Bioelectrochemistry 95 (2014) 7–14

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Simple laccase-based biosensor for formetanate hydrochloride quantification in fruits

Francisco Wirley Paulino Ribeiro [a,b], Maria Fátima Barroso [a,c], Simone Morais [a,*], Subramanian Viswanathan [a], Pedro de Lima-Neto [b], Adriana N. Correia [b], Maria Beatriz Prior Pinto Oliveira [c], Cristina Delerue-Matos [a]

[a] REQUIMTE, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida 431, 4200-072 Porto, Portugal
[b] GELCORR, Departamento de Química Analítica e Físico-Química, Centro de Ciências, Universidade Federal do Ceará, Bloco 940 Campus do Pici, 60455-970, Fortaleza, CE, Brazil
[c] REQUIMTE, Serviço de Bromatologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

ARTICLE INFO

Article history:
Received 28 May 2013
Received in revised form 12 September 2013
Accepted 15 September 2013
Available online 8 October 2013

Keywords:
Enzymatic biosensor
Gold nanoparticles
Laccase
Formetanate hydrochloride
Fruits

ABSTRACT

This work describes the development of an electrochemical enzymatic biosensor for quantification of the pesticide formetanate hydrochloride (FMT). It is based on a gold electrode modified with electrodeposited gold nanoparticles and laccase. The principle behind its development relies on FMT's capacity to inhibit the laccase catalytic reaction that occurs in the presence of phenolic substrates. The optimum values for the relevant experimental variables such as gold nanoparticles electrochemical deposition (at −0.2 V for 100 s), laccase immobilization (*via* glutaraldehyde cross-linking), laccase concentration (12.4 mg/mL), substrate selection and concentration (5.83×10^{-5} M of aminophenol), pH (5.0), buffer (Britton–Robinson), and square-wave voltammetric parameters were determined. The developed biosensor was successfully applied to FMT determination in mango and grapes. The attained limit of detection was $9.5 \times 10^{-8} \pm 9.5 \times 10^{-10}$ M ($0.02 \pm 2.6 \times 10^{-4}$ mg/kg on a fresh fruit weight basis). Recoveries for the five tested spiking levels ranged from 95.5 ± 2.9 (grapes) to 108.6 ± 2.5% (mango). The results indicated that the proposed device presents suitable characteristics in terms of sensitivity (20.58 ± 0.49 A/μM), linearity (9.43×10^{-7} to 1.13×10^{-5} M), accuracy, repeatability (RSD of 1.4%), reproducibility (RSD of 1.8%) and stability (19 days) for testing of compliance with established maximum residue limits of FMT in fruits and vegetables.

1. Introduction

The extensive use of carbamate pesticides in modern agriculture has raised serious public concern regarding the environment and food safety. The presence of residues is a concern for consumers because carbamates are known to have potential harmful effects to other non-targeted organisms. Together with other pesticides, carbamates have been included in the list of known endocrine disruptor compounds [1]. The major concerns are their toxic effects such as interfering with the reproductive systems and foetal development [2].

The carbamate formetanate hydrochloride (3-dimethylamino-methyleneaminophenyl methylcarbamate hydrochloride; FMT) is an insecticide and acaricide, widely applied in grapes, mango, potato, onion, citrus, bean, watermelon, pepper and tomato [3]. It is in the priority list released by the United States Environmental Protection Agency (US EPA Regulating pesticides, accessed at: http://www.epa.gov/pesticides/regulating/index.htm (September 2012)). Recently, significant developments have been achieved for its residue analysis in fruit samples and focus was put towards sample preparation and analytical detection based on ultraperformance liquid chromatography tandem mass spectrometry (LC–MS/MS) [4]. Indeed, in industrialized countries LC–MS and LC–MS/MS methods are the techniques of choice for identification and determination of carbamates. However, in developing countries the mass spectroscopy instrumentation is financially prohibitive for comparable wide usage, yet the need for pesticide analysis is arguably even greater than for the industrialized nations because of the agrarian basis of the former countries [5]. Thus, the development of reliable and fit-for-purpose methods of analysis for pesticides with the use of simple and relatively inexpensive instrumentation is an appropriate objective to strive for.

Electrochemical biosensors have been intensively developed and applied for numerous analytical purposes, becoming a promising alternative methodology for pesticide monitoring in environmental and food samples [6,7]. The application of biosensors is intended to be more environmentally friendly, cheaper, easier and faster than conventional methodologies while being amenable to integration for *in situ* determination. Several strategies have been tested for the construction of the biosensors [6,8,9]. One of the most successful approaches is based on the use of enzymes immobilized or incorporated onto the electrode since pesticides are designed to inhibit various enzymes. Acetylcholinesterase [6,9–11], alkaline phosphatase [12], peroxidase [13], tyrosinase [14,15], glutathione-S-transferase [16],

* Corresponding author. Tel.: +351 228340500; fax: +351 228321159.
E-mail address: sbm@isep.ipp.pt (S. Morais).

1567-5394/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bioelechem.2013.09.005

laccase (Lac) [17,18] and organophosphorus hydrolase [19] have been tested for electroanalysis.

Lac is a copper oxidoreductase that catalyses the oxidation of numerous compounds such as ortho-, meta- and para-diphenols, phenol, aminophenols, aryl diamine and others, with concomitant reduction of oxygen to water [20–22]. Lac from the white rot fungus *Trametes versicolor* was reported as an attractive biocatalyst for electrochemical applications such as enzymatic biofuel cell cathodes [23,24] because of its high redox potential as well as its good stability. Despite its interesting properties, Lac-based biosensors are scarce [17,18,25–27].

Since the last decade, nanomaterials have been intensively used to improve the electroanalytical properties of enzymatic biosensors [28]. The enormous advantages of using nanomaterials, such as gold nanoparticles (AuNPs), are the enhancement of the conductivity, electrocatalytic capacity, biocompatibility and the facility of the electron transfer between proteins and the electrode surface. Furthermore, AuNPs improve the immobilization of the biomolecules which retain their bioactivity [28,29].

The present work aims to develop a rapid, reliable and sensitive Lac-based biosensor for detection of FMT pesticide in fruits. No report concerning FMT quantification by AuNPs-based biosensors was found. Regarding electrochemical biosensors, only our previous work [18] concerning the development of a Lac/Prussian blue film/graphene doped carbon paste modified electrode for detection of several pesticides in potato and tomato samples was reported so far. The electrochemical behavior of FMT was also characterized by Subbalakshmamma et al. [30] applying the outdated technique of differential pulse polarography. The principle behind the proposed biosensor relies on the FMT capacity to inhibit the Lac catalytic reaction that occurs in the presence of phenolic substrates. The construction of the biosensor was based on the immobilization of Lac, *via* glutaraldehyde (Glu) cross-linking, onto a gold electrode (AuE) previously modified with AuNPs. The developed Lac-based biosensor was applied to quantify FMT residues in mango and grapes. These tropical fruits were selected because their established maximum residue limits (MRLs) correspond to the lowest (0.02 mg/kg in mango) and the highest (1.0 mg/kg in grapes) allowable values for residues of FMT [31,32]. Moreover, there is a general lack of validated procedures based on biosensors for application in food samples, and none concerning tropical fruits.

