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Determination of thiodicarb using a biosensor based on alfalfa sprout peroxidase immobilized in self-assembled monolayers

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ABSTRACT

A biosensor based on alfalfa sprout (*Medicago sativa*) homogenate as a source of peroxidase is proposed for the determination of thiodicarb by square-wave voltammetry. This enzyme was immobilized in self-assembled monolayers of L-cysteine on a gold electrode. Several parameters were investigated to evaluate the optimum conditions for operation of the biosensor. The analytical curve was linear for thiodicarb concentrations of 2.27×10^{-6} to 4.40×10^{-5} mol L^{-1} with a detection limit of 5.75×10^{-7} mol L^{-1}. The lifetime of the Au-alfalfa sprout–SAMs was 20 days (at least 220 determinations). The average recovery of thiodicarb from samples of vegetable extracts ranged from 99.02 to 101.04%. The results obtained for thiodicarb in vegetable extracts using the proposed method are in close agreement with those using a high performance liquid chromatography procedure at the 95% confidence level.

1. Introduction

Several toxic organic compounds (herbicides, fungicides, acaricides, and insecticides) have been used in agriculture to control diseases and obtain high yields. Residues of these pesticides may enter the food chains through air, water and soil and cause numerous health problems to ecosystems and humans. They can produce bone marrow diseases, infertility, nerve disorders, and immunological and respiratory diseases [1]. Therefore, the presence of residues of pesticides in natural waters and in foods is of major concern for public health reasons [2].

Carbamate compounds are used as pesticides on a large scale worldwide due to their wide ranging biological activity. These pesticides include a broad-spectrum of insecticides widely used as acaricides, molluscicides, nematocides and helmithicides [3,4]. People involved in the manufacture or application of carbamates may be exposed to these pesticides in the general environment or in the workplace [5]. Several carbamate compounds are highly toxic to humans and other mammals. Carbamate acts by inhibiting the cholinesterase enzymes, especially acetylcholinesterase, leading to an accumulation of acetylcholine in nerve synapses, triggering a series of parasympathomimetic effects. Carbamates are reversible inhibitors of colinesterases, but poisoning can be very serious [6].

Thiodicarb (IUPAC: 3,7,9,13-tetramethyl-5,11-dioxa-2,8,14-trithia-4,7,9,12-tetra-azapentadeca-3,12-diene-6,10-dione) is a carbamate pesticide with a relatively narrow spectrum of activity closely related to its first metabolite, methomyl. It acts against slugs (on ingestion) as well as lepidopterous pests, controlling larvae at different stages as well as eggs in many instances. It is neurotoxic through the inhibition of cholinesterase causing paralysis followed by death. This pesticide is used in seed treatment plants and for the control of various pests in cotton, soybeans, tomatoes, peanuts, corn and leafy vegetables, and other crops [7].

The analytical methods which are most commonly employed in the determination of environmental pesticides are high performance liquid chromatography (HPLC) and gas chromatography (GC) with various detectors [8–10]. These methods require homogenization, extraction, clean-up of the sample, concentration and analytical determination, often involving time consuming procedures. Biosensors offer an alternative means to determine pesticides in environmental and food matrices [11] and have the advantage of low cost, high sensitivity, easy operation and the possibility for the construction of simple portable devices for fast screening purposes [12]. Enzyme biosensors use the inhibitor effects of harmful compounds on the enzyme activity [13]. The ability to detect these toxic compounds via their inhibiting action on the esterase enzymes has resulted in the development of many biosensors based on esterases [14,15]. Biosensors based on the inhibition of oxidoreductase enzymes (tyrosinase and peroxidase) by carbamate pesticides [16–18] have also been described, the pres-

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ence of these inhibitor compounds causing a constant diminution in the current response of the biosensor.

The immobilization of enzymes is an indispensable part of the development of biosensors. The enzyme can be attached via the formation of self-assembled monolayers (SAMs), which involves the deposition of stable layers, spontaneously organized into ordered monolayers, directly onto the transducer surface (e.g. Au, Si, Pt). The major advantages are the simplicity and sensitivity of the process, and the strong and stable biomolecule attachment. The formation of SAMs of alkanethiolates (e.g. L-cysteine, mercaptopropionic acid, cystamine) on gold has been used as an efficient method to prepare an electrochemically active surface and ensure the orientation of the enzymes in the immobilization procedure [19,20].

