Biosensor based on multi-walled carbon nanotubes paste electrode modified with laccase for pirimicarb pesticide quantification

Thiago M.B.F. Oliveira [a,c], M. Fátima Barroso [a], Simone Morais [a,*], Pedro de Lima-Neto [c], Adriana N. Correia [c], Maria B.P.P. Oliveira [b], Cristina Delerue-Matos [a]

[a] REQUIMTE, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. Bernardino de Almeida 431, 4200-072 Porto, Portugal
[b] REQUIMTE, Dep. Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
[c] 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

ABSTRACT

This study focused on the development of a sensitive enzymatic biosensor for the determination of pirimicarb pesticide based on the immobilization of laccase on composite carbon paste electrodes. Multi-walled carbon nanotubes (MWCNTs) paste electrode modified by dispersion of laccase (3%, w/w) within the optimum composite matrix (60:40%, w/w, MWCNTs and paraffin binder) showed the best performance, with excellent electron transfer kinetic and catalytic effects related to the redox process of the substrate 4-aminophenol. No metal or anti-interference membrane was added. Based on the inhibition of laccase activity, pirimicarb can be determined in the range 9.90×10^{-7} to 1.15×10^{-5} mol L^{-1} using 4-aminophenol as substrate at the optimum pH of 5.0, with acceptable repeatability and reproducibility (relative standard deviations lower than 5%). The limit of detection obtained was 1.8×10^{-7} mol L^{-1} (0.04 mg kg^{-1} on a fresh weight vegetable basis). The high activity and catalytic properties of the laccase-based biosensor are retained during *ca.* one month. The optimized electroanalytical protocol coupled to the QuEChERS methodology were applied to tomato and lettuce samples spiked at three levels; recoveries ranging from $91.0 \pm 0.1\%$ to $101.0 \pm 0.3\%$ were attained. No significant effects in the pirimicarb electro-analysis were observed by the presence of pro-vitamin A, vitamins B1 and C, and glucose in the vegetable extracts. The proposed biosensor-based pesticide residue methodology fulfills all requisites to be used in implementation of food safety programs.

1. Introduction

The production and application of agrochemicals have been so intense in the last years that the pesticide sector has been one of the world's major industries, with world expenditures higher than 32.5 billion dollars per year [1]. Among the different chemical classes of pesticides available worldwide, carbamates are largely used on crops due to their broad spectrum of biological activity. Although carbamates present low bioaccumulation potentials and short-term toxicity, they are considered hazardous to human and environment health, being included in the list of known endocrine disruptor compounds [2] and in the priority list released by the United States Environmental Protection Agency [3]. Pirimicarb ((2-Dimethylamino-5,6-dimethylpyrimi-din-4-yl)*N,N*-dimethylcarbamate); PMB) is a carbamate insecticide that is classified by the U.S. EPA as "likely to be carcinogenic to humans" [4]. It has been commonly detected in food surveys [5]. Furthermore, pesticides can persist and accumulate in the environment, interfering in the equilibrium of ecosystems and changing important biological functions of the living organisms, even at very low concentrations [6].

Biomonitoring is an essential tool for rapid and cost-effective screening [7]. New devices and protocols, with negligible waste generation or no hazardous substances, and *in situ* real-time monitoring capability are particularly needed for addressing the challenges of green analytical chemistry [8]. For this reason, biosensor technology has been considered as a key tool for the complete implementation of the new European Union directives, such as the Water Framework Directive, and other related directives [7]. Carbonaceous materials are among the most interesting transducers that convert a biological recognition process into a measurable electroanalytical signal for biosensors development [9,10]. In recent years, carbon nanotubes, and multi-walled (MWCNTs) in particular, have received great attention as nanomaterials [11–13]. Their main advantages are enhanced electron transfer rate, excellent electrical conductivity, reduced electrode surface fouling, high mechanical strength and good stability while lowering the over potential of analytes [9–14].

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

MWCNTs still need to be further explored due to their tremendous potential to improve the sensitivity and efficiency of biosensors for carbamates detection [9–14]. Few reports concerning the PMB electrochemical determination were found [15–18], none regarding detection by MWCNTs-based biosensors.

Concerning the use of MWCNTs, Lin and Chen [19] determined PMB in nature water based on the enhancement of electrochemiluminescent behavior of tris(2,2′-bipyridyl)ruthenium [Ru(bpy)$_3^{2+}$] at a MWCNTs/nafion composite film-modified electrode. One important drawback of the developed sensor is the long incubation time needed (16 h) to prepare the electrode.

Enzymatic analytical methods, especially those based on electrochemical procedures, are strongly encouraged [20]. For enzymatic biosensors construction, MWCNTs singular advantages arise from the high surface area, excellent electron transfer rate, strong electrocatalytic activity and abundant acidic sites offering the capacity for high-density loading of enzymes [14,21]. Previous reports concerning the application of MWCNTs for pesticides detection are based mainly on the use of acetylcholinesterase and some on tyrosinase [22–25]. Concerning carbamates detection by MWCNTs-based enzymatic biosensors, only the work of Chen et al. [23] was found. Authors immobilized acetylcholinesterase on a glassy carbon electrode, modified with MWCNTs and Prussian Blue films, and determined carbofuran in spiked beverages. Laccase (*p*-diphenol: dioxygen oxidoreductase, E.C. 1.10.3.2) are dimeric or tetrameric glycoproteins from the small group of enzymes called blue oxidases, which are detected in many plants and secreted by numerous fungi [8]. These enzymes are considered excellent biocatalysts [26] and have been studied due to their ability to oxidize various aromatic substances, particularly polyphenols, as well as, anilines [27,28]. Although their high activity, selectivity and specificity [26], the studies concerning the development of laccase-based biosensors for pesticides detection are very scarce [29], none regarding pirimicarb, and laccase-based biosensors need clearly to be further exploited. The enzyme immobilization step is critical, since the biocatalyst has to remain active to perform an efficient biorecognition of the substrate, whose non-covalent functionalization is one of the best way to preserve the integrity of the biological element [30,31]. Studies made with composite electrodes prepared by dispersion of carbonaceous materials with mineral oil as binder showed excellent electrocatalytic activity to various substrates [11–13,26,27].