2. Materials and methods

2.1. Reagents

Lac enzyme from *T. versicolor* (EC 1.10.3.2; ≥0.5 U/mg), FMT (certified purity higher than 99.6%), hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4$), Glu and the tested phenolic substrates: 4-aminophenol (4-AMP), gallic acid, caffeic acid, rutin and dopamine were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade. The ultrapure water (18.2 MΩ/cm) was produced by a Milli-Q Simplicity 185 system (Millipore, Molsheim, France). A 0.04 M Britton–Robinson buffer (BR) was used as electrolyte.

2.2. Electrochemical measurements

A potentiostat/galvanostat, AUTOLAB model PGSTAT 30 (Metrohm-Eco Chemie, The Netherlands) controlled by a computer through the Model GPES version 4.9 software (General Purpose Electrochemical System, Metrohm-Eco Chemie) was utilized for all electrochemical measurements. A conventional electrochemical cell consisting of an Ag/AgCl saturated electrode as the reference electrode, a graphite rod as the auxiliary electrode, and a polycrystalline AuE (BASi MF-2014, surface area 2.0 mm^2) as a working electrode were used. Square wave voltammetry (SWV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were applied to evaluate the construction of the enzymatic biosensor. The voltammetric experiments were carried out using 5.83×10^{-5} M of 4-AMP as the redox mediator in 0.04 M BR buffer (pH 5.0), over the potential range of 0.0 to 0.8 V. The optimal SWV parameters were a frequency of 40 Hz, amplitude of 30 mV and scan increment of 2 mV. EIS experiments were performed in the presence of 1.0 mM $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ (1:1) in 0.1 M KCl using a frequency range from 10^{-1} to 10^5 Hz with an amplitude perturbation of 5 mV.

2.3. Electrochemical deposition of AuNPs onto the gold electrode

Firstly, the AuE was polished with 0.3 and 0.05 μm alumina powder and sonicated for 5 min in ultrapure water. Then, the AuE was immersed in piranha solution (H_2O_2:H_2SO_4, 1:3, v/v) for 10 min, and electrochemically cleaned in 0.5 M H_2SO_4 solution by applying a potential scanning from −0.2 to 1.5 V at 100 mV/s. The electrochemical deposition of AuNPs onto the pretreated AuE was carried out at −0.2 V for 100 s in a solution containing 1.0 mM $HAuCl_4$ and 0.1 M KNO_3. The performance of the AuNPs/AuE was evaluated in 1.0 mM $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ (1:1) in 0.1 M KCl by using the SWV technique.

2.4. Immobilization of laccase onto the AuNPs/AuE

The modified AuE (AuNPs/AuE) was dried with a gentle stream of nitrogen. Then, 5 μL of 12.4 mg/mL Lac and 5 μL of 2% Glu solution were deposited onto the AuNPs/AuE to promote cross-linking (Scheme 1). Next, the electrode was placed at 4 °C overnight. Finally, the $Lac_{(Glu)}$/AuNPs/AuE was washed with BR buffer pH 5.0 and stored at 4 °C until use.

2.5. Formetanate hydrochloride quantification

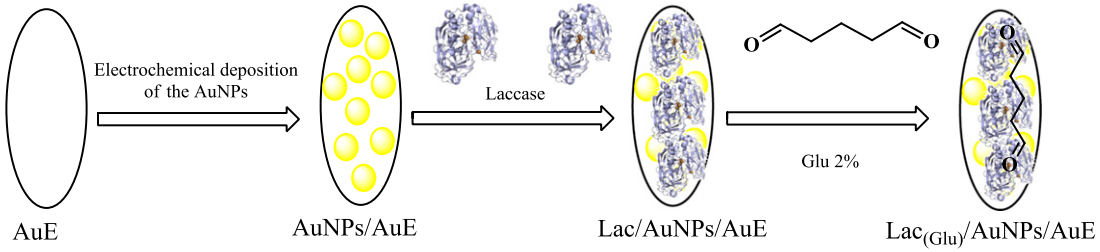
FMT quantification by the developed enzymatic biosensor was carried out as follows: i) measurement of the base current (I_p°) in a solution containing 5.83×10^{-5} M of 4-AMP in BR buffer pH 5.0 (in the absence of the inhibitor pesticide); ii) addition of an aliquot (10 μL) of FMT standard (5.0×10^{-4} M) in the electrochemical cell and incubation for 120 s; iii) measurement of the new peak current (I_p) which will be smaller than I_0. The inhibition percentage (% IR) was calculated using the following Eq. (1):

$$\%\,IR = \left[1 - \left(I_p / I_p^\circ\right)\right] \times 100 \quad (1)$$

where I_p° and I_p are the peak currents before and after the standard addition of FMT, respectively.

2.6. Application to fruit samples

Fruit samples were acquired from local supermarkets (Oporto, Portugal). Samples of mango and grape were taken, chopped and homogenized in accordance with the guidelines of the European Council Directive [33]. Pesticide extraction was performed by the Quick, Easy, Cheap, Effective, Rugged and Safe — QuEChERS method [4,34,35]. An aliquot of 15 g of homogenized sample was quantitatively transferred to a QuEChERS tube containing the buffer–salt mixture of 6 g $MgSO_4$/1.5 g NaCl/1.5 g $C_6H_5Na_3O_7 \cdot 2H_2O$ (UCT, Bristol, USA). Next, 15 mL of acetonitrile was added and the QuEChERS tube was shaken vigorously during 3 min. After centrifugation in a 2.16 Sartorius centrifuge (Sigma, Goettingen, Germany), for 5 min at 4500 rpm, the solvent layer was reduced to 2 mL by evaporation under vacuum in a Büchi B-940 rotary evaporator (Büchi, Switzerland). Then, it was transferred onto a column cleanup (containing 150 mg $MgSO_4$, 150 mg primary secondary amine sorbent and 50 mg octadecyl; UCT Enviro-Clean Bristol, PA), shaken and centrifuged as mentioned above. The supernatant was then evaporated to dryness with a gentle stream of nitrogen. Immediately before electroanalysis, the residue was re-dissolved with the supporting electrolyte (5.83×10^{-5} M 4-AMP, 0.04 M BR buffer solution at pH 5).



Scheme 1. Schematic representation of the preparation of the laccase-based biosensor.

Validation of the pesticide residue methodology was performed by recovery assays at five spiking levels (0.49–1.70 mg/kg on a fresh fruit weight basis (w/w)). All measurements were made in triplicate by the standard additions method. For recovery data, the interval of confidence (μ) was determined according to the following Eq. (2) [36]:

$$\mu = \bar{x} \pm ts/\sqrt{n}. \quad (2)$$

Where $\bar{x}$ is the mean concentration of FMT found (mg/kg) in the selected fruits, *t* is the coefficient of the Student's *t* distribution (4.271) at the 95% confidence level, s is the standard deviation of the concentration (mg/kg) and n is the number of determinations.

3. Results and discussion

3.1. Biosensor development

3.1.1. Electrochemical deposition of AuNPs onto the gold electrode

The electrodeposition of AuNPs onto the AuE surface was performed at −0.2 V based on the rapid, easy and reproducible procedure proposed by Park et al. [37]. The deposition time was optimized in order to reach maximum sensitivity of the modified AuE using 1.0 mM $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ (1:1) in 0.1 M KCl. The peak current increased with the increment of the deposition time from 0 until 100 s, and from this value onward the peak intensity decreased (till 250 s which was the higher duration tested). Some authors [38,39] have correlated higher deposition times with the formation of AuNP aggregates, which results in the diminution of the surface area, and increases the resistance as well as the double layer capacitance of the modified electrode. Therefore, 100 s was selected as the optimum value for the electrochemical deposition of AuNPs onto the AuE.