In this study, a homogenate of alfalfa sprouts was used as the peroxidase enzyme source. The peroxidases constitute a broad class of enzymes that are widely distributed in the animal and plant kingdoms. Most peroxidases are heme proteins and contain iron(III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. The catalytic cycle involves native peroxidase (Fe^{3+}), hydrogen peroxide and a donor substrate (e.g. phenolic compounds and catecholamines). Peroxidases also catalyze a diversity of oxygen transfer reactions including sulfoxidations, olefin epoxidations and allylic, benzylic, and propargylic hydroxylations. The catalytic cycle of peroxidase occurs in three stages: the ferric form of the enzyme is oxidized by hydrogen peroxide (called compound I), which is the radical oxyferryl. Through the transfer of an electron from the substrate, compound I is then reduced to the form known as compound II and returns to its initial phase with a new electron transfer from one substrate molecule to the enzyme [21–23]. When an inhibitor is introduced into the reaction medium, it can coordinate with the compound I intermediate, resulting in a decrease in enzymatic activity and consequent decrease in the signal chain [24]. Several organic and inorganic compounds, such as metals, p-aminobenzoic acid, cysteine, glutathione, hydroxylamine, sulfides, thiouracils, dichromate, thiols, diethyldithiocarbamate and cyanide, have been reported to inhibit the action of peroxidase [17,25–27].

There are few studies employing bioelectrodes for the determination of pesticides in real samples. For this reason, in this paper we describe a biosensor based on Au-alfalfa sprout–SAMs which was applied to the determination of thiodicarb in vegetable samples. This toxic compound can be determined from its inhibitory effect on peroxidase activity. Lastly, the results obtained with the proposed bioelectroanalytical method were compared with those obtained using the well-established HPLC method for thiodicarb analysis.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and were used without further purification and all solutions were prepared with deionized water. Adrenaline, dopamine, caffeic acid, guaiacol, hydroquinone, methyldopa, L-cysteine, cyanamide, ethylic alcohol, glutaraldehyde and thiodicarb were purchased from Sigma. Alumina slurry (water suspension of Al_2O_3 of 0.3 and 0.05 μm) was obtained from Aratec, Brazil. A "piranha solution" was prepared with a 3:1 (v/v) mixture of H_2SO_4 with purity >95% (Sigma) and H_2O_2 30% (v/v) (Sigma). Phosphate buffer (0.1 mol L^{-1}, pH 7.0) solution was used as the supporting electrolyte. A 2.0×10^{-2} mol L^{-1} hydroquinone stock solution was prepared daily in 0.1 mol L^{-1} phosphate buffer solution at pH 7.0. The alfalfa sprouts (*Medicago sativa*) were purchased from a local producer in Florianópolis/SC, Brazil and used as a source of peroxidase enzyme.

2.2. Apparatus

Square-wave voltammetry experiments were performed using an Autolab PGSTAT12 potentiostat/galvanostat (Eco Chemie, The Netherlands) connected to data processing software (GPES, software version 4.9.006, Eco Chemie). All experiments were carried out using a conventional three-electrode system: the working electrode used was a gold (Au) electrode (surface area 2.0 mm^2) modified with a self-assembled monolayer and immobilized alfalfa sprout peroxidase (Au-alfalfa sprout–SAMs); an Ag/AgCl (3.0 mol L^{-1} KCl) electrode was used as the reference and a platinum wire as the auxiliary electrode. Electrochemical experiments were performed in an electrochemical cell containing 5.0 mL of the supporting electrolyte at room temperature (25.0 ± 0.5 °C). The ultrasound bath cleaner used was 1400A Unique with time control and a frequency of 55 Hz. A Hewlett-Packard (Boise, ID, USA) model 8452A UV-visible spectrophotometer with a quartz cell (optical path of 1.00 cm) was used for determination of the peroxidase activity.

2.3. Obtainment of peroxidase homogenate

Alfalfa sprouts (*M. sativa*) represent a low cost source of enzyme peroxidase. For production of the raw extract, a portion of the vegetable matter (25 g) was homogenized in a mixer with 100 mL of 0.1 mol L^{-1} phosphate buffer solution, at pH 7.0. The extract was then rapidly filtered and the resulting supernatant solution was maintained at 4 °C. This solution was used as the peroxidase source in the construction of the Au-alfalfa sprout–SAMs.

2.4. Determination of peroxidase activity

The enzymatic activity of alfalfa sprout peroxidase was determined in triplicate using a spectrophotometric method [28], based on the change of absorbance at 470 nm due to the formation of tetraguaiacol (the product of guaiacol oxidation). Peroxidase activity was measured in a reaction medium containing 0.1 mol L^{-1} phosphate buffer (pH 7.0); 0.2 mL of enzyme solution, 2.7 mL of 0.05 mol L^{-1} guaiacol solution and 0.1 mL of 10×10^{-3} mol L^{-1} hydrogen peroxide solution at 25 °C. The kinetic evolution of the absorbance at 470 nm was measured for 1 min. One unit of peroxidase was defined as the amount of enzyme sufficient to produce 0.001 units of absorbance per min. Control experiments (blank runs) were carried out using the same procedure, but in the absence of peroxidase.

2.5. Preparation of the Au-alfalfa sprout–SAMs

Before SAM deposition, the gold electrode was subjected to a pre-treatment cleaning. Firstly, a mechanical pre-treatment was carried out where the electrode surface was manually polished with aqueous slurries of alumina (0.3 and 0.05 μm) for approximately 2 min each. The sensing surface was then thoroughly rinsed with deionized water and cleaned in an ultrasonic bath with water for 1 min to remove any residual alumina particles that may be trapped on the surface of the electrode. Next, the electrode was immersed in piranha solution [H_2O_2:H_2SO_4 (1:3, v/v)] for around 10 min and rinsed with deionized water. The electrode was then cleaned electrochemically by cycling the electrode potential between 0.0 and +1.7 V vs. Ag/AgCl (3.0 mol L^{-1} KCl) electrode until the characteristic voltammograms for a clean electrode were observed [29].

