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ORIGINAL PAPER

Acetylcholinesterase-based biosensors for quantification of carbofuran, carbaryl, methylparaoxon, and dichlorvos in 5% acetonitrile

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Abstract Amperometric acetylcholinesterase biosensors have been developed for quantification of the pesticides carbofuran, carbaryl, methylparaoxon, and dichlorvos in phosphate buffer containing 5% acetonitrile. Three different biosensors were built using three different acetylcholinesterase (AChE) enzymes—AChE from electric eel, and genetically engineered (B394) and wild-type (B1) AChE from *Drosophila melanogaster*. Enzymes were immobilized on cobalt(II) phthalocyanine-modified electrodes by entrapment in a photocrosslinkable polymer (PVA-AWP). Each biosensor was tested against the four pesticides. Good operational stability, immobilisation reproducibility, and storage stability were obtained for each biosensor. The best detection limits were obtained with the B394 enzyme for dichlorvos and methylparaoxon (9.6×10^{-11} and 2.7×10^{-9} mol L^{-1}, respectively), the B1 enzyme for carbofuran (4.5×10^{-9} mol L^{-1}), and both the B1 enzyme and the AChE from electric eel for carbaryl (1.6×10^{-7} mol L^{-1}). Finally, the biosensors were used for the direct detection of the pesticides in spiked apple samples.

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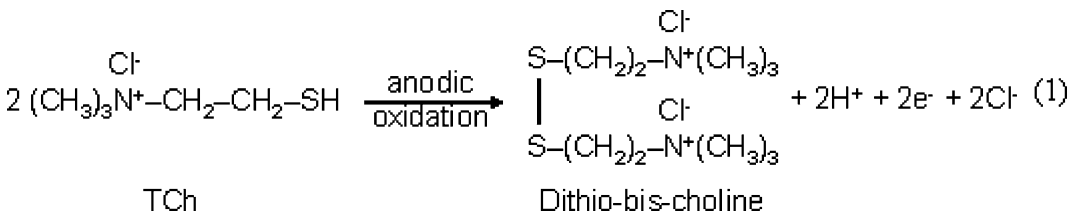
Introduction

Organophosphorus and carbamate pesticides are toxic compounds intensively used in agriculture [1]. Their presence in water and food is a potential hazard to human health. Thus, there is a growing interest in their fast and accurate identification and quantification. Standard procedures, based on liquid chromatography (LC) or gas chromatography (GC) are currently used for detection of wide range of pollutants with high sensitivity, reliability, and precision [2–4]. Despite their advantages, these methods require expensive instrumentation and highly trained personnel; they are, moreover, time consuming and not easily adapted to field analysis.

In order to address these problems, biosensors based on inhibition of the enzyme acetylcholinesterase (AChE) have been developed [5–9]. It is well known that organophosphorus and carbamate pesticides quantitatively inhibit cholinesterase. For this reason, amperometric biosensors based on inhibition of AChE activity have been extensively applied to rapid, simple, and selective analysis of pesticides.

The activity of the enzyme is measured by anodic oxidation of the thiocholine (TCh) produced by hydrolysis of acetylthiocholine (ATCh) (Eq. 1). The decrease in biosensor response is correlated with the amount of pesticide present in the sample and the time of incubation, as had been described in some research reports [10–12].



The use of redox mediators such as tetracyanoquinodimethane (TCNQ) and cobalt(II) phthalocyanine (CoPC) is interesting due to the decrease of the applied potential from 700 mV to 100 mV vs. Ag/AgCl, hindering the oxidation of other compounds [5, 13, 14].

Organophosphorus and carbamate pesticides are characterised by their low solubility in water. However, pesticides are highly soluble in organic solvents. Extraction of pesticides from solid matrices (fruits, vegetables, etc.) is thus carried out with such solvents [10, 15–19]. Some advantages are offered by the development of biosensors working in organic solvents, such as the change in substrate specificity and a wider voltage window due to higher resistance to redox reactions [20–22]. Nevertheless, enzyme activity is strongly affected by the presence of organic solvents that interact with the aqueous layer around the enzyme molecule and, usually, a decrease in activity is reported [23]. Hence, the inhibition measured depends on both the pesticide and the organic solvent [10, 15, 16, 18, 24].