3.1.2. Immobilization of laccase onto the AuNPs/AuE

The electrochemical behavior of 4-AMP at a bare AuE and Lac(Glu)/AuNPs/AuE is shown in Fig. 1. In the absence of Lac, the electrochemical behavior of 4-AMP corresponds to the formation of a quinone–imine intermediate [40], which is represented by a quasi-reversible couple involving $2H^+$ and 2e−, recorded at *ca.* 0.24 V (anodic process) and 0.18 V (cathodic process) (Scheme 2).

Concerning the immobilization of the enzyme on the AuNPs/AuE sensor, two different strategies were tested. Firstly, Lac was directly immobilized onto the AuNPs/AuE by drop coating (5 μL of 12.4 mg/mL Lac). The cyclic voltammogram of the prepared Lac/AuNPs/AuE showed uniquely a reduction peak. This behavior indicated that the 4-AMP oxidation was catalyzed by Lac. However, in the successive scans, both oxidation and reduction peaks of 4-AMP were observed indicating that leaching of the enzyme from the modified electrode occurred. Therefore, this immobilization procedure was discarded.

Glu is a linear five carbon dialdehyde which possesses unique characteristics for effective protein cross-linking. It has been widely used for enzyme immobilization [41] and was successfully applied in this study. The presence of the enzyme (concentration of 12.4 mg/mL Lac) at the electrode promotes the disappearance of the 4-AMP oxidation process in the presence of dioxygen while the reduction current increased. When the dioxygen was removed from the solution, the electrocatalytic peak was not observed; the resulting voltammograms were similar to those obtained with the bare AuE (Fig. 1b). Lac reduced dioxygen to water, with concomitant oxidation of 4-AMP to quinone–imine [20,22]. Subsequently, the generated product is electrochemically reduced on the Lac-biosensor surface (Fig. 1) being the onset at 0.28 V and the peak apex potential at 0.13 V. This peak was further used as the electroanalytical signal for FMT quantification. The shape of the cyclic voltammograms obtained with the enzymatic biosensor (Fig. 1a) demonstrates that the electrochemical reaction is mainly controlled by a diffusion process. In order to confirm this phenomenon the influence of the scan rate was studied in the range of 1 to 100 mV/s. The results

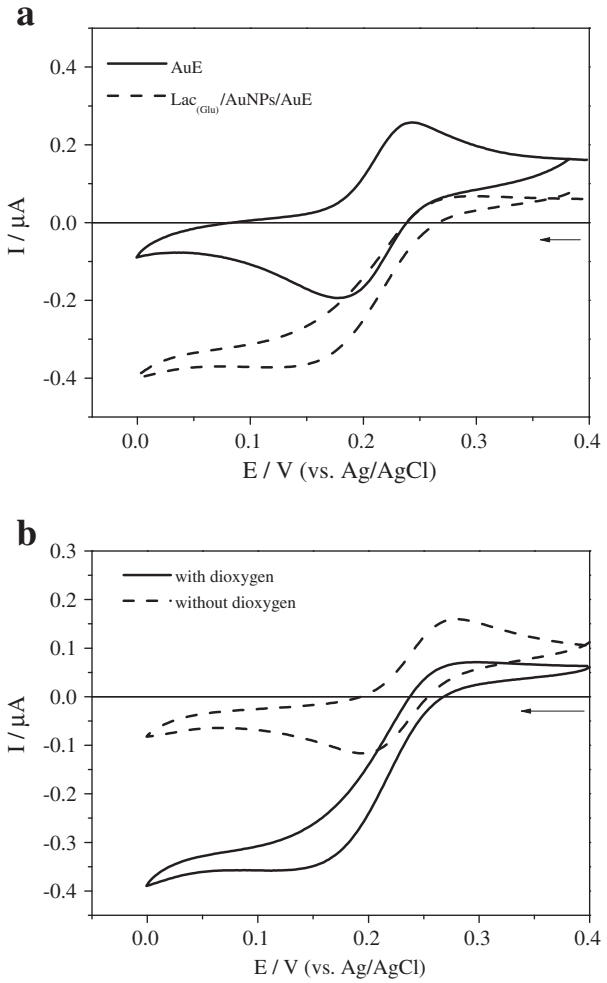
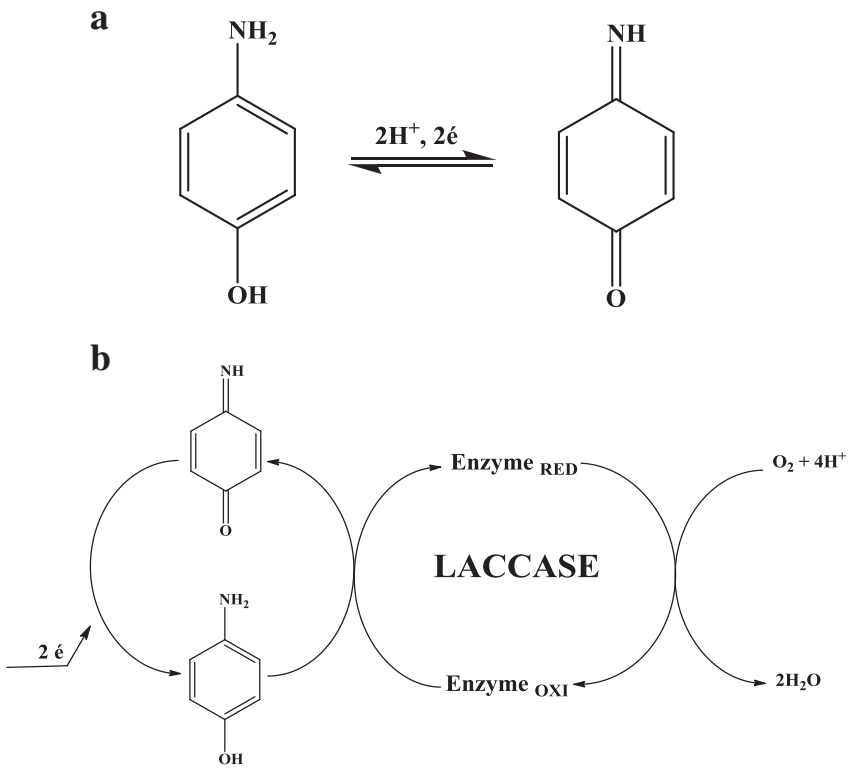


Fig. 1. Cyclic voltammograms of 4-AMP (5.83×10^{-5} M BR buffer pH 5.0): a) at bare AuE and $Lac_{(Glu)}$/AuNPs/AuE ($i_p/A = 2.78 \times 10^{-7} \pm 2.69 \times 10^{-9} + 1.68 \times 10^{-6} \pm 2.33 \times 10^{-8}$ $(\nu/Vs^{-1})^{1/2}$; n = 5; $r^2 = 0.983$; and $\log i_p/A = -5.90 \pm 7.07 \times 10^{-3} + 0.21 \pm 2.12 \times 10^{-3}$ $\log \nu/Vs^{-1}$; n = 5; $r^2 = 0.985$ for 1 to 100 m Vs^{-1}); b) at $Lac_{(Glu)}$/AuNPs/AuE in the presence (solid line) and absence (dashed line) of dioxygen. Scan rate of 10 mV s^{-1}.