After cleaning, the gold substrate was immediately immersed in a solution of 1.0×10^{-3} mol L^{-1} L-cysteine dissolved in ethanol for 2 h at room temperature to form the SAMs. Subsequently, the substrate was rinsed with pure ethanol to remove weakly reacted components and dried at room temperature. The peroxi-

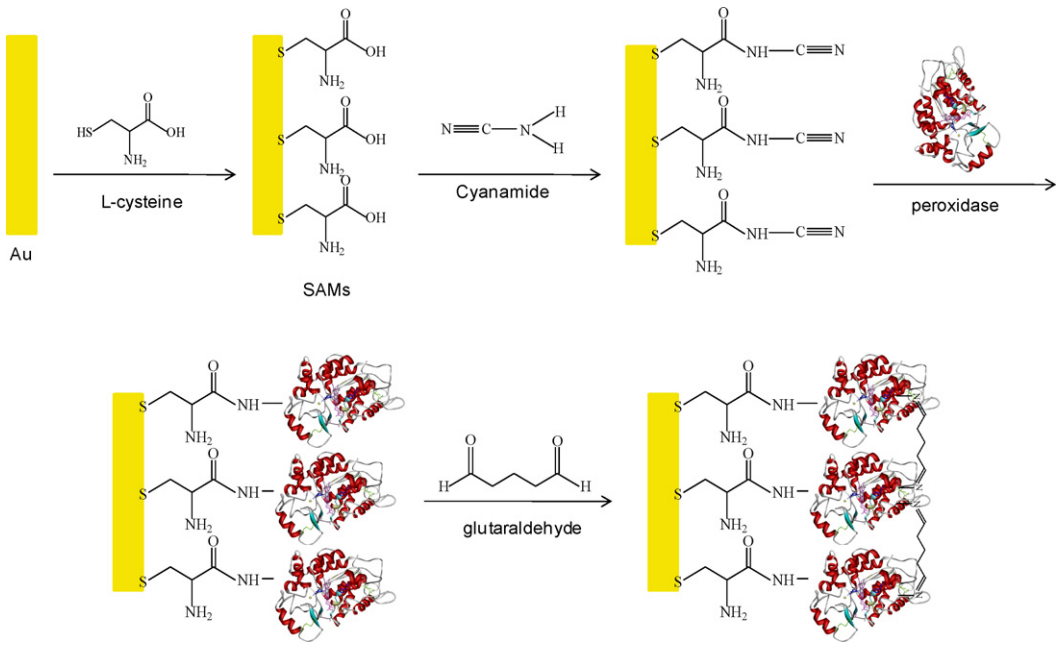


Fig. 1. Schematic representation of the formation of the Au-alfalfa sprout–SAMs.

dase enzyme (60.0 units mL^{-1}) obtained from alfalfa sprouts was covalently anchored on the SAM electrode through the activation of terminal carboxylic acid groups of L-cysteine. For this, the gold-modified substrate was treated with a solution of carbodiimide (0.36 mol L^{-1}) in phosphate buffer solution (pH 7.0) for 1 h and dried at room temperature. Finally, the resulting electrode was dipped in a 2.5% solution of glutaraldehyde for a period of 1 h to form crosslinks in order to avoid desorption of the enzyme. When not in use the biosensor was stored at room temperature.

2.6. Application of the biosensor to the analysis of thiodicarb

The vegetables were purchased from a local market in Florianópolis, SC, Brazil. To determine the pesticide residue in the vegetable samples (apple, potato and strawberry), 20 g of each chopped vegetable was extracted with 50 mL of ethanol and the mixture was mechanically macerated for 5 min, using a mortar and pestle. The extract was filtered three times in a Buchner funnel attached to a Buchner flask and a vacuum pump, collected and stored at 4 °C. The determination of thiodicarb in the vegetable samples followed the method of standard addition: an accurate volume of 20 μL of the sample was transferred to the glass cell containing 5 mL of the phosphate buffer solution at pH 7.0 by micropipette. The measurements were performed after successive additions of the reference thiodicarb. All measurements were performed in triplicate.

Square-wave voltammetry measurements were performed in an unstirred and non-deaerated 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) in a 5.0 mL glass cell. The square-wave voltammograms were recorded by applying in all cases a potential of +0.2 to −0.4 V vs. Ag/AgCl at 25.0 ± 0.5 °C. All potentials were measured and reported vs. Ag/AgCl (3.0 mol L^{-1} KCl) after a suitable initial stirring time of 60 s in 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) in order to homogenize the solution before current monitoring.