The aim of this work was the development of a fast, simple, and very highly sensitive amperometric biosensor based on AChE for direct measurements of carbofuran, carbaryl, methylparaoxon, and dichlorvos in the presence of acetonitrile. The strategy was based on use of a commercially available AChE from the electric eel and two genetically modified AChEs derived from *Drosophila melanogaster* (B1 and B394). Low-cost screen-printed electrodes (SPEs) were used for tests. The acetonitrile–buffer concentration was chosen in order to use the highest acetonitrile concentration that enabled maintenance of the integrity of the enzyme. Finally, biosensors were used for the direct detection of pesticides in spiked apple samples.

Experimental

Chemicals and reagents

AChE (EC 3.1.1.7, type V-S, 1000 U/mg) from the electric eel was purchased from Sigma (Switzerland). Wild-type (B1) and genetically modified (B394) AChE from *Drosophila melanogaster* were kindly supplied by Protein Bio Sensor (PBS, Toulouse, France). Acetylthiocholine iodide (ATChI), acetylthiocholine chloride (ATChCl), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and phosphate buffer (PBS, 0.1 mol L^{-1} K_2HPO_4/KH_2PO_4 pH 7 containing 0.1 mol L^{-1} KCl) were obtained from Sigma.

HPLC-grade acetonitrile was supplied by Carlo Erba (Italy) and photocrosslinkable poly(vinyl alcohol) (azide-unit pendant water-soluble photopolymer, PVA-AWP) was purchased from Toyo Gosei (Japan). The pesticides carbofuran, carbaryl, methylparaoxon, and dichlorvos were obtained from Chem Service (West Chester, PA, USA).

The pastes used for screen-printing, i.e. Electrodag PF 410, 423SS, and 6037SS, were obtained from Acheson (Erstein, France). Transparent poly(vinyl chloride) (PVC) sheets (200 mm×100 mm×0.5 mm), supplied by SKK (Germany), were used as support for the SPEs. CoPC was obtained from Gwent Electronic Materials (UK).

Apparatus

Spectrophotometric measurements were performed using a Hewlett–Packard model 8451A diode-array spectrophotometer. Amperometric measurements were carried out with a 641 VA potentiostat (Metrohm, Switzerland) connected to a BD40 (Kipp and Zonen, The Netherlands) X-t recorder. SPEs were fabricated using a semi-automatic DEK 248 printing machine (UK).

Manufacture of biosensors

The biosensors were manufactured in groups of 24 (reference, working, and auxiliary electrodes) on PVC sheets. The following layers were consecutively printed: a silver conducting film, a carbon pad, an Ag/AgCl layer on the reference electrode, a CoPC layer on the working electrode, and, finally, an insulating layer. After each deposition the electrodes were dried at 60°C for 40 min. A schematic representation of the manufactured electrodes is presented in Fig. 1. As can be seen, the working electrode was a 4 mm-diameter disk, the auxiliary electrode was a 16 mm×1.5 mm curved line, and the Ag/AgCl pseudo-reference electrode was a 5 mm×1.5 mm straight line.

Three different kinds of biosensor were prepared using AChE from the electric eel, and wild-type (B1) and

