employing a frequency of 30 Hz, a pulse amplitude of 50 mV and a scan increment of 4 mV. Therefore, these experimental conditions were applied in the following experiments.

### 3.3. Repeatability, reproducibility and stability of the biosensor

The repeatability of the proposed biosensor was examined by measuring the current response in a phosphate buffer solution (0.1 mol $L^{-1}$; pH 7.0) containing $5.3 \times 10^{-4}$ mol $L^{-1}$ hydroquinone and $1.9 \times 10^{-4}$ mol $L^{-1}$ hydrogen peroxide, taking several separate measurements using the same sensor. The relative standard deviation was 5.5% for eight successive assays.

The reproducibility was also investigated by utilising four separate biosensors prepared and used independently under the optimised conditions described previously. The biosensor showed an acceptable reproducibility with a relative standard deviation of 8.7%.

The long-term stability of the biosensor was evaluated by measuring the voltammetric current response in triplicate over a period of eight weeks. The current response was recorded in $3.6 \times 10^{-4}$ mol $L^{-1}$ of hydroquinone and $1.9 \times 10^{-4}$ mol $L^{-1}$ hydrogen peroxide in a 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0). The biosensor was dry-stored and maintained at room temperature (25 °C). The control chart (Fig. 5) shows that the mean value of the measures for each week remained within the limits of statistical control, with normal random fluctuations. This result may be due to the efficiency of the immobilisation of the peroxidase on the nanoclay.

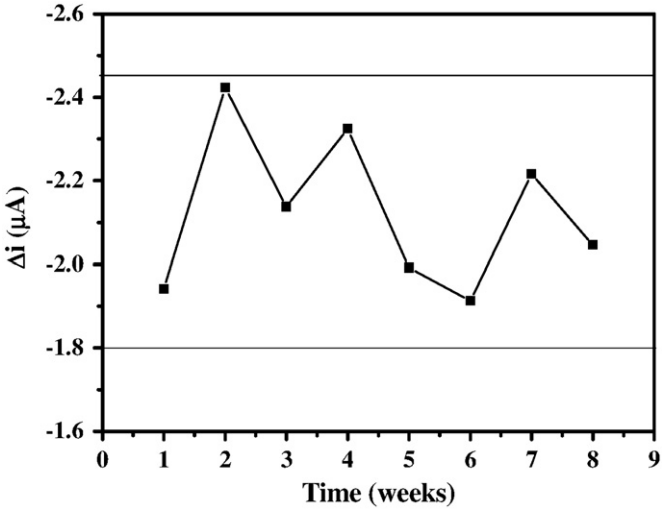


**Fig. 5.** Study of the stability of the biosensor stored at room temperature in $3.6 \times 10^{-4}$ mol $L^{-1}$ hydroquinone in 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0).

### 3.4. Analytical curves of hydroquinone and glyphosate

Once the best working conditions for the proposed biosensor were selected, a calibration curve was constructed using SWV in the potential range of −0.5 to +0.2 V vs. Ag/AgCl. Fig. 6 shows the square-wave voltammograms (A) and analytical curve (B) for