Thus, the aim of this study was to report an easy, fast and very effective way to prepare an electrochemical biosensor for PMB quantification based on laccase and a composite carbon paste transducer (multi-walled carbon nanotubes and graphite powder-based pastes with different compositions were tested), without adding any metal or anti-interference membrane. Moreover, as far as the authors know, informations about the electrochemical behavior of the selected substrate 4-aminophenol (4-AMP) on modified electrodes with laccase are inexistent. Considering that studies dealing with practical applications of enzymatic biosensors using food samples are limited [29,32], the electroanalytical performance of the developed device in vegetable crops was also evaluated.

2. Experimental

2.1. Reagents

Spectroscopic grade graphite powder (UCP 2-200, from Ultracarbon, Spain) and MWCNTs, average diameter of 10 nm and average length of 1.5 μm, purified to more than 95% C (DropSens, Spain) were used. Laccase from *Trametes versicolor* (0.5 U mg^{-1},

CAS: 80498-15-3) and 4-aminophenol were purchased from Sigma-Aldrich (Germany). Pirimicarb pesticide (≥98% purity; Pestanal®, CAS: 23103-98-2) was from Fluka (Germany). β-carotene (pro-vitamin A), tiamine (vitamin B1), ascorbic acid (vitamin C) and paraffin oil binder were obtained from Merck (Germany). D(+)-Glucose anhydrous was from Scharlau (Spain). Other chemicals were reagent grade and used without further purification.

Ultrapure water ($\rho = 18$ MΩ cm^{-1}) from a Simplicity 185 apparatus (Millipore, Molsheim, France) was used to prepare the solutions. A 0.04 mol L^{-1} Britton–Robinson (BR) buffer solution was employed as supporting electrolyte and the pH was adjusted to the desired value by adding appropriate amounts of 1.0 mol L^{-1} NaOH stock solution.

2.2. Electrochemical assays

Cyclic voltammetry (CV) and square-wave voltammetry (SWV) were performed using an Autolab electrochemical system (Eco Chemie, Ultrecht, The Netherlands) equipped with PGSTAT-30 and GPES software. Electrochemical impedance spectroscopy (EIS) experiments were done using the same equipment, but with the FRA software. The electrochemical cell was assembled with a conventional three-electrode system: the developed enzymatic biosensor as the working electrode, a Ag/AgCl/KCl (3.0 mol L^{-1}) reference electrode, and a rod of glassy carbon counter electrode. All experiments were performed at room temperature.

Performance of the biosensor and optimization of the analytical procedure were performed using 4.75×10^{-5} mol L^{-1} of 4-AMP as substrate, over the potential range of −0.4 to 1.4 V, after a brief deaeration (2–3 min.) with high purity nitrogen. Qualitative informations concerning the influence of the pH of the medium, kinetic, catalytic effect, voltammetric profile and mass transport at electrode/solution interface were obtained by CV and EIS. Experiments by EIS were performed using a frequency range from 10^{-1} to 10^5 Hz with an amplitude perturbation of 5 mV, and applying 0.20 (in the absence of TvL) and −0.01 V (in the presence of TvL) and using 4.75×10^{-5} mol L^{-1} 4-AMP solution (BR buffer, pH 5) as redox mediator. For the quantification of PMB by the proposed enzymatic biosensor, the SWV parameters, i.e., the frequency, pulse amplitude and the height of the potential step, were optimized based on maximum value of peak current (I_p), displacement of the potential peak (E_p), and alterations on half-peak width ($\Delta E_{p/2}$), since their values exert considerable influence on kinetic, mechanism redox, sensibility and selectivity of the electrochemical procedure [33,34].

2.3. Preparation of the biosensor

Different carbon transducers, namely, multi-walled carbon nanotubes-based paste (MWCPE) and graphite powder-based paste (CPE) electrodes were tested. MWCPE was prepared by mixing MWCNTs and paraffin oil binder at different ratios. The paste was carefully hand-mixed in a mortar, packed into a handmade cavity of a Teflon® tube (1.0 mm internal diameter) and then provided by a stainless steel piston. The surface was smoothed against a plain white paper while a slight manual pressure was applied to the piston. CPE were prepared in a similar way using spectroscopic grade graphite powder. The surface of the resulting paste electrodes was smoothed and rinsed carefully with ultrapure water prior to each measurement.