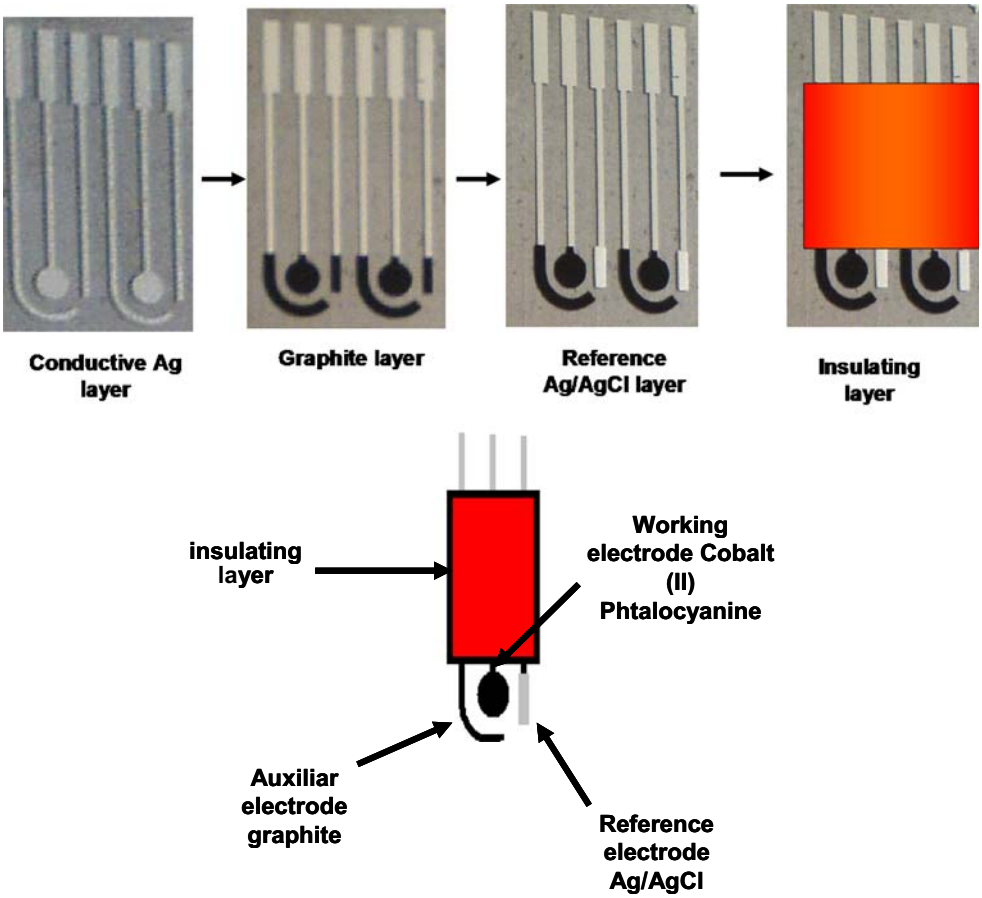


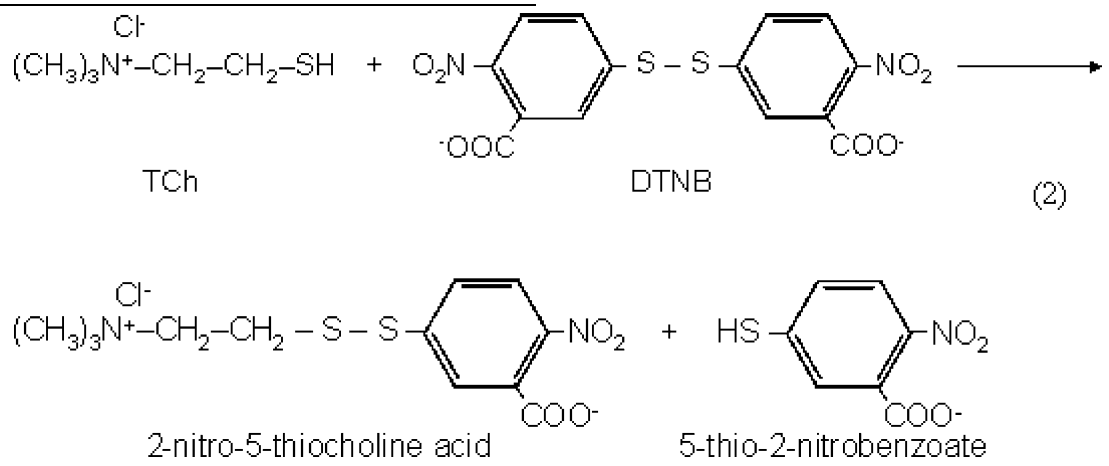
Fig. 1 Schematic representation of the screen-printing process

genetically modified (B394) AChE from *Drosophila melanogaster*. Each enzyme was immobilised on the working electrode surface by entrapment in a polymeric matrix of PVA-AWP, 3 μL of enzymatic solution containing 30% enzyme and 70% PVA-AWP were manually spread on the working electrode surface in order to immobilise 1 mU enzyme per electrode. Afterwards the electrodes were exposed to neon light (15 W) for 3 h at 4°C to promote photo-polymerisation between azide groups. After drying for 72 h at 4°C the biosensors were ready to use.