2.7. HPLC analysis

The validation of the proposed analytical method was carried out on a chromatographic system consisting of a Varian Pro Star chromatograph with a 210 ternary pump, and a Varian Pro Star 325 UV-Vis detector was used together with a C18 reverse phase column (250 mm × 4.6 mm × 5 μm) (Varian). The injection volume was 20 μL. The mobile phase was a water/acetonitrile mixture (65:35) and elution of carbamate was monitored at 220 nm. The flow rate was 1 mL min^{-1} and the time required for analysis of one sample was around 18 min [30].

3. Results and discussion

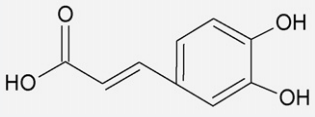
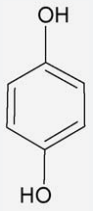
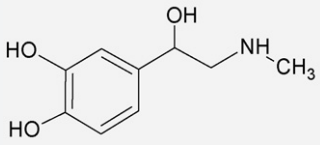
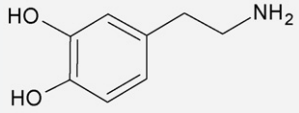
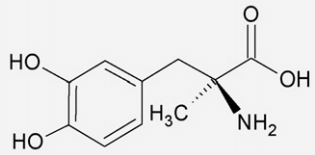
3.1. Formation of Au-alfalfa sprout–SAM biosensor surface

The biosensor based on alfalfa sprout peroxidase was constructed on SAMs of L-cysteine. The thiol group in L-cysteine allowed the assembly on a gold electrode through the strong sulfur–gold interaction. A short alkyl chain alkanethiol, usually no more than three carbons, should be used to attach the redox center of the enzyme as closely as possible to the metallic base of the electrode to facilitate the electron transfer [31]. The terminal carboxylic group of alkanethiolate SAMs is frequently used to immobilize proteins due to its reactivity with certain chemical groups in these biomolecules. To immobilize peroxidase tightly onto the Au-SAM electrode surface, the amino groups of the enzyme were coupled to the acidic groups of L-cysteine through the formation of imides using a carbodiimide solution. The reaction of glutaraldehyde through the formation of Schiff's bases is commonly applied in the covalent binding of enzymes to their primary amino groups [32]. Fig. 1 shows a schematic representation of the formation of the self-assembled monolayers and immobilization of the peroxidase covalently crosslinked with glutaraldehyde at the Au-alfalfa sprout–SAM biosensor surface.

3.2. Study of phenolic compounds

In order to investigate the affinity of this enzyme for different phenolic compounds, caffeic acid, dopamine, epinephrine, hydroquinone and methyldopa were selected and investigated. The biosensor responses for these substrates were obtained at 1.53×10^{-3} mol L^{-1} in 0.1 mol L^{-1} phosphate buffer solutions (pH 7.0). Table 1 shows a comparison of the relative responses (%) obtained. As can be seen, the proposed biosensor showed decreas-

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

Phenolic compound	Structure	Relative response (%)
Caffeic acid		100
Hydroquinone		77
Epinephrine		67
Dopamine		44
Methyldopa		11

ing sensitivity in the order: caffeic acid (100%), hydroquinone (77%), epinephrine (67%), dopamine (44%) and methyldopa (11%). In this study, hydroquinone was selected for optimization of the biosensors.

3.3. Influence of the pH and enzyme concentration

The influence of the pH was studied in the range of 6.0–8.0 using 1.0×10^{-3} mol L^{-1} hydroquinone and 2.0×10^{-5} mol L^{-1} hydrogen peroxide solutions. As seen in Fig. 2A, when the pH was increased from 6.0 to 7.0 increases in the enzyme biosensor response were observed. On the other hand, when the pH was increased from 7.0 to 8.0 decreases in the enzyme biosensor response were recorded. The current response resulting from the enzyme-catalyzed reaction achieved a maximum value at pH 7.0. Consequently, this pH was selected as the working pH.

The enzymatic activity of the bioactive layer of the biosensor is dependent on the concentration of enzyme used. The effect of peroxidase enzyme concentration (in the range of 20.0–80.0 units mL^{-1}) on the biosensor response was investigated (Fig. 2B). According to the optimization studies, the best response was obtained for a concentration of 60.0 units mL^{-1}, and this concentration was subsequently used in the study.

3.4. Optimization of the SWV parameters

Square-wave voltammetry was used for the study of the electrocatalytic performance of the biosensor in terms of the electrochemical reduction of hydroquinone. To obtain the best experimental working conditions for the Au-alfalfa sprout–SAMs, frequency, pulse amplitude and scan increment were investigated.