Two different strategies for the immobilization of the enzyme on the different carbon paste electrodes were tested: (i) by drop coating a TvL solution (5.0 U mL^{-1}) onto the electrode surface; (ii) by adding different amounts of TvL into the pastes (1 to 10%, w/w). The biosensors construction was evaluated based on the feasibility to

incorporate the enzyme into the paste, the intensity of background/faradaic currents, easy handling and renewal of the biosensor surface.

2.4. PMB quantification

PMB was quantified based on the inhibition of the catalytic reaction of the substrate 4-AMP performed by TvL present on the biosensor. A base current (I_o) was measured with the biosensor in a solution containing 4.75×10^{-5} mol L^{-1} 4-AMP (0.04 mol L^{-1} Britton–Robinson at pH 5), and in the absence of the pesticide (inhibitor). This initial response corresponds to the blank signal. Then, an aliquot of PMB standard was added and, after stirring, a second base current (I) was generated being smaller than I_o due to inhibition of the catalytic reaction. As the current (I) is proportional to the concentration of the substrate (4-AMP), a decrease in enzyme activity leads to a change in the base signal, which is related to the amount of PMB [8]. The inhibition percentage (IR, %; equation 1) was employed to obtain PMB analytical curves in the range from 9.90×10^{-7} to 2.0×10^{-5} mol L^{-1}.

$$\%IR = [1 - (I/I_o)]100 \tag{1}$$

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) [35,36]; LOD in fresh weight (mg kg^{-1}) basis was calculated based on an extract redissolution volume of 10 mL, a vegetable mass of 10 g and the molecular mass of PMB (238.29 g/mol). Repeatability and precision of the proposed electroanalytical procedure were evaluated by the relative standard deviations (RSD) of 10 pesticide determinations (at 1.96×10^{-6} mol L^{-1}) performed on the same day (intraday) and on different days (interday) in 4.75×10^{-5} mol L^{-1} 4-AMP (0.04 mol L^{-1} BR buffer, pH 5). Reproducibility tests were also made through the comparison of the responses obtained by five different biosensor surfaces (from the same batch) to the same pesticide concentration (1.96×10^{-6} mol L^{-1}). The repeatability assays were carried out with different measurements on the same biosensor surface.

2.5. Application to vegetable samples

Vegetable samples were obtained from local markets (Oporto region, Portugal) and were taken in accordance to the guidelines of the EU [37]. Samples were chopped, homogenized, and analyzed as soon as possible after their acquisition. An aliquot of 10 ± 0.05 g of homogenized sample was quantitatively transferred to a "Quick, Easy, Cheap, Effective, Rugged and Safe" (QuEChERS, [38]) tube containing 6 g MgSO$_4$/1.5 g NaCl/1.5 g C$_6$H$_5$Na$_3$O7.2H$_2$O (UCT, Bristol, USA) of buffer–salt mixture. Next, 10 mL of acetonitrile were added and the QuEChERS tube was vortexed vigorously during 1 min. After centrifugation in a 2.16 Sartorius centrifuge (Sigma, Goettingen, Germany), for 3 min at 4000 rpm, the solvent layer was evaporated under vacuum in a Büchi B-940 rotary evaporator (Büchi, Switzerland), and then with a gentle stream of nitrogen to complete dryness. Immediately before electroanalysis, the residue was re-dissolved with 10 mL of the supporting electrolyte (0.04 mol L^{-1} BR buffer solution at pH 5) and 4.75×10^{-5} mol L^{-1} 4-AMP.

Validation of the pesticide residue methodology based on QuEChERS extraction and the developed biosensor was performed by recovery assays, of fortified chopped and homogenized samples of tomato and lettuce, at three spiking levels (0.47–2.36 mg kg^{-1}). All measurements were performed in triplicate by the standard additions method.

Table 1

Electrochemical parameters obtained with the different composite carbon paste electrodes tested by cyclic voltammetry at a scan rate of 50 mV s^{-1} in 4.75×10^{-5} mol L^{-1} 4-AMP (BR buffer solution, pH 5).

Parameters	MWCNT: graphite powder: oil binder (%, w/w/w)		
	0:60:40	30:30:40	60:0:40
I_{pa} (A)	3.70×10^{-7}	9.41×10^{-7}	2.27×10^{-6}
E_{pa} (V)	0.295	0.264	0.260
$-I_{pc}$ (A)	2.91×10^{-7}	7.11×10^{-7}	1.66×10^{-6}
$-E_{pc}$ (V)	0.152	0.180	0.188
ΔE_p (V)	0.143	0.084	0.072
I_{pa}/I_{pc}	1.28	1.32	1.37