Spectrophotometric measurements

For the determination of both enzymatic activities and inhibition constants, spectrophotometric measurements were performed.

Determination of AChE activity was carried out by following the Ellman method, which is based on the fact that the enzymatic reaction product (TCh) reacts quantitatively and irreversibly with DTNB producing a yellow compound (5-thio-2-nitrobenzoate) that can be spectrophotometrically detected at 412 nm [25]. The reaction is represented in Eq. 2.



The assay was performed as follows. PBS (700 μL), 90 μL distilled H_2O, 100 μL 7.57 mmol L^{-1} DTNB (in PBS), 100 μL 10 mmol L^{-1} ATChI (in 0.9% NaCl), and 10 μL enzyme were added to a spectrophotometric cell. The kinetic spectrometric measurements were performed within 1 min and the enzymatic activity, expressed in mU mL^{-1}, was calculated.

The procedure used for the determination of inhibition constants (k_i) has already been described [26, 27]. It was adapted from the methodology described by Segel [28]. Basically, the enzymes were incubated for various times with different pesticide concentrations and the residual enzyme activity was then determined according to the Ellman spectrophotometric method. In this work, the procedure for the k_i calculation in the presence of the pesticides studied was as follows. Aliquots of 100 μL from an enzyme–pesticide solution, incubated at 30°C in a temperature-controlled bath, were taken at fixed time intervals and added to a spectrophotometric cell containing 700 μL PBS and 100 μL 7.57 mmol L^{-1} DTNB solution. Then, 100 μL 10 mmol L^{-1} ATChI substrate were added and the absorbance versus time spectrophotometric curve was recorded. Parallel assays without inhibitor (control) and without enzyme-inhibitor mixture (blank) were carried out.

Amperometric measurements

The activity of each AChE enzyme immobilised on the SPEs was determined by electrochemically monitoring the oxidation of the TCh, the product of the enzymatic hydrolysis of the ATChCl. It is correlated with the AChE activity on the biosensor.

Amperometric measurements were carried out as follows. The biosensor was immersed in a thermostatted cell (30°C) containing PBS, under constant magnetic stirring. A potential of 100 mV vs. Ag/AgCl pseudo-reference electrode was applied to the working electrode. After the current stabilisation, ATChCl solution was added and the difference of the current between the baseline and the plateau was measured (I_1).

In order to find the saturation concentration of ATChCl, the procedure describe above was followed by adding different concentrations of ATChCl, until no changes in the amperometric signal were observed. Thus, the saturation concentration for each kind of biosensor was obtained.

To evaluate the operational stability of the electrode, the response of the biosensor was measured in triplicate by injecting the same quantity of the substrate and rinsing the cell with PBS between measurements. The reproducibility of the biosensors was evaluated by measuring the response of ten SPEs fabricated by the same procedure. The storage stability for each kind of biosensor was evaluated by measuring the response of biosensors prepared in the same way and stored under the same conditions.

Four different acetonitrile–PBS concentrations were tested in order to select the highest acetonitrile percentage that could be used without influencing the catalytic activity of immobilised AChE. The procedure was performed in three steps. The initial response of the electrode on injection of ATChCl was recorded (I_1). The biosensor was then incubated for 10 min in buffer solution containing acetonitrile, and, finally, the residual response of the electrode was recorded again (I_2). The tested acetonitrile concentrations were 1, 5, 10, and 20% (*v*/*v*). I_1 and I_2 were obtained at saturation concentration of ATChCl. The residual enzyme activity (%*Ar*) was calculated according to Eq. 3:

$$\%Ar = \frac{I_2}{I_1} \times 100 \tag{3}$$

Likewise, in order to check that the selected acetonitrile concentration did not affect the enzymatic activity, the saturation concentration of the substrate was again measured.

Pesticide detection was carried out in the same three-step procedure described above. Biosensors were incubated for 10 min in the cell containing PBS–acetonitrile and a given pesticide concentration. The relative inhibition percentage (%*I*) was calculated according to Eq. 4:

$$\%I = \frac{I_1 - I_2}{I_1} \times 100 \tag{4}$$

After each amperometric measurement and biosensor incubation, both the cell and the biosensor were washed with PBS.