Scheme 2. Schematic representation of the electrochemical reactions of the substrate 4-AMP (a) in the absence of laccase and (b) at the laccase based biosensor in the presence of dioxygen.

displayed a linear correlation between i_p/A and scan rate $\nu^{1/2}/(Vs^{-1})^{1/2}$ (Randles–Sevcik equation [42]) which are indicative of a diffusion controlled redox process. However, the correlation between the log i_p/A and log $\nu/(Vs^{-1})$ (caption of Fig. 1) showed a slope of 0.21, which is lower than 0.5, the value predicted for a diffusion control process, suggesting also the existence of complex kinetic processes [42].

3.2. Electrochemical characterization of the $Lac_{(glu)}$/AuNPs/AuE biosensor

Since EIS is an effective tool for the characterization of the interface properties of the electrode surface during different modification steps, it was also applied in this study. The obtained Nyquist diagrams at the bare AuE, AuNPs/AuE and $Lac_{(Glu)}$/AuNPs/AuE are exhibited in Fig. 2a.

The equivalent electrical circuit used to fit the electrochemical impedance data comprises the resistance of the solution (R_s/Ω), the Warburg impedance (Z_w/Ω), the double-layer capacitance (C_{dl}/F), and the electron transfer resistance (R_{ct}/Ω), and it is presented in Fig. 2b. The Nyquist plots include a semicircle at high frequency region and a linear relationship at low frequency region. At high frequencies, the diameter of the semicircular portion is equal to R_{ct}/Ω which controls the electron transfer kinetics of the redox process at the electrode interface. At lower frequencies, the linear part is typical of a mass diffusion-limited electron-transfer process, which is represented by Z_w [37]. According to the standard complex function representation, impedance can be described as a real $Z'(\omega)$ and an imaginary part $Z''(\omega)$ (Eqs. (3) to (5)) [43]:

$$Z(\omega) = Z'(\omega) + iZ''(\omega) \tag{3}$$

$$Z'(\omega) = R_s + (R_{ct} + Z'_w)\left[(Z'_w C_{dl} + 1)^2 + \omega^2 {C_{dl}}^2 (R_{ct} + Z'_w)^2 \right]^{-1} \tag{4}$$

$$-Z''(\omega) = \left[\omega C_{dl}(R_{ct} + Z'_w)^2 + \omega (Z'_w)^2 C_{dl} + Z'_w\right]\left[(Z'_w C_{dl} + 1)^2 + \omega^2 {C_{dl}}^2 (R_{ct} + Z'_w)^2\right]^{-1} \tag{5}$$

where, $\omega = 2\pi f$ is the angular frequency, f is the ac-frequency. At high frequencies, as ω approaches infinity $\omega \to \infty$, $R_{ct} \ggg Z_w$ and $Z'(\omega)$ and $Z''(\omega)$ are represented by Eqs. (6) and (7), respectively.

$$Z'(\omega) = R_s + R_{ct}\left(1 + \omega^2 {C_{dl}}^2 {R_{ct}}^2\right)^{-1} \tag{6}$$

$$Z''(\omega) = -\omega C_{dl} {R_{ct}}^2 \left(1 + \omega^2 {C_{dl}}^2 {R_{ct}}^2\right)^{-1}. \tag{7}$$

The maximum frequency value, $\omega_{max} = 2\pi f_{max}$ can be used to calculate C_{dl} using the Eq. (8) [44]:

$$C_{dl} = (2\pi f_{max} R_{ct})^{-1}. \tag{8}$$

The observed R_{ct} of the AuNPs/AuE is smaller than the one obtained with the bare AuE, being 350Ω and 960Ω, respectively. These values indicate that the deposition of AuNPs onto the AuE improves the conductivity and facilitates electron transfer. When Lac was immobilized onto the AuNps/AuE *via* Glu crosslinking, a higher R_{ct} value was attained confirming that Lac was successfully immobilized onto the AuNps/AuE.

3.3. Optimization of the experimental parameters

Since the FMT inhibition process is directly related to the kinetic activity of the enzyme, Lac concentration was optimized. Current peak measurements were performed in 4-AMP (5.83×10^{-5} M in BR buffer, pH 5.0) using different concentrations (6.2–24.8 mg/mL) of Lac immobilized on AuNPs/AuE. An increase in the peak current was clearly observed until 12.4 mg/mL of Lac, decreasing sharply thereafter. This pattern of variation may be due to the increase of the thickness of the interface electrode/solution, which may hamper the charge transference process. Thus, a Lac concentration of 12.4 mg/mL was selected for the preparation of the biosensor.

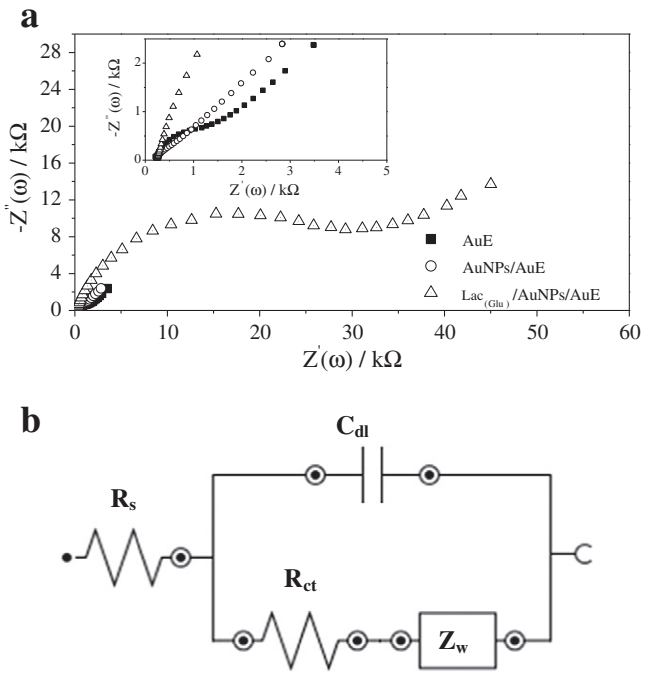


Fig. 2. a) Nysquist plot of electrochemical impedance spectroscopy for the different modified electrodes, for a frequency range of 10^{-1} Hz to 10^{5} Hz, applied potential + 0.19 V (*vs* Ag/AgCl) using 1.0×10^{-3} M $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1:1) in 0.1 M KCl; b) Equivalent electrical circuit comprising the resistance of the solution (R_s/Ω), the Warburg impedance (Z_w/Ω), the double-layer capacitance (C_{dl}/F), and the electron transfer resistance (R_{ct}/Ω).

The catalytic activity of Lac extends between the acidic to mildly basic and therefore the optimization of pH is a key factor for biosensing. Also, the changes in the pH affect the protonation mechanism involved in the electrochemical phenol reaction. The effects on the electrocatalytic activity of Lac were evaluated by SWV and CV over a pH range of 4.0 to 7.0 (Fig. 3). The data showed that the current response resulting from the enzyme-catalyzed reaction is strongly pH dependent. The maximum peak current is clearly evidenced at pH 5.0, which was selected as the optimum value. These results are in agreement with those found by other authors [25,26] and indicate that the immobilization step did not negatively affect the enzyme activity. Moreover, the increase in pH caused a linear displacement of the peak potential to more negative values. This electrochemical behavior demonstrates that the protonation of the 4-AMP is the determinant kinetic step in the redox mechanism. Using the experimental data (E_p *vs.* pH), a slope ($\partial Ep/\partial pH$) of 77.6 mV/pH was obtained. This value is quite different from the theoretical value predicted by the Nernst equation (59 mV/pH) indicating that the electron transfer process may not be a simple one electron one proton process.