3. Results and discussion

3.1. Composition and electrochemical performance of the MWCPE and CPE electrodes

The more adequate proportion for analytical applications of MWCNTs: graphite powder: oil binder (%, w/w/w) was evaluated based on I_p, E_p and $\Delta E_{p/2}$ values (Table 1). Three different compositions (0:60:40, 30:30:40 and 60:0:40%, w/w/w) were tested to confront the probable advantages of MWCPE compared to conventional CPE. The pastes based on spectroscopic graphite powder were consistent with a little proportion of paraffin (15%, w/w), but MWCNTs-based pastes with oil content lower than 40% (w/w) were difficult to pack into the Teflon® tube. Also, the MWCPE-based pastes with oil content $\geq 50\%$ (w/w) were difficult to handle. Thus, a proportion of 40% (w/w) of paraffin was selected. 4-AMP displayed sharp and well-defined anodic and cathodic peaks (Fig. S1, Supplementary Material) with all electrode compositions tested. The best results were obtained for the electrode containing only MWCNTs and paraffin (without graphite) in a ratio of 60:40 (%, w/w). For this composition, a significant increase of the currents (ca. 6 times) and of the catalysis of the anodic peak, improvement of the reversibility with increase of the anodic-to-cathodic current ratio, lower separation between the peak potentials (ΔE_p), besides lowering the overvoltage for the redox system, can be observed in Table 1 and Fig. S1 (Supplementary Material) pointing the important role of MWCNTs on the electron-tranfer kinetics. The observed potential window for the MWCPE composed of MWCNTs and paraffin binder (60:40%, w/w) was also higher (-0.3 to 1.3 V) than those exhibited by the other composite carbon electrodes. These advantages can be associated with the nanometric dimensions and high area, electronic structure and topological defects present on the nanotubes surface [39]. Banks and Compton [40] reported that the enhanced electrocatalytic activity is due to the presence of edge-plane like sites located at the end and in the defects areas of the tubes. Britto and co-workers [41] demonstrated by ab-initio calculation that the improvement in the electron transfer is due to the curvature of the tubes which originate changes in the energy bands close to the Fermi level, where the presence of pentagonal defects produce regions with charge density higher than those observed in the region hexagonal graphite, either in planar or in tubular structures. Thus, the performance observed for MWCPE (60:40%, w/w) suggested that it exhibited the best configuration for analytical applications.

3.2. Modification of the MWCPE and CPE with TvL

The different composite carbon paste electrodes were evaluated as TvL-based biosensors. Even knowing that the best electroanalytical performance was previously showed by the paste
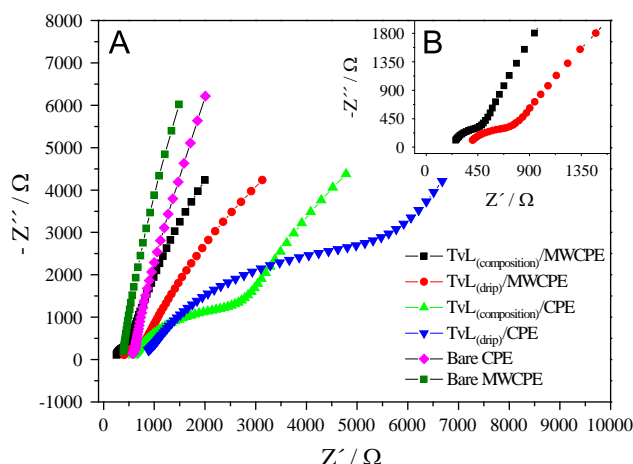
Fig. 1. (A) Nyquist plot of electrochemical impedance spectroscopy for the bare and the different modified electrodes, for a frequency range of 10^5 to 10^{-1} Hz, and applying 0.20 (in the absence of TvL) and -0.01 V (in the presence of TvL), and using 4.75×10^{-5} mol L^{-1} 4-AMP solution (BR buffer, pH 5). (B) Enlarged image of the Nyquist plot of electrochemical impedance spectroscopy for TvL$_{(drop\ coating;\ 5.0\ U\ mL^{-1})}$/MWCPE and TvL(composition; 3% w/w)/MWCPE, under the same conditions previously described.

containing MWCNTs, both carbonaceous materials (MWCNTs and spectroscopic graphite powder) were tested as transducers to evaluate any synergistic effect on the development of the devices. Two strategies were tested for immobilizing the enzyme, by drop coating of a TvL solution onto the electrode surface, and adding different amounts of TvL into the pastes (1 to 10%, w/w).

The interface properties of the devices and their performance as biosensors were characterized by EIS applying 0.20 (in the absence of TvL) and -0.01 V (in the presence of TvL). Results are presented in Fig. 1. The composite carbon materials used in this work did not contain any metal or anti-interference membrane. The properties attained with the developed devices were exclusively due to the integrated characteristics of TvL and the selected carbonaceous materials. In EIS, the semicircle diameter observed at high frequencies in the Nyquist diagram equals the interfacial electron transfer resistance (R_{et}), which controls the electron transfer kinetics of the redox process at the electrode surface [9]. Both bare CPE and MWCPE exhibited a linear relation between real (Z') and imaginary ($-Z''$) impedance, suggesting a diffusion controlled process, as well as confirming the high electrical conductivity of the carbonaceous materials. After successful modification with TvL, independently of the applied procedure, a capacitive arc appeared indicating an increase in the interfacial electron transfer resistance. Capacitive arcs were also observed at bare electrodes probably due to the relatively high amount of paraffin employed. The biosensors based on CPE displayed higher R_{et} when compared to those constructed with MWCPE, corroborating the prior information concerning charge transfer obtained by voltammetric experiments. For the electrodes modified by drop coating of a TvL solution on their surface (TvL$_{(drop\ coating)}$/CPE and TvL$_{(drop\ coating)}$/MWCPE), the arcs of capacitance were higher than those of the electrodes modified by the addition of enzyme in the composition of the paste itself (TvL$_{(composition)}$/CPE and TvL$_{(composition)}$/MWCPE). This phenomenon suggested that when TvL (5.0 U mL^{-1}) is immobilized on the electrodes surface, the R_{et} tends to increase, due to the growth of the thickness of the interface electrode/solution, which difficult the charge transference process. When TvL is embedded directly in the paste (3%, w/w), this limitation is overcome.