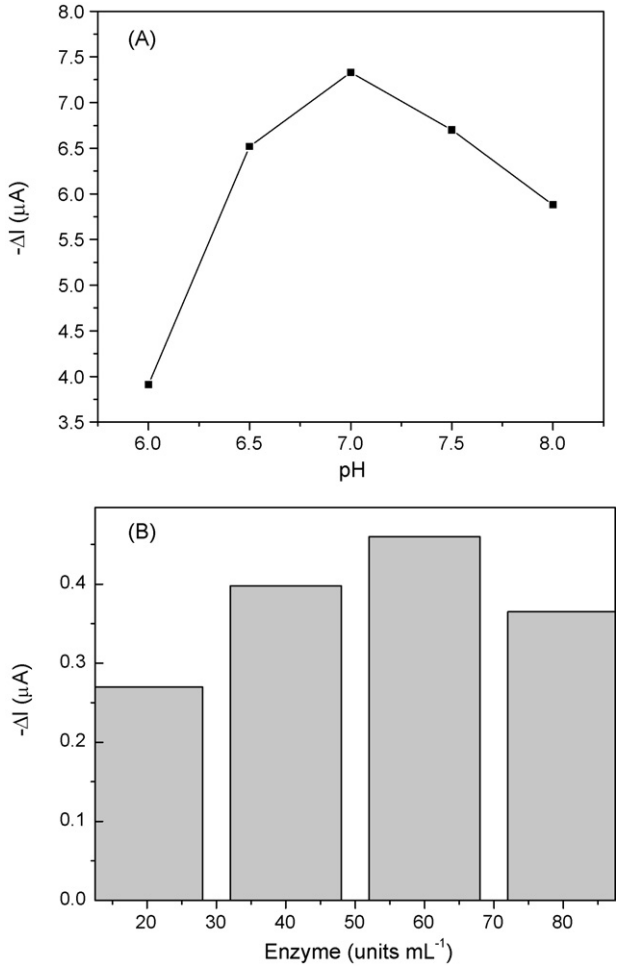


Fig. 2. (A) Influence of the pH and (B) enzyme concentration.

These parameters affect the sensitivity and signal intensity of the analytical methodology. Square-wave voltammetry parameters (frequency of 10–100 Hz, pulse amplitude of 10–100 mV and scan increment of 5.0–12.5 mV) were studied in relation to the Au-alfalfa sprout–SAM biosensor response to 6.37×10^{-4} mol L^{-1} hydroquinone and 2.0×10^{-5} mol L^{-1} hydrogen peroxide solutions at pH 7.0. In the investigation process, each variable was changed while the other two were kept constant. The maximum sensitivity of the biosensor response to the frequency parameter was obtained at 30.0 Hz. The best performance of the biosensor varying the pulse amplitude was obtained at 100.0 mV. In addition, the highest analytical signal obtained on varying the scan increment was at 12.5 mV. The best experimental conditions were selected for application in the following experiments.

3.5. Repeatability, reproducibility and stability of the biosensor

The repeatability of the current response obtained using the same biosensor was examined in phosphate buffer solutions (0.1 mol L^{-1}; pH 7.0) containing 1.6×10^{-4} mol L^{-1} hydroquinone solution and 2.0×10^{-5} mol L^{-1} hydrogen peroxide solution. The relative standard deviation was 1.76% for 10 successive measurements, indicating that the biosensor has a high level of repeatability.

Five Au-alfalfa sprout–SAM electrodes were constructed using the same procedure and were independently used for the determination of hydroquinone under the optimized conditions described previously. All the biosensors showed an acceptable reproducibility with a relative standard deviation of approximately 7.60%, indicat-