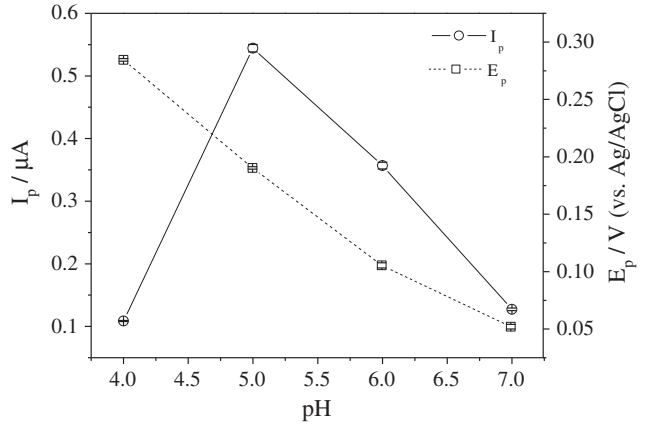


Fig. 3. Influence of pH on peak current (left y-axis) and peak potential (right y-axis) of 4-AMP (5.83×10^{-5} mol L^{-1}; BR buffer pH 5.0) using the enzymatic biosensor. Regression equation: Ep = 0.585 ± 0.004–0.0776 ± 0.0007 pH. Square-wave voltammetric parameters: frequency 40 Hz, amplitude 30 mV and step 2 mV.

The selection of the more appropriate buffer was also studied at pH 5. Three alternatives were tested, namely, BR, acetate and phosphate buffer. The highest peak intensity and the best peak definition were attained using BR buffer, which as then considered as the optimum.

In order to reach maximum sensitivity, the influence of five phenolic substrates on the voltammetric response of the $Lac_{(Glu)}$/AuNPs/AuE was studied by SWV and CV. 4-AMP, gallic acid, caffeic acid, rutin and dopamine were tested at a fixed concentration level (5.83×10^{-5} M). The electrochemical characterization performed by CV indicated that the enzyme catalyzed reaction occurred for all the compounds. Comparatively, 4-AMP was the substrate that presented the higher steady state cathodic current value. Based on the SWV peak currents, the following order was established: 4-AMP > dopamine > caffeic acid > rutin > gallic acid. These analytical results are in accordance with previous studies which refer that Lac preferentially catalyze para-substituted monophenols (4-AMP) instead of para-substituted diphenols

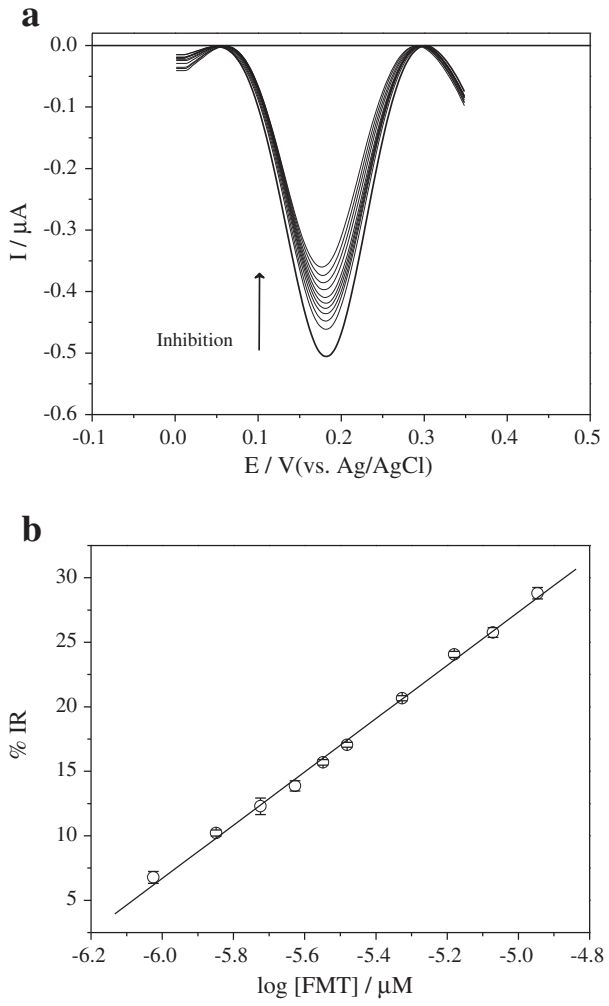


Fig. 4. a) Square-wave voltammograms of 5.83×10^{-5} mol/L 4-AMP (BR buffer pH 5.0) obtained with the $Lac_{(Glu)}$AuNPs/AuE in the absence of formetanate hydrochloride (a) and with the following formetanate hydrochloride concentrations: (b) 0.943, (c) 1.42, (d) 1.89, (e) 2.36, (f) 2.83, (g) 3.30, (h) 4.72, (i) 6.60, (j) 8.49, (k) 11.3 μM. Square-wave voltammetric conditions: frequency 40 Hz, pulse amplitude 30 mV and scan increment 2 mV. b) Analytical curve of FMT obtained by inhibition of the enzymatic catalysis (inhibition percentage % IR). Regression equation: % IR = 130.2 ± 2.6 + 20.58 ± 0.49 log [FMT]/μM (n = 10, $r^2 = 0.9993$).

Table 1
Biosensors based on oxidoreductase enzymes for carbamate pesticides detection.

Electrode	Substrate	Pesticide	LOD[a]/M	Sample	Ref.
Tyrosinase/graphite	Phenol	Ziram	7.4×10^{-8}	Apple	[48]
		Diram	1.3×10^{-6}		
		Zinc diethyldithiocarbamate	1.7×10^{-6}		
Tyrosinase/screen-printed carbon electrode	Cathecol	Sodium diethyldithiocarbamate	2.0×10^{-6}	River water	[14]
Tyrosinase/bovine serum albumin/Pt	Cathecol	Carbaryl	2.0×10^{-7}	Synthetic aqueous solutions	[49]
Peroxidase/SAM/Au	Hydroquinone	Thiodicarb	5.8×10^{-7}	Potato, apple and strawberry	[50]
CHcych-PPO/CPE[b]	Hydroquinone	Thiodicarb	1.6×10^{-7}	Lettuce, grape and peach	[51]
Lac/carbon ceramic electrode	Escurletin	Methomyl	2.0×10^{-7}	Carrot, cucumber, lettuce, pepper, potato and tomato	[25]
Lac/Pt-BMI.BF4-MMT/CPE[c]	Dopamine	Methomyl	2.4×10^{-7}	Carrot and tomato	[26]
Lac/multi-walled carbon nanotube paste electrode	4-Aminophenol	Pirimicarb	1.8×10^{-7}	Tomato and lettuce	[17]
Lac/Prussian blue film/ graphene doped carbon paste modified electrode	4-Aminophenol	Carbofuran	1.0×10^{-7}	Tomato and potato	[18]
		Carbaryl	5.5×10^{-9}		
		Formetanate	7.0×10^{-8}		
		Pirimicarb	3.1×10^{-8}		
		Ziram	5.2×10^{-9}		
$Lac_{(Glu)}$/AuNPs/AuE	4-Aminophenol	Formetanate	9.5×10^{-8}	Mango and grape	This work

[a] LOD — limit of detection.
[b] CPE/CHcych-PPO: carbon paste electrode/polyphenol oxidase immobilized in chitosan and cyanuric chloride.
[c] CPE/Pt-BMI.BF4-MMT/Lac: carbon paste electrode/platinum nanoparticles/1-butyl-3-methylimidazolium tetrafluoborate ionic liquid supported in montmorillonite/Laccase.