Overall, as it can be observed in Fig. 1B, the best conditions were observed for biosensors composed by MWCNTs, regardless

of the technique of immobilization employed. TvL$_{(composition)}$/MWCPE displayed improved conductivity, accelerated electron transfer and the lowest R_{et} (308 Ω) allowing highly sensitive detection of 4-AMP. TvL$_{(composition)}$/MWCPE was selected as the more suitable device for further analytical applications.

3.3. Electrochemical behavior of 4-AMP on the TvL- based biosensors

The detection of PMB through the use of the tested biosensors (TvL$_{(composition)}$/MWCPE and TvL$_{(composition)}$/CPE) is based on PMB ability to inhibit the natural catalytic reaction performed by laccase for a given substrate. No studies were found regarding the mechanism of inhibition of laccase activity using PMB as inhibitor. Laccase catalyses the reaction of oxidation of a variety of substrates, particularly phenolic compounds, in expense of reduction oxygen directly to water without intermediate formation of hydrogen peroxide [26–28]. As far as the authors know, informations about the redox behavior of the substrate 4-AMP on modified electrodes with this enzyme are inexistent. Results attained with the TvL$_{(composition)}$/MWCPE (3% of TvL, w/w) are presented in Fig. 2. The electrochemical behavior of 4-AMP corresponds to a redox process which occurs in two steps. The first step is fast, and is related to the formation of a quinone-imine intermediate, which is represented by a quasi-reversible couple involving 2H$^+$ and 2e$^-$ [42,43], here recorded at 0.243 V (anodic process) and 0.173 V (cathodic process) (Fig. 2, voltammogram a). This intermediate easily hydrolyzes to quinone at pH=5. The second step is slow, irreversible and concerns the formation of p-benzoquinone, parallel to the detachment of nitrogen as ammonia [43] (Fig. S2, Supplementary Material). Subsequently, the produced p-benzoquinone is reduced to hydroquinone at -0.070 V on the TvL-biosensor surface (Fig. 2, voltammograms b to m). At this potential, the amount of electroactive interfering substances is greatly reduced. This fact highlights the electroanalytical feature of the developed TvL$_{(composition)}$/MWCPE biosensor for quantification of PMB, since the redox process of this pesticide occurs usually at high oxidation [44] or reduction [15] potentials. Studies performed by CV showed a linear relationship between the peak current and the square root of scan rate indicating that the mass transport of the analyte at the biosensor/solution interface is controlled by diffusion [21,22,45]. Over time, the quasi-reversible couple has a gradual reduction in its peak current, while the reduction of
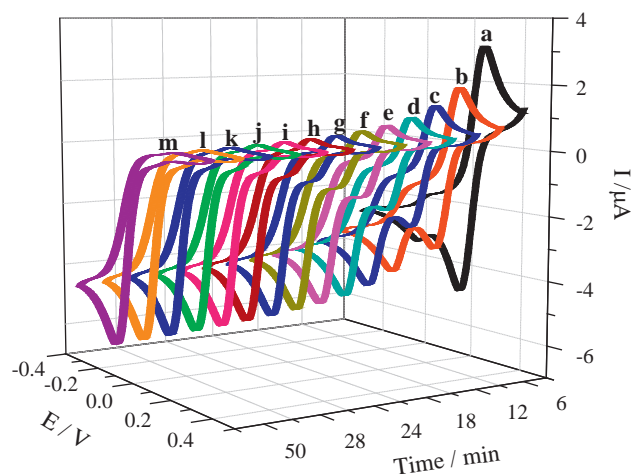

Fig. 2. Tridimensional cyclic voltammograms of 4.75×10^{-5} mol L^{-1} 4-AMP (BR buffer, pH 5) obtained with the TvL$_{(composition)}$/MWCPE (3% of TvL, w/w) at different incubation times: 0 (a), 5 (b), 10 (c), 15 (d), 20 (e), 25 (f), 30 (g), 35 (h), 40 (i), 45 (k), 50 (j), 55 (l), and 60 min. Scan rate of 50 mV s^{-1}.
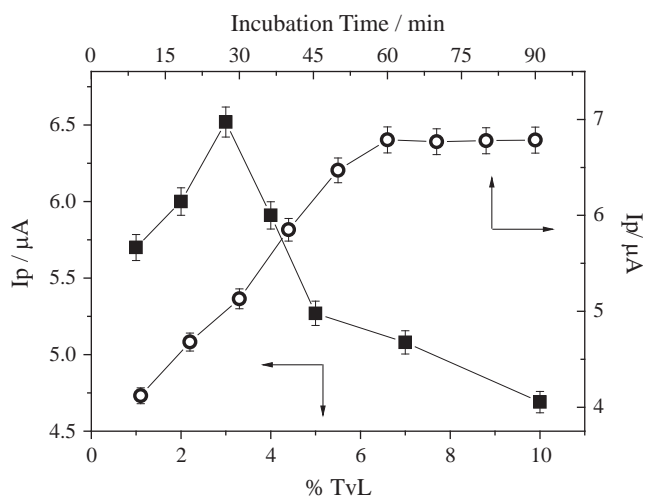
Fig. 3. Relation between I_p and amount of TvL (%, w/w) in the composition of the biosensor (■), for an incubation time of 60 min; and between I_p and the incubation time (○), for the TvL(composition)/MWCPE (3% of TvL, w/w). Cyclic voltammetric conditions: 4.75×10^{-5} mol L^{-1} 4-AMP (BR buffer, pH 5); scan rate of 50 mV s^{-1}.

p-benzoquinone (at -0.070 V) is enhanced and stabilized along time which was used as analytical signal. Although the results presented in Fig. 2 are related to the electrochemical behavior of 4-AMP on the TvL(composition)/MWCPE, similar data were observed for TvL(composition)/CPE. However, the incubation time necessary to stabilize the TvL(composition)/CPE enzymatic response was higher while the intensity of the peak current were about ten times lower than the obtained with TvL(composition)/MWCPE.