Pesticide quantification in apple samples

In order to eliminate pesticide residues on the samples, four middle-sized apples were washed, dried, and kept under refrigeration for 1 week. Later, the apples were cut into two parts and each half was treated with a known amount of each pesticide. First, the skin was removed and spiked with a known amount of pesticide. Afterwards, the skin was stirred with 5 mL acetonitrile for 10 min to allow pesticide extraction. The solvent was then removed and evaporated. Finally, 1 mL acetonitrile was added to the pesticide residue and an aliquot of this solution was measured with the AChE biosensor to determine the percentage inhibition. The concentration of each pesticide was determined using the calibration curve. Found pesticide concentrations were compared with the results obtained for a blank which was made with the same procedure without apple skin. Verification that the skin without addition of pesticides did not inhibit the enzyme was done with the other half samples.

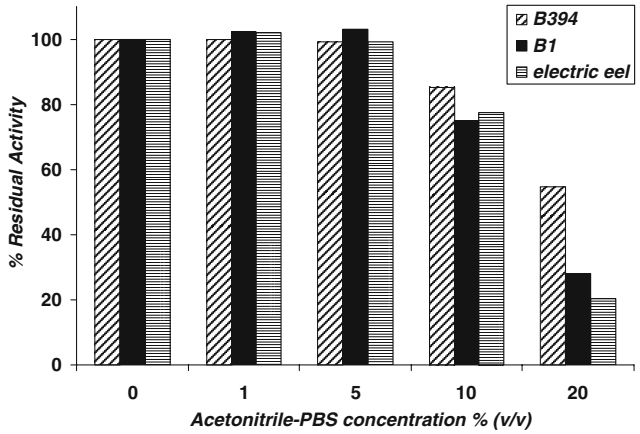


Fig. 2 Percentage of residual enzyme activity for each kind of biosensor after incubation for 10 min in 1, 5, 10, and 20% (*v*/*v*) acetonitrile

Results and discussion

Characterisation of the biosensors

Immobilisation reproducibility and both operational and storage stability for each kind of biosensor were tested.

In order to evaluate the reproducibility of the amperometric signal among biosensors of the same set, thirteen biosensors with each enzyme were tested. The reproducibility of the sensors was quite good: residual standard deviation (R.S.D.) values of 3.6, 4.1, and 7.1%, respectively, were obtained with the B394, B1 and electric eel biosensors.

In order to evaluate the operational stability of the electrode, the response of the biosensor was repeatedly measured by injecting the same quantity of the substrate (final concentration in the cell 1 mmol L^{-1}) and rinsing the cell with PBS between assays. After twelve successive substrate injections, average amperometric signals were 404±6, 399±6, and 401±6 nA for the B394, B1, and electric eel biosensors, respectively. The results obtained showed good operational stability for each kind of biosensor.

The storage stability was tested by keeping the electrodes in sealed plastic bags at 4°C. Amperometric measurements were performed during 10 consecutive days with each kind of biosensor, obtaining low R.S.D. values: 2.3% for both B394 and B1 biosensors and 2.9% for the electric eel biosensor. Furthermore, the same study was carried out over the course of seven months. In this case, results showed a decrease of 10% in the amperometric response for B394 and B1 biosensors and a decrease of 13% for the electric eel biosensor. Therefore, AChE biosensors prepared by entrapment in PVA-AWP polymer were more stable than those prepared by sol–gel encapsulation or entrapment in PVA-SbQ polymer [8, 10, 18].

Substrate saturation is usually desired for the determination of enzyme activity. In order to operate under substrate-saturation conditions, several calibration curves of biosensors at various ATChCl concentrations were obtained. No changes in the amperometric response were obtained when using a substrate concentration higher than 1 mmol L^{-1} (final concentration in the cell). Thus, the following experiments were performed under conditions of enzyme saturation with substrate (1 mmol L^{-1} ATChCl in the electrochemical cell). The time necessary to reach the plateau, after substrate addition, was 2 min.