(dopamine and caffeic acid) [45,46]. Still, as far as the authors know, studies regarding electroanalytical procedures based on enzymatic biosensors using the selected substrate 4-AMP are almost nonexistent [17,18].

The 4-AMP concentration (in BR buffer pH 5.0) was optimized, varying from 5.12×10^{-6} to 1.57×10^{-4} M. Two regions of linearity (i_p *vs.* 4-AMP concentration) were observed: from 5.12×10^{-6} to 5.83×10^{-5} M (with a sensitivity of $5.38 \times 10^{-3} \pm 4.15 \times 10^{-5}$ A/M; n = 11; $r^2 = 0.9982$) and from 6.74×10^{-5} to 1.57×10^{-4} M of 4-AMP (with a sensitivity of the $4.15 \times 10^{-3} \pm 9.50 \times 10^{-5}$ A/M; n = 9; $r^2 = 0.9974$). The slope variation may be caused by the existence of diffusional limitations at high concentrations. Still, the lower sensitivity observed at the higher 4-AMP levels is appropriate for electroanalytical purposes. The 4-AMP concentration of 5.83×10^{-5} M was chosen considering the higher sensitivity reached.

To attain the best electroanalytical signal, the parameters of SWV were also optimized. Briefly, the frequency was ranged from 10 to 100 Hz, the amplitude from 5 to 50 mV and the step from 1 to 4 mV. A frequency of 40 Hz, a pulse amplitude of 30 mV and a staircase step of 2 mV were selected as the most adequate for subsequent electroanalytical FMT determination.

Table 2
Recovery of formetanate hydrochloride (FMT) from spiked mango and grape samples using the developed $Lac_{(Glu)}$/AuNPs/AuE biosensor at optimal conditions.

Mango					
$[FMT]_{added}$/(mg/kg)	0.49	0.73	0.97	1.46	1.70
$[FMT]_{found}$/(mg/kg)	0.53	0.70	0.94	1.47	1.77
CI[a]/(mg/kg)	±0.03	±0.06	±0.15	±0.10	±0.01
Recovery/%	108.6	96.1	96.8	100.9	104.0
BIAS/%	8.6	−3.9	−3.1	0.1	4.0
RSD[b]/%	2.5	3.7	6.3	2.7	0.2
Grapes					
$[FMT]_{added}$/(mg/kg)	0.73	0.97	1.22	1.46	1.70
$[FMT]_{found}$/(mg/kg)	0.72	0.93	1.19	1.44	1.69
CI[a]/(mg/kg)	±0.10	±0.24	±0.09	±0.10	±0.01
Recovery/%	98.9	95.5	98.0	98.7	99.8
BIAS/%	−1.1	−4.5	−2.0	−1.3	0.2
RSD[b]/%	5.9	2.9	6.2	3.9	1.2

[a] CI — confidence interval of the mean concentration of FMT found (mg/kg) in the selected fruits.
[b] RSD — relative standard deviation (%).

3.4. Formetanate hydrochloride quantification

Using the developed electrochemical $Lac_{(Glu)}$/AuNPs/AuE biosensor, the electrocatalytic peak corresponding to the quinone–imine reduction was used to indirectly quantify FMT. The principle supporting the analytical application of the developed biosensor relies on FMT's capacity to inhibit the Lac catalytic reaction. Applying the optimized operational parameters, analytical curves with a sensitivity of 20.58 ± 0.49 A/μM were attained for FMT concentrations ranging from 0.943 to 11.3 μM (Fig. 4). The standard deviation of the intercepts and the average of slopes of the straight lines from the analytical curves were used to determine the detection (LOD) and quantification limits (LOQ) [36,47]. The attained LOD and LOQ were $9.5 \times 10^{-8} \pm 9.5 \times 10^{-10}$ M (0.02 ± 2.6×10^{-4} mg/kg (w/w)) and $3.2 \times 10^{-7} \pm 3.2 \times 10^{-9}$ M (0.08 ± 7.9×10^{-4} mg/kg (w/w)), respectively.

FMT is extensively applied for the protection of fruit and vegetable crops with MRLs ranging from 0.02 (mango) to 1.0 mg/kg (w/w) (grapes) in fruits, and 0.05 (potato) to 2.0 mg/kg (w/w) (pepper) in vegetables [4,31,32]. The LOD calculated on a fresh weight basis is enough for the method to be used for residue monitoring purposes in fruits and vegetables, considering the established MRLs. Still, if needed, a significant enhancement in LOD can be achieved by augmenting the sample weight to be extracted and/or redissolving the crop residue in a lower volume of supporting electrolyte. Table 1 presents an overview of oxidoreductase enzyme-based biosensors for carbamate pesticides detection. The attained results, coupled with the high simplicity of the biosensor construction, compare favorably with those reported previously for other biosensors and carbamate pesticides (Table 1). No study regarding FMT quantification based on AuNPs-based biosensors was found. Concerning electrochemical biosensors, only our previous work [18] regarding the application of a Lac/Prussian blue film/graphene doped carbon paste modified electrode to several pesticides in potato and tomato samples was reported so far. The electrochemical behavior of FMT was also characterized by Subbalakshmamma et al. [30] applying differential pulse polarography.

The intra-day repeatability and the reproducibility of the developed Lac-based biosensor were evaluated by relative standard deviations (RSD). The repeatability was assessed by performing 10 measurements in the same day. Regarding reproducibility, three prepared biosensors were tested. RSD values of 1.4 and 1.8% were reached for repeatability and the reproducibility, respectively. The stability of the $Lac_{(Glu)}$/AuNPs/AuE biosensor responses was also assessed during 19 d

over a period of almost four weeks. The biosensor exhibited *ca.* 94% of its initial response. Globally, the developed device presents interesting characteristics, such as, easy preparation, good repeatability and reproducibility, appropriate stability, and short time of analysis, which is clearly acceptable for analytical purposes.

3.5. Application of the $Lac_{(Glu)}$/AuNPs/AuE biosensor in fruits

The developed biosensor was applied for the analysis of FMT in fruit samples (mango and grapes). The accuracy of the proposed methodology was evaluated by recovery assays performed at five spiking levels (0.49–1.70 mg/kg (w/w)) by the standard addition method. The obtained recoveries (Table 2) ranged from 96.1 to 108.6% and from 95.5 to 99.8% for mango and grape samples, respectively. RSD values less than 6.3% were achieved. These results confirm that the optimized protocol fulfils all analytical characteristics, including accuracy and precision, and can be used in the implementation of food safety programs. However it is important to highlight that in agreement with previously reported studies concerning pesticides quantification [14,17,18,25,26,48–51], enzymatic biosensors usually display a lower specificity than the established chromatographic methods [4] since other carbamates may also inhibit the enzymatic catalysis.