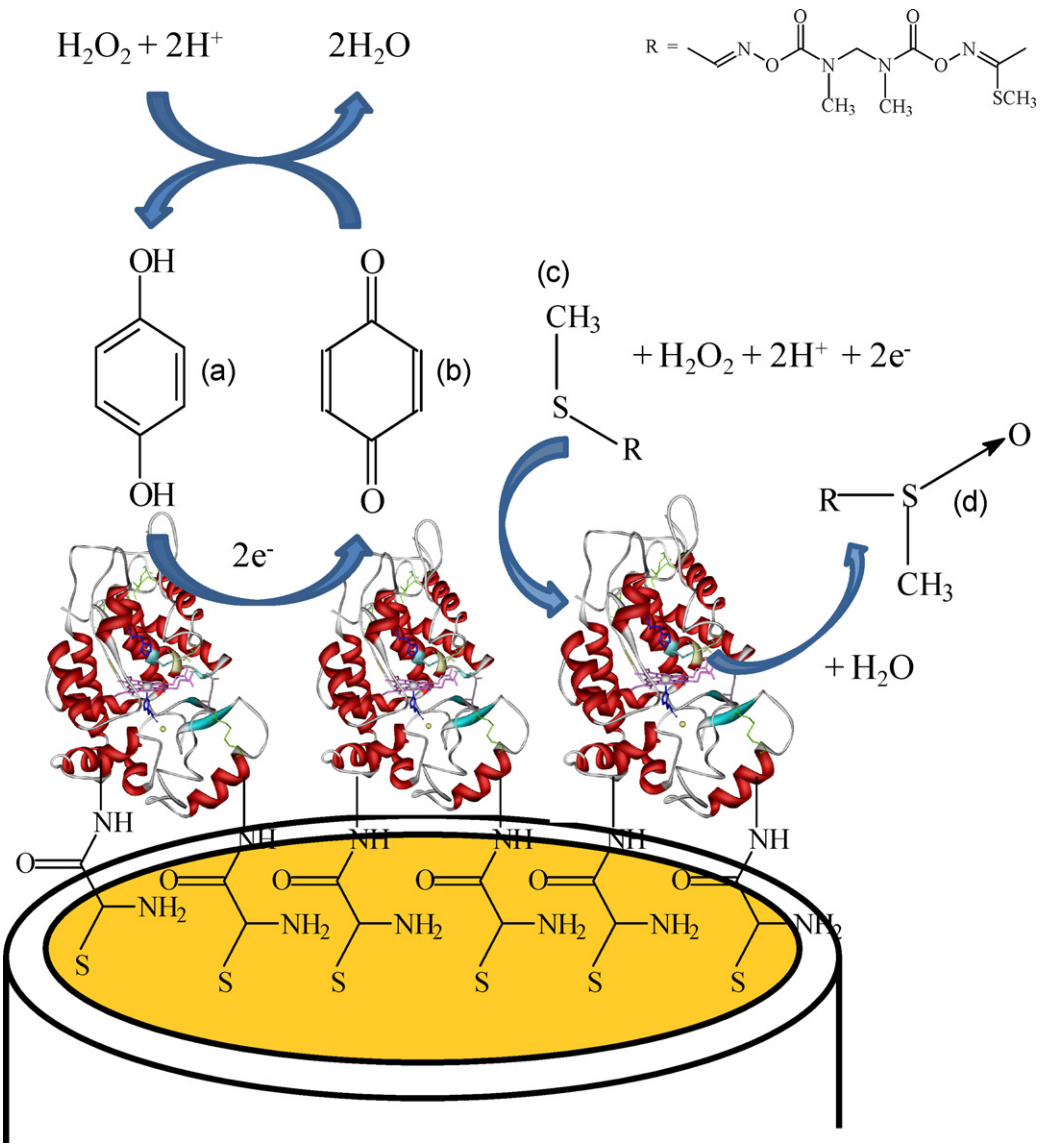


Fig. 3. Schematic representation of the reaction involving hydroquinone on the surface of the biosensor and the reaction between peroxidase and thiodicarb. (a) hydroquinone, (b) *p*-benzoquinone, (c) thiodicarb and (d) sulfoxide compound.

ing that the results obtained with the proposed biosensor have a high level of reproducibility.

The stability and lifetime of the biosensor were investigated over a period of 30 days maintained at room temperature. The current response was recorded in a 1.6×10^{-4} mol L^{-1} hydroquinone and 2.0×10^{-5} mol L^{-1} hydrogen peroxide solution in phosphate buffer solution (0.1 mol L^{-1}, pH 7.0). The Au-alfalfa sprout–SAM electrode was stable for at least 20 days (at least 220 determinations) without a change in the response.

3.6. Analytical curves of hydroquinone and thiodicarb

After establishing the optimal conditions for hydroquinone determination, an analytical curve was constructed using square-wave voltammetry employing the proposed biosensor in the potential range of +0.12 to −0.38 V vs. Ag/AgCl. A lower working potential will prevent the oxidation of possible interferents present which would oxidize at more positive potentials. The analytical curve obtained for hydroquinone was linear in the range of 9.75×10^{-5} to 1.28×10^{-3} mol L^{-1} ($-\Delta I = 0.26$ (± 0.02) + 2.48 (± 0.05) $\times 10^3$ [hydroquinone]; $r = 0.9986$), where ΔI is the resultant peak current in μA and [hydroquinone] is the hydroquinone concentration in mol L^{-1}. Fig. 3 shows a schematic representation of the reaction involving hydroquinone on the surface of the biosensor containing immobilized peroxidase. Initially, the hydroquinone (a) is oxidized by peroxidase in the presence of hydrogen peroxide to *p*-benzoquinone (b) and this product is then electrochemically reduced back to hydroquinone at a potential of −0.072 V.

In this study, the reaction between the *p*-benzoquinone produced in this enzymatic process and the thiodicarb added led to a decrease in the steady-state current. This decrease in the current has also been observed in the determination of sulfides and carbamate using biosensors based on the inhibition of peroxidase and tyrosinase [16,26]. Table 2 shows biosensors based on oxidoreductase enzymes (tyrosinase, laccase and peroxidase), developed by several authors, for the determination of a variety of analytes through methods of inhibition [26,33–39]. These biosensors have been proposed as alternative devices for the detection of pesticides. Furthermore, the use of redox enzymes has the advantage of the application of a low potential in relation to the reference electrode minimizing interferences [40].

Table 2
Biosensors containing oxidoreductases for analyte determination by inhibition process.