3.4. Optimization of the enzyme percentage and incubation time of the MWCPE-based biosensor

The inhibition process is directly related to the kinetic activity of the enzyme. Current peak measurements were performed by CV in 4.75×10^{-5} mol L^{-1} 4-AMP (BR buffer, pH 5) using different percentages (1–10%, w/w) of TvL in the composition of the TvL(composition)/MWCPE and a stabilization time fixed at 60 min. Fig. 3 summarizes the results attained. An increase of the peak current is clearly observed until 3% of TvL (w/w), decreasing sharply for higher percentages, probably due to the increase of carbon paste resistivity. Also, for a TvL(composition)/MWCPE containing 3% of TvL (w/w), 4-AMP peak current increases with the incubation time until 60 min, remaining constant thereafter. Thus, a percentage of 3% (w/w) of the enzyme and an incubation time of 60 min were selected for the preparation of the biosensor surface. Thereafter, and concerning analytical application of the constructed TvL(composition)/MWCPE biosensor, no incubation/stabilization time between each SWV measurement was applied.

3.5. Optimization of experimental electroanalytical conditions

The catalytic activity of TvL extends between the acidic to mildly basic and therefore the optimization of pH is a key factor for biosensing applications. In this work, the pH of the electrolyte was optimized in the 2–8 range using the TvL(composition tion)/MWCPE biosensor (3%, w/w) response in 4.75×10^{-5} mol L^{-1} 4-AMP substrate (Fig. S3, Supplementary Material). The results showed that the increase of pH promotes an increase of the current up to pH 5 and an almost linear displacement of E_p to more negative values. Moreover, the complete catalysis of the 4-AMP redox process was only observed in the pH range 4–6 when 60 min was applied as optimum incubation time. This

optimum working range is in accordance to those found by other authors using electrochemical and spectroscopic techniques [20,46,47]. The optimum pH value was selected as 5.

Considering the inherent advantages of SWV, such as, minimization of the contribution from the capacitive charging current to the current signal, this technique was selected for PMB electroanalytical quantification. The SWV parameters were optimized in order to reach maximum electroanalytical sensitivity. The frequency (10–150 s^{-1}) promoted a linear increase of I_p until 50 s^{-1} remaining almost constant for higher values. Regarding the effect of the pulse amplitude (5–50 mV), the current increased until 30 mV, and simultaneously a displacement of E_p to more negative values, as well as an increase of $\Delta E_{p/2}$, were observed. The increase of the potential step (1–7 mV) influenced proportionally and positively the peak current, but an increase of the noise/signal ratio also occurred. A frequency of 50 s^{-1}, an amplitude of 30 mV and a staircase step of 2 mV were considered the most suitable for subsequent electroanalytical PMB quantification.

3.6. PMB quantification

The current obtained in the electrochemical reduction of the *p*-quinone to hydroquinone on the TvL(composition)/MWCPE (3%, w/w) biosensor was used to determine indirectly PMB in trace concentrations. When PMB is added to the electrochemical cell containing a 4-AMP standard solution, the decrease in the resulting peak current is related to the inhibitory effect of PMB on TvL activity. Using the optimum conditions, PMB calibration data were attained (Fig. 4). The analytical curve obtained (insert of the Fig. 4), IR=$-2.79 \pm 0.51 + 4.65 \pm 0.06$ [PMB, μmol L^{-1}], $n=10$, is linear in a concentration range of 9.90×10^{-7} to 1.15×10^{-5} mol L^{-1}, with a linear correlation coefficient of 0.9994. The LOD and LOQ were calculated, respectively, as 3 and 10 times the standard deviation estimated for the regression equation ($S_{Y/X}$) dividing by the slope of the calibration equation [36,37]. LOD and LOQ of 1.8×10^{-7} and 6.0×10^{-7} mol L^{-1}, respectively, were reached. The results achieved with the proposed TvL(composition)/MWCPE (3%, w/w) biosensor compare favorably with those reported by other authors for this pesticide. Few reports concerning the PMB electrochemical determination were
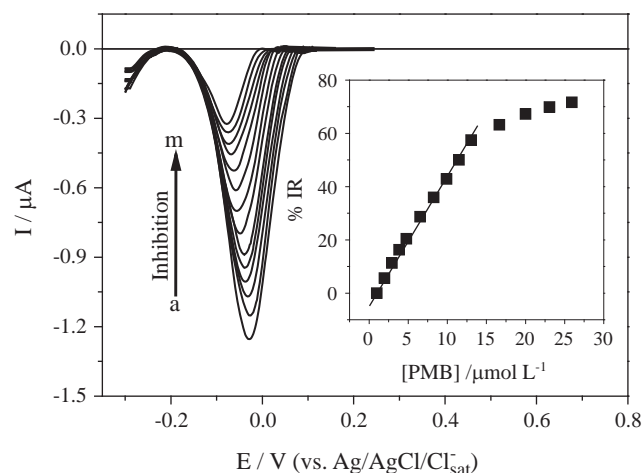