Acetonitrile concentration

Pesticide extraction from different matrices is usually carried out with ethyl acetate, chloroform, benzene, hexane, or acetone. These solvents are not compatible with screen-printed materials and/or enzymes and/or electrochemical measurements, however. Acetonitrile has also been used for extraction of pesticides and good results have also been obtained when working with AChE SPEs [10, 15, 17].

In this work, the influence of four acetonitrile concentrations on the catalytic activity of immobilised AChE was studied by measuring the response of the sensor after 10 min incubation in phosphate buffer containing acetonitrile. Figure 2 shows the percentage residual enzyme activity for the three different biosensors. As expected, the AChE activity was shown to be much lower when working in 20 and 10% acetonitrile. This decrease in the residual activity could be related to enzyme denaturation or to enzyme leakage due to solubilisation of the PVA-AWP polymer in acetonitrile. The smallest residual activity was found with the electric eel biosensor, because the mutations in B1 and B394 enzymes protect their active site.

Table 1 ATChCl saturation concentration, percentage of residual enzyme activity, and Michaelis–Menten constant for the three AChE biosensors found before and after incubation in PBS containing 5% acetonitrile

	Before incubation in 5% acetonitrile			After incubation in 5% acetonitrile		
	B394	B1	Electric eel	B394	B1	Electric eel
ATChCl saturation concentration (mmol L^{-1})	1.05±0.05	1.05±0.05	1.00±0.05	1.00±0.05	1.00±0.05	0.99±0.05
%Ar	112.6±3.4	118.3±5.9	117.1±5.9	108.6±3.3	107.6±5.1	107.9±5.3
*K*m′ (mmol L^{-1})	0.21±0.01	0.18±0.02	0.16±0.01	0.20±0.02	0.12±0.01	0.09±0.01

Confidence intervals are calculated at the 95% confidence level

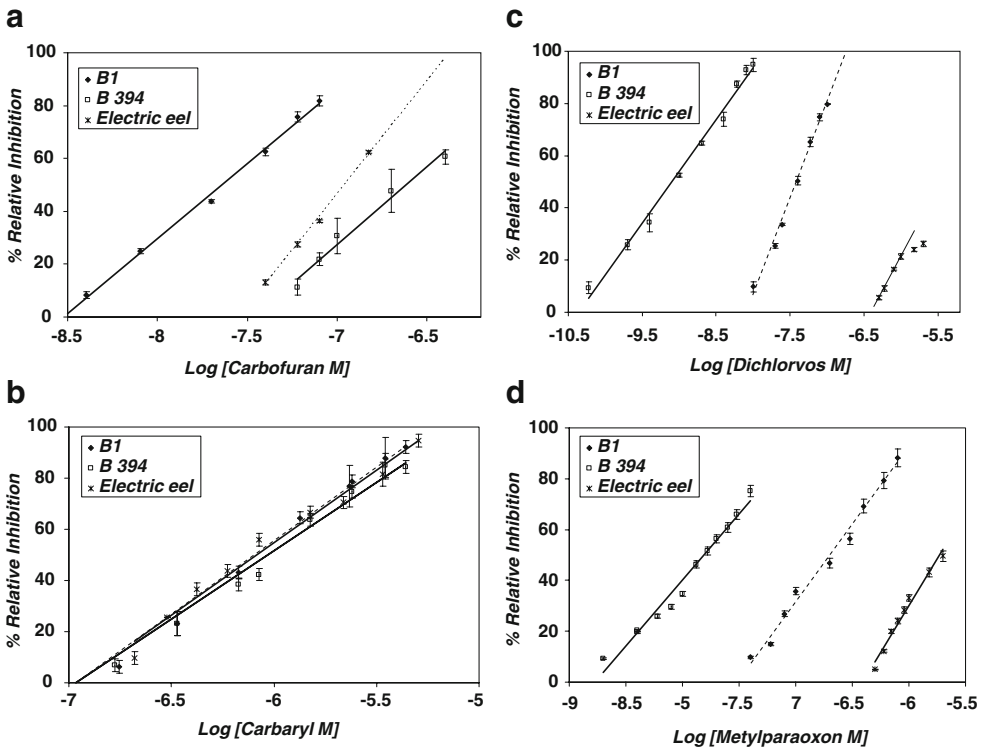


Fig. 3 Curves for inhibition of AChE biosensors by the pesticides (**a**) carbofuran, (**b**) carbaryl, (**c**) dichlorvos, and (**d**) methylparaoxon after incubation for 10 min

Nevertheless, using 1 and 5% acetonitrile the residual activities were statistically the same as those obtained when only incubating in PBS. Thus, buffer solution containing 5% acetonitrile was selected for the assays.