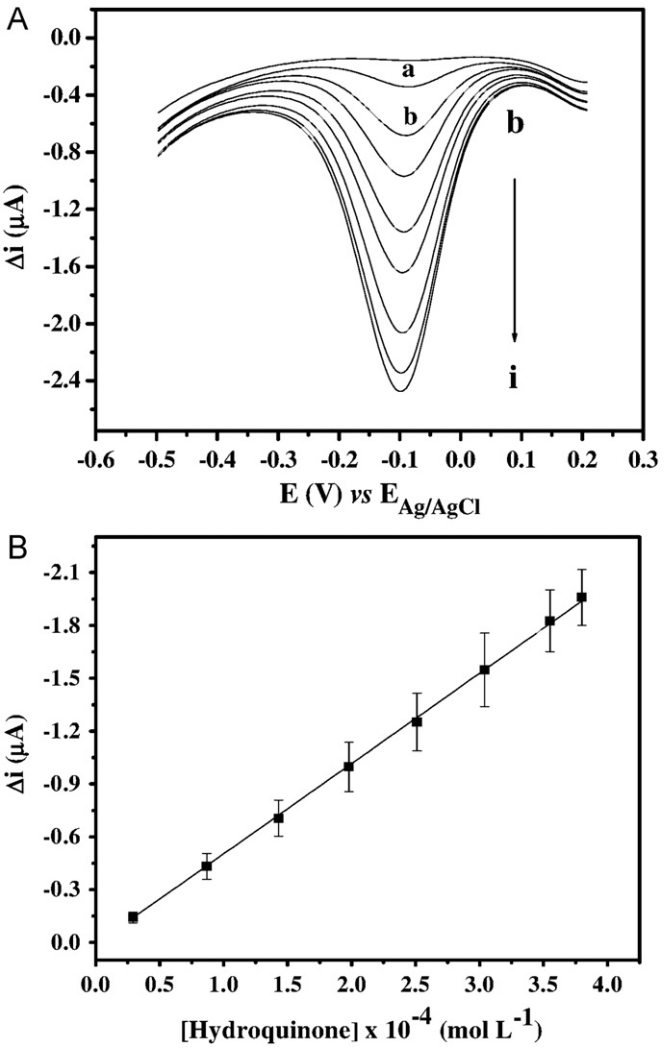


**Fig. 6.** (A) Square-wave voltammograms obtained using the developed biosensor in (a) phosphate buffer solution (0.1 mol $L^{-1}$, pH 7.0), and with the addition of standard solutions of hydroquinone in the following concentrations: (b) $2.92 \times 10^{-5}$ mol $L^{-1}$, (c) $8.67 \times 10^{-5}$ mol $L^{-1}$, (d) $1.43 \times 10^{-4}$ mol $L^{-1}$, (e) $1.98 \times 10^{-4}$ mol $L^{-1}$, (f) $2.51 \times 10^{-4}$ mol $L^{-1}$, (g) $3.04 \times 10^{-4}$ mol $L^{-1}$, (h) $3.55 \times 10^{-4}$ mol $L^{-1}$ and (i) $3.80 \times 10^{-4}$ mol $L^{-1}$; (B) Analytical curve for hydroquinone.

hydroquinone. The calibration curve was linear from $2.92 \times 10^{-5}$ to $3.80 \times 10^{-4}$ mol $L^{-1}$ ($\Delta i = -9.269 \times 10^{-9}$ ($\pm 3.350 \times 10^{-8}$) + 0.005 ($\pm 2.880 \times 10^{-4}$) [hydroquinone]; $r = 0.9997$), where Δi is the peak resultant current in μA, and [hydroquinone] is the concentration of hydroquinone in mol $L^{-1}$.

In this study, the detection principle of the herbicide was based on the inhibition of the peroxidase activity. In its catalytic cycle, the ferric form of the peroxidase is oxidised by hydrogen peroxide (a), which is called compound I (radical oxyferryl). Through the transfer of an electron from the substrate (b), compound I is reduced to the form known as compound II and returns to its native form with a another electron transfer from the substrate molecule to the enzyme (c), as shown in Fig. 2 [24]. Studies show that when the peroxidase is exposed to an inhibitor, it can coordinate with compound I, inhibiting the electrocatalytic activity of the enzyme and consequently decreasing the current response of the biosensor [31]. Songa et al. suggest that the $^{+}NH_2$ group of glyphosate coordinates with the oxygen atoms in the carbonyl groups of peroxidase, forming an enzyme–glyphosate complex [32]. The inhibitory effect of glyphosate on the enzymatic activity was followed by the oxidation of the substrate (hydroquinone), catalysed by the enzyme to *p*-benzoquinone, in

which the electrochemical reduction to hydroquinone was monitored at a constant potential. The decrease in the cathodic peak current for the hydroquinone produced after incubation in the glyphosate solution was used to measure the corresponding inhibitory effect. The square-wave voltammograms and the analytical curve for glyphosate were obtained using the proposed biosensor at a potential of −0.1 V vs. Ag/AgCl. Fig. 7(A) shows the square-wave voltammograms, where the voltammogram (a) corresponds to the blank measurement obtained in 0.1 mol $L^{-1}$ phosphate buffer solution (pH 7.0), (b) is the peak current obtained for $1.89 \times 10^{-4}$ mol $L^{-1}$ of hydroquinone in the absence of the inhibitor (base signal), and the other voltammograms correspond to the increasing concentrations of glyphosate. These relative percentages of inhibition are correlated with the glyphosate concentration. Fig. 7(B) shows that the analytical curve obtained for glyphosate was linear at concentrations from 0.10 to 4.55 mg $L^{-1}$ (IR=59.7266 (±0.23484)+30.83801 (±0.28615) [glyphosate], $r$=0.9955), where IR is the relative inhibition percentage, and [glyphosate] is the log glyphosate concentration in mg $L^{-1}$, with a detection limit of 30 μg $L^{-1}$ and a quantification limit of 90 μg $L^{-1}$.

The detection limit (DL) and quantification limit (QL) were calculated using the standard deviation of the lower level of concentration ($s$) and the slope of analytical curve ($S$) as follows:

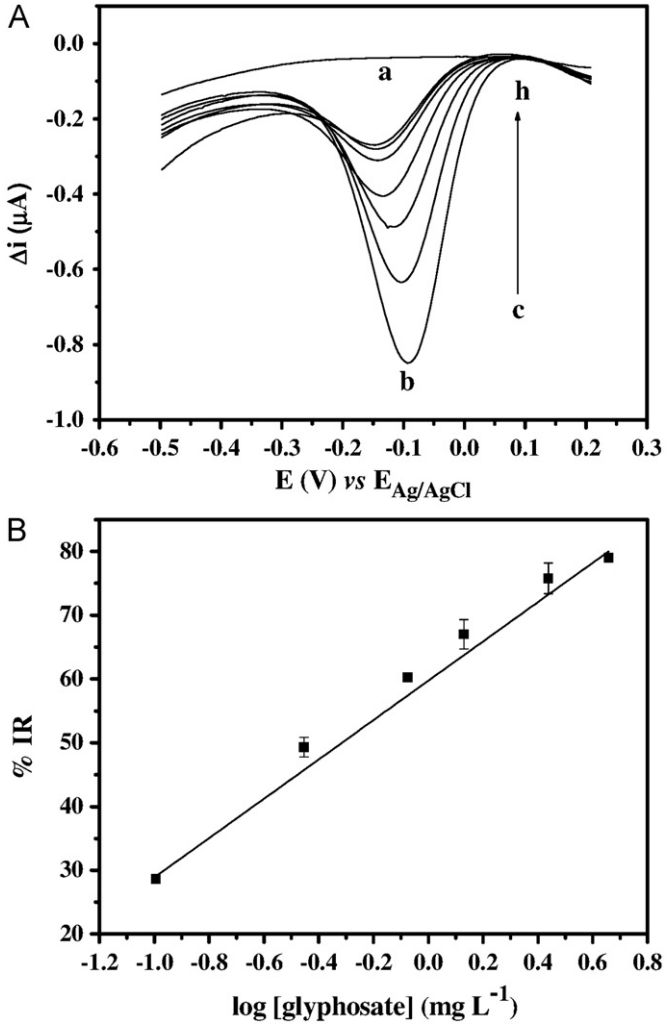


**Fig. 7.** (A) Square-wave voltammograms obtained using the developed biosensor in (a) phosphate buffer solution (0.1 mol $L^{-1}$, pH 7.0), (b) $1.89 \times 10^{-4}$ mol $L^{-1}$ hydroquinone and standard solutions of glyphosate in the following concentrations: (c) 0.10 mg $L^{-1}$, (d) 0.35 mg $L^{-1}$, (e) 0.84 mg $L^{-1}$, (f) 1.35 mg $L^{-1}$, (g) 2.74 mg $L^{-1}$, (h) 4.55 mg $L^{-1}$. (B) Analytical curve for glyphosate.

**Table 1**
Recovery of glyphosate in water samples using the biosensor and IEC.

| Fortified | Glyphosate (mg $L^{-1}$) | | | |
|---|---|---|---|---|
| | Found | | Recovery (%) | |
| | IEC | Biosensor[a] | IEC | Biosensor |
| 0.20 | Not found | 0.22 ± 0.013 | Not recovered | 108.9 |
| 1.00 | 0.80 ± 0.027 | 0.97 ± 0.017 | 79.6 | 96.9 |
| 1.75 | 1.39 ± 0.096 | 1.66 ± 0.048 | 79.5 | 94.9 |

[a] $n$=3, confidence level of 95%.

DL=3.3$s$/$S$ and QL=10$s$/$S$. According to the Brazilian National Health Surveillance Agency (ANVISA), through Ordinance N° 518/2004, the maximum allowed value of glyphosate in drinking water is 500 μg $L^{-1}$. For application in water analysis, the biosensor was sufficiently sensitive because the DL and QL are below the value allowed by ANVISA.

### 3.5. Recovery study and quantification of glyphosate

The atemoya biosensor was applied in the determination of glyphosate in natural water samples. Recovery measurements and quantification of glyphosate were performed by SWV, in triplicate, using water samples fortified with three concentration levels, 0.20, 1.00 and 1.75 mg $L^{-1}$, of herbicide. An ion exchange chromatography (IEC) method was also used in the quantification of the samples. The results obtained by both methods are presented in Table 1.

The results of the recovery rates in both methods were compared with the concentrations of glyphosate added to the water samples. Table 1 shows recovery values of 94.9–108.9% of glyphosate for the proposed biosensor and from 79.5% to 79.6% for IEC. According to Student's t-test, at a 95% confidence level, there are no significant differences between the recoveries obtained using the biosensor and the added concentrations of the glyphosate in water samples. The average recoveries demonstrate the accuracy of the biosensor. The lowest concentration of glyphosate was not recovered by the IEC, indicating that low levels would not be detected by this method. The biosensor showed better recovery percentages of glyphosate for all fortification levels evaluated when compared with the IEC.

The IEC has the advantage over the biosensor method of being a simpler method that only requires the filtration of water samples. However, the main disadvantages are the higher cost of the chromatographic instrument and the higher detection limit.

## 4. Conclusions

Atemoya peroxidase was successfully immobilised on nanoclay and associated with carbon nanotubes, which led to an efficient biocatalyst. The biosensor constructed from this material combined with the inhibition effect of the biocide on the enzyme activity proved to be satisfactory for the determination of glyphosate in water samples. Furthermore, the biosensor construction was simple, inexpensive and displays long-term stability, a satisfactory linear range with low limit detection and good repeatability and reproducibility for glyphosate quantification.

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