Enzyme	Analyte	Linear range (mol L^{-1})	Detection limit (mol L^{-1})	References
Tyrosinase	Ziram	0.2×10^{-6} to 2.2×10^{-6}	0.074×10^{-6}	[33]
	Diram	4.0×10^{-6} to 44×10^{-6}	1.3×10^{-6}	
	Zinc diethyldithiocarbamate	4.0×10^{-6} to 40×10^{-6}	1.7×10^{-6}	
Tyrosinase	L-cysteine	6.0×10^{-5} to 8.0×10^{-4}	4.4×10^{-6}	[34]
Tyrosinase	Fluoride	1×10^{-6} to 20×10^{-6}	–	[35]
Polyphenol oxidase	Benzoic acid	25×10^{-6} to 100×10^{-6}	25×10^{-6}	[36]
Peroxidase	L-ascorbic acid	2.0×10^{-4} to 5.5×10^{-3}	2.2×10^{-5}	[37]
Peroxidase	Sulfides	0.5×10^{-6} to 12.7×10^{-6}	0.3×10^{-6}	[26]
Peroxidase	Phenylhydrazine	2.5×10^{-7} to 1.1×10^{-6}	8.2×10^{-8}	[38]
Laccase	L-cysteine	4.99×10^{-5} to 4.54×10^{-3}	-	[39]
Peroxidase	Thiodicarb	2.27×10^{-6} to 4.40×10^{-5}	5.75×10^{-7}	This study

Table 3
Linearity and regression equations obtained for thiodicarb as a function of hydroquinone concentration.

[hydroquinone] (mol L^{-1})	Linearity range (mol L^{-1})	Equation (Ipc (mA) = A – B[thio][a])	Correlation coefficient (r)
1.96×10^{-4}	2.27×10^{-6} to 4.40×10^{-5}	$Ipc = 0.5602 - 6.3376 \times 10^{3}$ [thio]	0.9997
5.77×10^{-4}	4.37×10^{-6} to 5.78×10^{-5}	$Ipc = 1.3072 - 1.1533 \times 10^{4}$ [thio]	0.9995
1.12×10^{-3}	1.85×10^{-5} to 8.20×10^{-5}	$Ipc = 2.3700 - 2.0239 \times 10^{4}$ [thio]	0.9995

[a] [thio]: Thiodicarb.

Thus, the influence of hydroquinone concentrations of 1.96×10^{-4}, 5.77×10^{-4} and 1.12×10^{-3} mol L^{-1} on the linearity of the thiodicarb analytical curves was investigated and the results are shown in Table 3. Good linearity and better correlation coefficients were observed at lower hydroquinone concentrations. Therefore, a hydroquinone concentration of 1.96×10^{-4} mol L^{-1} was used in this study.

The square-wave voltammograms and analytical curve for thiodicarb were obtained employing the biosensor. Fig. 4A shows the square-wave voltammograms obtained using the biosensor in (a) 0.1 mol L^{-1} phosphate buffer solution (pH 7) and (b) 1.96×10^{-4} mol L^{-1} hydroquinone, and the other voltammograms correspond to increasing additions of thiodicarb (from bottom to top). As shown in Fig. 4B, the analytical curve obtained for thiodicarb was linear for concentrations of 2.27×10^{-6} to 4.40×10^{-5} mol L^{-1} ($-\Delta I = 0.56\,(\pm 0.001) - 6.33\,(\pm 0.05) \times 10^{3}$ [thiodicarb]; $r = 0.9997$) where ΔI is the resultant peak current in μA and [thiodicarb] is the thiodicarb concentration in mol L^{-1}, with a detection limit of 5.75×10^{-7} mol L^{-1} and quantification limit of 1.92×10^{-6} mol L^{-1}.

Consequently, when the thiodicarb was added to the hydroquinone solution, a decrease in the cathodic peak current was observed which was proportional to the increase in the thiodicarb concentration, demonstrating that thiodicarb has an inhibitory effect on the reaction. This sulfur-containing compound can inhibit the activity of the enzyme through binding with the heme group in peroxidase at the non-catalytic site of the enzyme, changing its structure [41]. On the other hand, as previously described, peroxidases can also catalyze the transfer of oxygen from hydrogen peroxide (e.g. sulfoxidation, epoxidation). Therefore, these results suggest that there may also be a sulfoxidation reaction between the enzyme and thiodicarb. Fig. 3 also shows a schematic representation of the possible reaction between peroxidase and thiodicarb. When the thiodicarb (c) is added, the peroxidase catalyzes the sulfoxidation of thiodicarb with an initial electron transfer from the substrate to compound I followed by oxygen atom transfer from the reduced species of compound I, so-called compound II, to produce the sulfoxide (d) [42].