4. Conclusions

A novel approach for FMT quantification in fruit samples was developed. The transducer was prepared by electrodeposition of AuNPs onto a gold electrode and immobilization of Lac *via* Glu covalent attachment. The proposed electroanalysis is based on the FMT capacity to inhibit the Lac catalytic reaction that occurs in the presence of 4-AMP. Bearing in mind that applications of enzymatic biosensors to food samples are quite limited, the usefulness of the developed device was also assessed in fruits. The biosensor displayed a good electroanalytical performance for FMT quantification in mango and grapes, with satisfactory sensitivity, linearity, accuracy, repeatability, reproducibility and stability. The proposed, easy to prepare and inexpensive, Lac-based biosensor may be an interesting alternative for food safety screening purposes. It can serve as a device for the screening of hundreds of samples rapidly complementing or replacing the well-established chromatographic methods and allowing for a faster estimation of contaminations. Further studies are being conducted in order to adjust the optimized electroanalytical parameters to screen-printed electrodes. The use of these disposable electrodes widens the field of possible applications (*e.g.* in the environment) since the necessary equipment has reduced dimensions making *in situ* analysis possible.

Acknowledgments

The authors wish to thank the Brazilian and Portuguese research funding institutions CAPES and FCT, respectively, for the financial support through the project No. 313/11 (Proc. 4.4.1.00) “Determinação eletroanalítica de pesticidas em amostras de águas naturais e de alimentos utilizando ultramicroelectrodos/biossensores”. M. F. Barroso is grateful to FCT for the pos-doc fellowship SFRH/BPD/78845/2011.

References

[1] U. Schulte-Oehlmann, J. Oehlmann, F. Keil, Before the curtain falls: endocrine-active pesticides — a german contamination legacy, Rev. Environ. Contam. Toxicol. 213 (2011) 137–159.
[2] L.M. Frazier, Reproductive disorders associated with pesticide exposure, J. Agromedicine 12 (2007) 27–37.
[3] C.D.S. Tomlin, The Pesticide Manual: A World Compendium, Bristish Crop Protection Council, England, 1997. 623–624.
[4] L.V. Podhorniak, A. Kamel, D.M. Rains, Determination of formetanate hydrochloride in fruit samples using liquid chromatography-mass selective detection or -tandem mass spectrometry, J. Agric. Food Chem. 58 (2010) 5862–5867.
[5] S. Morais, O. Tavares, P.B. Paíga, C. Delerue-Matos, Determination of chlorfenvinphos in soils by microwave-assisted extraction and stripping voltammetry with an ultramicroelectrode, Anal. Lett. 40 (2007) 1085–1097.
[6] J.S.V. Dyk, B. Pletschke, Review on the use of enzymes for the detection of organochlorine, organophosphate and carbamate pesticides in the environment, Chemosphere 82 (2011) 291–307.
[7] M. LeDoux, Analytical methods applied to the determination of pesticide residues in foods of animal origin. A review of the past two decades, J. Chromatogr. A 1218 (2011) 1021–1036.
[8] V. Somerset, P. Baker, E. Iwuoka, Chapter 9 — mercaptobenzothiazole-on-gold organic phase biosensor systems: 3, in: Margarita Stoytcheva (Ed.), Thick-Film Biosensors for Organophosphate and Carbamate Pesticide Determination, Pesticides — Strategies for Pesticides Analysis, ISBN: 978-953-307-460-3, 2011, pp. 185–204, (InTech, Rijeka).
[9] Y. Qu, Q. Sun, F. Xiao, G. Shi, L. Jin, Layer-by-layer self-assembled acetylcholinesterase/PAMAM-Au on CNTs modified electrode for sensing pesticides, Bioelectrochemistry 77 (2010) 139–144.
[10] I. Cesarino, F.C. Moraes, M.R.V. Lanza, S.A.S. Machado, Electrochemical detection of carbamate pesticides in fruit and vegetables with a biosensor based on acetylcholinesterase immobilised on a composite of polyaniline–carbon nanotubes, Food Chem. 135 (2012) 873–879.
[11] P. Raghu, B.E.K. Swamy, T.M. Reddy, B.N. Chandrashekar, K. Reddaiah, Sol–gel immobilized biosensor for the detection of organophosphorous pesticides: a voltammetric method, Bioelectrochemistry 83 (2012) 19–24.
[12] F. Garcia-Sanchez, A. Navas Dias, M.C. Ramos Peinado, C. Belledone, Free and sol–gel immobilized alkaline phosphatase-based biosensor for the determination of pesticides and inorganic compounds, Anal. Chim. Acta. 484 (2003) 45–51.
[13] E.A. Songa, O.A. Arotiba, J.H.O. Owino, N. Jahed, P.G.L. Baker, E.I. Iwuoha, Electrochemical detection of glyphosate herbicide using horseradish peroxidase immobilized on sulfonated polymer matrix, Bioelectrochemistry 75 (2009) 117–123.
[14] J. Wang, V.B. Nascimento, S.A. Kane, K. Rogers, M.R. Smyth, L. Angnes, Screen-printed tyrosinase-containing electrodes for the biosensing of enzyme inhibitors, Talanta 43 (1996) 1903–1907.
[15] J. Shim, J. Woo, S. Moon, G. Kim, A preparation of a single-layered enzyme-membrane using asymmetric pBPPO base film for development of pesticide detecting biosensor, J. Membr. Sci. 330 (2009) 341–348.
[16] T.I.S. Oliveira, M. Oliveira, S. Viswanathan, M.F. Barroso, L. Barreiros, O.C. Nunes, J.A. Rodrigues, P. Lima-Neto, S.E. Mazzeto, S. Morais, C. Delerue-Matos, Molinate quantification in environmental water by a glutathione-S-transferase based biosensor, Talanta 106 (2013) 249–254.
[17] T.M.B.F. Oliveira, M.F. Barroso, S. Morais, P. Lima-Neto, A.N. Correia, M.B.P.P. Oliveira, C. Delerue-Matos, Biosensor based on multi-walled carbon nanotubes paste electrode modified with laccase for pirimicarb pesticide quantification, Talanta 106 (2013) 137–143.
[18] T.M.B.F. Oliveira, M.F. Barroso, S. Morais, M. Araújo, C. Freire, P. Lima-Neto, A.N. Correia, M.B.P.P. Oliveira, C. Delerue-Matos, Laccase-Prussian blue film-graphene doped carbon paste modified electrode for carbamate pesticides quantification, Biosens. Bioelectron. 47 (2013) 292–299.
[19] R.P. Deo, J. Wang, I. Block, A. Mulchandani, K.A. Joshi, M. Trojanowicz, F. Scholz, W. Chen, Y. Lin, Determination of organophosphate pesticides at a carbon nanotube/organophosphorus hydrolase electrochemical biosensor, Anal. Chim. Acta 530 (2005) 185–189.
[20] S. Shleev, J. Tkac, A. Christenson, T. Ruzgas, A.I. Yaropolov, J.W. Whittaker, L. Gorton, Direct electron transfer between copper-containing proteins and electrodes, Biosens. Bioelectron. 20 (2005) 2517–2554.
[21] G.P. Shumakovich, S.V. Shleev, O.V. Morozova, P.S. Khohlov, I.G. Gazaryan, A.I. Yaropolov, Electrochemistry and kinetics of fungal laccase mediators, Bioelectrochemistry 69 (2006) 16–24.
[22] C. Tortolini, M.D. Fusco, M. Frasconi, G. Favero, F. Mazzei, Laccase-polyazetidine prepolymer-MWCNT integrated system: biochemical properties and application to analytical determination in real samples, Microchem. J. 96 (2010) 301–307.
[23] M. Ardhaoui, M. Zheng, J. Pulpytel, D. Dowling, C. Jolivalt, F.A. Khonsari, Plasma functionalized carbon electrode for laccase-catalyzed oxygen reduction by direct electron transfer, Bioelectrochemistry 91 (2013) 52–61.
[24] 1.-T. Kuwahara, T. Asano, M. Kondo, M. Shimomura, Bioelectrocatalytic O_2 reduction with a laccase-bearing poly(3-methylthiophene) film based on direct electron transfer from the polymer to laccase, Bioelectrochemistry 91 (2013) 28–31.
[25] S.C. Fernandes, I.C. Vieira, A.M.J. Barbosa, V.S. Ferreira, Methomyl detection by inhibition of laccase using a carbon ceramic biosensor, Electroanalysis 23 (2011) 1623–1630.
[26] E. Zapp, D. Brodani, I.C. Vieira, C.W. Scheeren, J. Dupont, A.M.J. Barbosa, V.S. Ferreira, Biomonitoring of methomyl pesticide by laccase inhibition on sensor containing platinum nanoparticles in ionic liquid phase supported in montmorillonite, Sens. Actuators, B 155 (2011) 331–339.
[27] M.I. Sabela, N.J. Gumede, P. Singh, K. Bisetty, Evaluation of antioxidants in herbal tea with a laccase biosensor, Int. J. Electrochem. Sci. 7 (2012) 4918–4928.
[28] R. Buiculescu, N.A. Chaniotakis, The stabilization of Au NP–AChE nanocomposites by biosilica encapsulation for the development of a thiocholine biosensor, Bioelectrochemistry 86 (2012) 72–77.
[29] K. Huang, J. Li, Y. Wu, Y. Liu, Amperometric immunobiosensor for α-fetoprotein using Au nanoparticles/chitosan/TiO_2-graphene composite based platform, Bioelectrochemistry 90 (2013) 18–23.
[30] M. Subbalakshmamma, S.J. Reddy, Electrochemical behavior of formetanate and chlordimeform pesticides, Electroanalysis 6 (1994) 612–615.
[31] EU pesticide residues database for all the EU-MRLs, Available at http://ec.europa.eu/food/plant/protection/pesticides/database(accessed January 2013).