Fig. 4. Square-wave voltammograms of 4.75×10^{-5} mol L^{-1} 4-AMP (0.04 mol L^{-1} BR buffer, pH 5) obtained with the TvL(composition)/MWCPE (3%, w/w) in the absence of PMB (a), and with the following concentrations of PMB: (b) 9.90×10^{-7}, (c) 1.96×10^{-6}, (d) 2.91×10^{-6}, (e) 3.84×10^{-6}, (f) 4.76×10^{-6}, (g) 6.54×10^{-6}, (h) 8.26×10^{-6}, (i) 9.91×10^{-6}, (j) 1.15×10^{-5}, (k) 1.30×10^{-5}, (l) 1.67×10^{-5}, (m) 2.00×10^{-5} mol L^{-1}. Square-wave voltammetric conditions: frequency 50 s^{-1}, pulse amplitude 30 mV and scan increment 2 mV. The insert refers to the analytical curve of PMB obtained by inhibition of the enzymatic catalysis (inhibition percentage IR, %).

found, none regarding detection by MWCNTs-based biosensors. A decade ago, Mena et al. [16] and Yáñez-Sedeño et al. [15] applied successfully the hanging mercury drop electrode to determine PMB in water samples reaching LOD values of 1.72×10^{-8} mol L^{-1} (on-line preconcentration by solid phase extraction was applied using molecularly imprinted polymers [16]) and 2.8×10^{-6} mol L^{-1}, respectively. Due to the generation of mercury residues, nowadays these approaches have to be obviously discarded. More recently, Sun and Fung [17] developed a piezoelectric quartz crystal sensor using an Ag-electrode modified by three different molecularly imprinted polymers. The complex procedure developed was based on the preparation of molecularly imprinted polymers particles by bulk and precipitation polymerizations in organic solvents (one of them being the toxic chloroform). Moreover, the developed sensor exhibited a linear range with quite higher concentrations than the one obtained in this study. Lin and Chen [19] used the capacity of PMB to enhance the chemical and electrochemiluminescent behavior of tris(2,2′-bipyridyl)ruthenium [Ru(bpy)$_3^{2+}$] immobilized on a multi-wall carbon nanotube/nafion composite film-modified electrode attaining a LOD of 5.0×10^{-9} mol L^{-1} in nature water. The main drawbacks of this sensor are the long immersion time needed (16 h) to obtain a uniform and dense film of Ru(bpy)$_3^{2+}$, and that thick films increase the background and noise levels of the baseline decreasing clearly the sensitivity. Bucur et al. [18] immobilized acetylcholinesterase by entrapment in a sol–gel matrix on 7,7,8,8-tetracyanoquinodimethane/modified screen-printed electrodes and sensitive detection of PMB was reached (LOD of 2.0×10^{-8} mol L^{-1}). The developed biosensor presented several operational problems, such as the complex and lengthy steps for its construction, low stability, long stabilization time between each measurement (20 min) and instability of the analytical signal due to considerable enzyme leakage (reduction of 53% current after 10 successive measurements).

The intra- and inter-day repeatability of the proposed electroanalytical protocol was evaluated by the relative standard deviations (RSD) of 10 pesticide determinations (at 1.96×10^{-6} mol L^{-1}) performed on the same day (intra-day) and on different days (inter-day) in 4.75×10^{-5} mol L^{-1} 4-AMP (0.04 mol L^{-1} BR buffer, pH 5). RSD values of 1.8% and 5.0% were reached for intra and interday, respectively. The RSD of the reproducibility assay performed with five different TvL modified MWCPE surfaces (from the same batch) was lower than 5% (4.9%). Satisfactory repeatability and reproducibility were attained.

The stability of the TvL modified MWCPE was also evaluated. TvL$_{(composition)}$/MWCPE was stored at 4 °C and the electrode surface was renewed before each assay. Measurements of the peak current of a 4.75×10^{-5} mol L^{-1} 4-AMP standard solution were performed during twenty different days over a period of one month. The device retained 92.4% of its initial current response. The results obtained proved that the enzyme integrity is preserved at the MWCPE during an acceptable period of time.

Overall, it can be concluded that the TvL modified MWCPE (3%, w/w) proposed in this work exhibits a considerable number of relevant advantages, over the electrochemical tools previously reported in the literature [15–19], namely, its easy way of preparation, high stability, acceptable repeatability and reproducibility, and relatively short time of analysis.