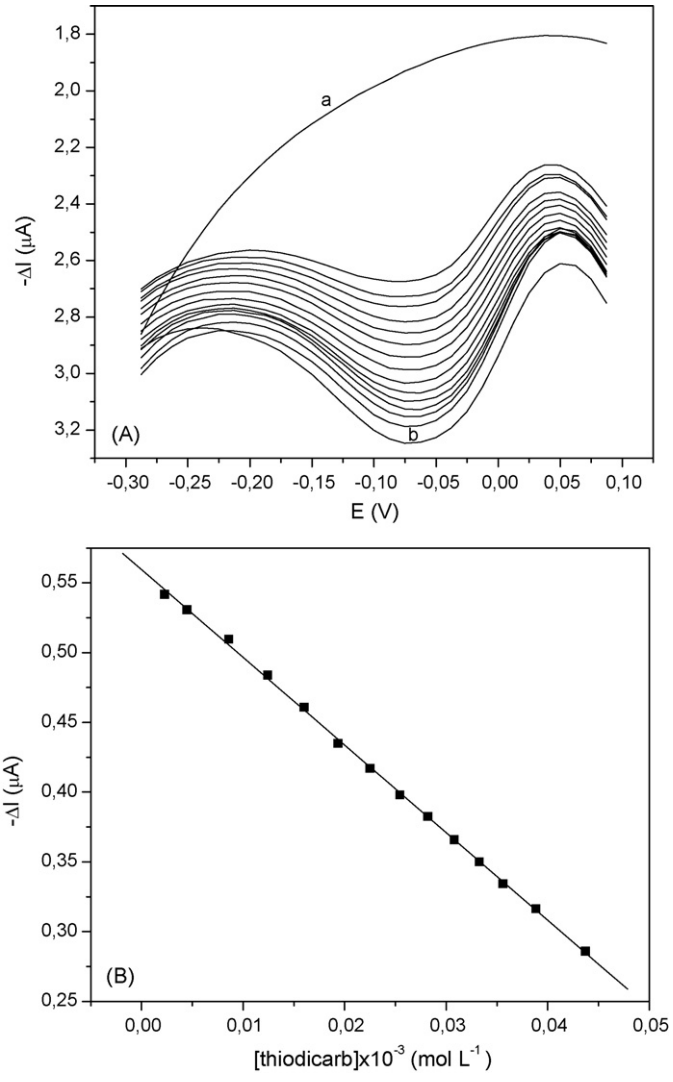


Fig. 4. (A) Square-wave voltammograms obtained using the proposed biosensor in (a) 0.1 mol L^{-1} phosphate buffer solution (pH 7), (b) 1.96×10^{-4} mol L^{-1} hydroquinone and other additions equivalent to thiodicarb concentrations of 2.26×10^{-6}, 4.45×10^{-6}, 8.58×10^{-6}, 1.24×10^{-5}, 1.60×10^{-5}, 1.93×10^{-5}, 2.24×10^{-5}, 2.54×10^{-5}, 2.81×10^{-5}, 3.08×10^{-5}, 3.32×10^{-5}, 3.55×10^{-5}, 3.88×10^{-5} and 4.37×10^{-5} mol L^{-1} (from bottom to top). (B) Analytical curve for thiodicarb.

3.7. Recovery study and thiodicarb determination

The recovery study was performed, in triplicate, using three vegetable samples (apple, potato and strawberry). The recovery experiments were performed by the standard addition technique.

Table 4
Recovery of thiodicarb in vegetable extracts using the proposed method.

Sample	Thiodicarb (mg L^{-1})		Recovery (%)
	Added	Found	
Potato	2.52	2.53	100.51
	3.30	3.29	99.91
	4.05	4.04	99.88
	2.52	2.54	100.66
Apple	3.30	3.28	99.60
	4.05	4.04	99.82
	2.52	2.55	101.04
Strawberry	3.30	3.31	100.23
	4.05	4.01	99.02

Table 5
Determination of thiodicarb (mg L^{-1}) in vegetable extract samples using the official method and the biosensor method.

Samples	Biosensor[a]	Official method	Er (%)[b]
Potato	0.80 ± 0.20	0.83 ± 0.10	+3.36
Apple	1.61 ± 0.40	1.60 ± 0.10	−0.72
Strawberry	2.41 ± 0.20	2.43 ± 0.10	+0.66

[a] $n = 3$, confidence level of 95%.
[b] Biosensor vs. official method.

The results obtained were compared with the thiodicarb standard concentrations added (2.52, 3.30 and 4.05 mg L^{-1}). The results presented in Table 4 show recovery values of 99.02–101.04%. These average recoveries demonstrate the accuracy of the proposed method and suggest an absence of matrix effects in these determinations.

The performance of the proposed method in the determination of thiodicarb in the three vegetable samples described above was evaluated. Analysis was carried out using the multiple standard additions method, in triplicate, and the results obtained using the biosensor were compared with those obtained using the high performance liquid chromatography method [42] (Table 5). According to Student's *t*-test, at a 95% confidence level, there are no significant differences between the results obtained using the standard method and with the biosensor. It can thus be concluded that the method is suitable for this application.

4. Conclusions

The proposed method using an Au-alfalfa sprout–SAM electrode was successfully applied in the selective determination of thiodicarb in vegetable extracts. There was no significant difference between the results obtained with this method and the official method and it can thus be concluded that the biosensor can be used for thiodicarb determination without matrix effects from the samples. This modified electrode also offers the advantages of good linear range, stability, absence of a derivatization step, low cost and rapid response time.

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