[32] ANVISA (Agência Nacional de Vigilância Sanitária, Brazil), Monographs of authorized pesticides, Accessed at http://portal.anvisa.gov.br/wps/wcm/connect/738132004745928 69ab9de3fbc4c6735/F40.pdf?MOD=AJPERES(accessed May 2013).
[33] European Council Directive 2002/63/CE, Establishing community methods of sampling for the official control of pesticide residues in and on products of plant and animal origin and repealing Directive 79/700/EEC. Off, J. Eur. Communities (2002) L187/30-31.
[34] M. Anastassiades, S.J. Lehotay, D. Stajnbaher, F.J. Schenck, Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce, J. AOAC Int. 86 (2003) 412–431.
[35] P. Paíga, S. Morais, T. Oliva-Teles, M. Correia, C. Delerue-Matos, S.C. Duarte, A. Pena, C.M. Lino, Development of QuEChERS-based extraction for ochratoxin A analysis in bread, Food Chem. 135 (2012) 2522–2528.
[36] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, Pearson Prentice Hall, United Kingdom, 2005.
[37] B. Park, D. Yoon, D. Kim, Formation and modification of a binary self-assembled monolayer on a nano-structured gold electrode and its structural characterization by electrochemical impedance spectroscopy, J. Electroanal. Chem. 661 (2011) 329–335.
[38] V. Carralero, M.L. Mena, A. González-Cortéz, P. Yáñez-Sedeño, J.M. Pingarrón, Development of a tyrosinase biosensor based on gold nanoparticles-modified glassy carbon electrodes application to the measurement of a bioelectrochemical polyphenols index in wines, Anal. Chim. Acta. 528 (2005) 1–8.
[39] Z. Huo, Y. Zhou, Q. Liu, X. He, Y. Liang, M. Xu, Sensitive simultaneous determination of catechol and hydroquinone using a gold electrode modified with carbon nanofibers and gold nanoparticles, Microchim. Acta 173 (2011) 119–125.
[40] H.J. Salavagione, J. Arias, P. Garcés, E. Morallón, C. Barbero, J.L. Vázquez, Spectroelectrochemical study of the oxidation of aminophenols on platinum electrode in acid medium, J. Electroanal. Chem. 565 (2004) 375–383.
[41] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, Glutaraldehyde: behaviour in aqueous solution, reaction with proteins, and application to enzyme crosslinking, BioTechniques 37 (2004) 790–802.
[42] J.K.D. Gosser, Cyclic Voltammetry: Simulation and Analysis of Reaction Mechanisms, UCH Publishers, New York, 1993. 97–103.
[43] C.M.A. Brett, A.M.O. Brett, Electrochemistry: Principles, Methods, and Applications, Oxford University Press Inc., New York, 1993.
[44] Q. Sheng, R. Liu, J. Zheng, Fullerene–nitrogen doped carbon nanotubes for the direct electrochemistry of hemoglobin and its application in biosensing, Bioelectrochemistry 94 (2013) 39–46.
[45] B. Haghighi, L. Gorton, T. Ruzgas, L.J. Jönsson, Characterization of graphite electrodes modified with laccase from *Trametes versicolor* and their use for bioelectrochemical monitoring of phenolic compounds in flow injection analysis, Anal. Chim. Acta. 487 (2003) 3–14.
[46] A. Jarosz-Wilkolazka, T. Ruzgas, L. Gorton, Amperometric detection of mono- and diphenols at *Cerrena unicolor* laccase-modified graphite electrode: correlation between sensitivity and substrate structure, Talanta 66 (2005) 1219–1224.
[47] I. Taverniers, M.D. Loose, E.V. Bockstaele, Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance, Trends Anal. Chem. 23 (2004) 535–551.
[48] M.T.P. Pita, A.J. Reviejo, F.J.M. de Villena, J.M. Pingarrón, Amperometric selective biosensing of dimethyl- and diethyldithiocarbamates based on inhibition processes in a medium of reversed micelles, Anal. Chim. Acta. 340 (1997) 89–97.
[49] X. Wang, L. Chen, S. Xia, Z. Zhu, J. Zhao, J. Chovelon, N.J. Renaul, Tyrosinase biosensor based on interdigitated electrodes for herbicides determination, Int. J. Electrochem. Sci. 1 (2006) 55–61.
[50] S.K. Mocceline, I.C. Vieira, F. de Lima, B.G. Lucca, A.M.J. Barbosa, V.S. Ferreira, Determination of thiodicarb using a biosensor based on alfalfa sprout peroxidase immobilized in self-assembled monolayers, Talanta 82 (2010) 164–170.
[51] F. Lima, B.G. Lucca, A.M.J. Barbosa, V.S. Ferreira, S.K. Moccelini, A.C. Franzoi, I.C. Vieira, Biosensor based on pequi polyphenol oxidase immobilized on chitosan crosslinked with cyanuric chloride for thiodicarb determination, Enzyme Microb. Technol. 47 (2010) 153–158.