3.7. Application to vegetable samples and interference studies

Nowadays, focus has been also put towards sample preparation. Scientists are exploring possible methods which are more environmental friendly, using less energy and less solvent while being cheaper, faster and producing higher yields. To satisfy these requirements, the QuEChERS [38] is a valuable option as already shown by this research team [48] and was applied with the TvL modified MWCPE (3%, w/w) at optimal operational conditions to tomato and lettuce samples. The accuracy of the proposed electroanalytical protocol was evaluated by recovery assays performed at three levels by the standard additions method (Table 2). The obtained recoveries ranged from $94.6 \pm 0.1\%$ to $101.0 \pm 0.3\%$ for tomato samples, and from $91.0 \pm 0.1\%$ to $98.7 \pm 0.3\%$ for lettuces. Lower recoveries were attained for the lower spiking level due possibly to analyte losses that may occur during the extraction process which are comparatively more significant at low levels. PMB is widely used for the protection of different crops with MRLs ranging from 0.05 (potato) to 1.0 mg kg^{-1} (lettuce and tomato) [49,50]. The LOD calculated on a fresh weight basis, 0.04 mg kg^{-1}, is sufficiently low for the method to be used for residue monitoring purposes, considering the established MRLs [49–51]. The sensitivity of the proposed method is sufficient to enable testing of compliance with food regulations and established maximum residue levels. Still, if necessary, a significant improvement in LOD can be yield by increasing the total mass of sample to be extracted or/and redissolving the vegetable residue in a lower volume of supporting electrolyte.

Considering that vegetables are important sources of β-carotene (pro-vitamin A), thiamine (vitamin B1), ascorbic acid (vitamin C) and glucose, these compounds were tested as possible interferences in the analytical response of the developed biosensor. SWV assays were carried out at optimal conditions in the presence of interference: substrate ratios of 1:10, 1:1 and 10:1 (w/w). Results expressed as bias (%) are exhibited in Fig. 5. Vitamin B1 and glucose showed no significant effect in the PMB electroanalysis (bias less than 7.0% for vitamin B1 and 5.0% for

Table 2
Recovery of pirimicarb from spiked tomato and lettuce samples using the TvL$_{(composition)}$/MWCPE (3%, w/w) at optimal operational conditions.

[PMB]$_{added}$ (mg kg^{-1})	Tomato		Lettuce	
	[PMB]$_{found}$ (mg kg^{-1})	Recovery (%)	[PMB]$_{found}$ (mg kg^{-1})	Recovery (%)
0.47	0.44 ± 0.01	94.6 ± 0.1	0.42 ± 0.01	91.0 ± 0.1
1.13	1.12 ± 0.02	98.8 ± 0.3	1.09 ± 0.01	95.8 ± 0.1
2.36	2.38 ± 0.03	101.0 ± 0.3	2.33 ± 0.02	98.7 ± 0.3

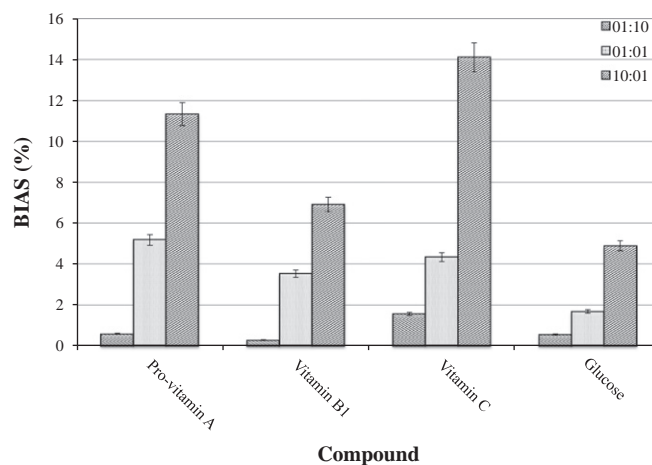**Fig. 5.** Responses of the proposed TvL$_{(composition)}$/MWCPE (3%, w/w) for 4.75×10^{-5} mol L^{-1} 4-AMP (0.04 mol L^{-1} BR buffer, pH 5) in the presence of pro-vitamin A, vitamins B1 and C, and glucose in different interference: substrate ratios (1:10, 1:1 and 10:1, w/w). Square-wave voltammetry conditions: frequency 50 s^{-1}, pulse amplitude 30 mV and scan increment 2 mV.

glucose at the higher ratio studied). Concerning the influence of vitamin A and vitamin C, bias varied between $0.59 \pm 0.01\%$ (1:10, interference: substrate ratio, w/w) to $11.33 \pm 0.01\%$ (10:1, interference: substrate ratio, w/w) and $1.56 \pm 0.02\%$ (1:10, interference: substrate ratio, w/w) to $14.11 \pm 0.01\%$ (10:1, interference: substrate ratio, w/w), respectively. However, the maximum ratio tested corresponds to an extreme condition which is seldom found in a 10 g mass of the analyzed vegetables. Thus, these results confirm the high selectivity and accuracy of the biosensor-based pesticide residue methodology proposed herein.

4. Conclusions

The obtained set of data demonstrated that the composite carbon paste consisting of 60:40% (w/w) MWCNTs and paraffin binder provides an interesting material for development of TvL-biosensors based on dispersion of the enzyme within the composite matrix. TvL$_{(composition)}$/MWCPE biosensor (3%, w/w) displayed the best performance for PMB pesticide quantification, with satisfactory linearity, detection and quantification limits coupled to good stability, repeatability and reproducibility. The high activity and catalytic properties of TvL is retained during *ca.* one month. The achieved sensitivity allowed the determination of PMB in QuEChERS vegetable extracts in the presence of pro-vitamin A, vitamins B1 and C, and glucose. The developed electroanalytical protocol is a promising tool for screening the presence of PMB in vegetable samples, complementing the traditional chromatographic techniques employed. However, it is important to highlight that in accordance with previously developed enzymatic biosensors [27,29,32], other carbamate pesticides, or even organophosphates, may also inhibit the enzymatic catalysis.

Acknowledgements

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.12.017.

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