In order to check that no changes in the substrate saturation concentration were produced due to biosensors incubation in acetonitrile, the amperometric responses of these biosensors to ATChCl were recorded after 10 min incubation in 5% acetonitrile solution. Results were compared with those obtained when incubating biosensors in PBS. In both cases, typical Michaelis–Menten kinetics were found. As can be seen in Table 1, the saturation concentration and the final residual activities were statistically the same with and without acetonitrile. The apparent Michaelis–Menten constant (*K*m′) was also determined in both cases, according to the Lineweaver–Burk plot. In a previous publication by Montesinos et al. [17], activation of AChE by acetonitrile was found. However, pesticides could be detected using the procedure detailed here without acetonitrile interference problems.

Detection of the pesticides

Pesticide inhibition measurements were performed under saturation substrate conditions (1 mmol L^{-1} ATChCl). Thus the shift in the biosensor response after contact with the pesticide solution is related only to enzyme–inhibitor interactions and does not depend on the mass transfer of the substrate and/or inhibitor from the bulk solution to the enzyme layer. With this procedure, the maximum sensitivity of pesticide determination is reached. An incubation time of 10 min was shown sufficient to attain the maximum inhibition rate whatever the pesticide concentration used. Inhibition experiments were carried out in phosphate buffer solution containing 5% acetonitrile.

Table 2 Inhibition constants (k_i) obtained for the *Drosophila melanogaster* and electric eel enzymes using carbofuran, carbaryl, dichlorvos, or methylparaoxon as inhibitors

Enzyme	k_i (μmol^{-1} min^{-1})			
	Carbofuran	Carbaryl	Dichlorvos	Methylparaoxon
B394	1.3	0.5	224	3.52
B1	6.00	0.196	1.9	0.44
Electric eel	4.00	0.065	0.036	0.14

Measurements performed in 0.1 mol L^{-1} PBS, pH 7

Table 3 Inhibition curves data, I_{10} values, and working ranges of pesticides analysed by different amperometric AChE-based biosensors in PBS containing 5% acetonitrile

Pesticide	*a*	*b* (mol L^{-1})	Correlation coefficient	I_{10} (mol L^{-1})	Dynamic range (mol L^{-1})
Biosensor with B1 enzyme					
Carbofuran	483±14	57±2	0.998	4.5×10^{-9}	4×10^{-9} –8×10^{-8}
Carbaryl	401±16	58±3	0.993	1.6×10^{-7}	9×10^{-8} –4×10^{-6}
Dichlorvos	597±22	74±39	0.996	1.1×10^{-8}	1×10^{-8} –1×10^{-7}
Methylparaoxon	454±16	60±2	0.988	4.4×10^{-8}	2×10^{-9} –4×10^{-6}
Biosensor with B394 enzyme					
Carbofuran	440±34	59±5	0.990	5.0×10^{-8}	6×10^{-8} –4×10^{-7}
Carbaryl	372±17	54±3	0.990	1.7×10^{-7}	1×10^{-7} –4×10^{-6}
Dichlorvos	430±13	42±2	0.996	9.6×10^{-11}	2×10^{-10} –1×10^{-8}
Methylparaoxon	455±20	52±2	0.977	2.6×10^{-9}	2×10^{-9} –4×10^{-6}
Biosensor with electric eel enzyme					
Carbofuran	646±26	86±4	0.998	3.8×10^{-8}	8×10^{-8} –1.5×10^{-7}
Carbaryl	394±18	56±3	0.992	1.6×10^{-7}	1×10^{-7} –5×10^{-6}
Dichlorvos	226±29	35±5	0.963	5.9×10^{-7}	8×10^{-7} –1.5×10^{-6}
Methylparaoxon	428±27	74±4	0.979	5.3×10^{-7}	5×10^{-7} –2×10^{-6}

%Relative inhibition = $a + b$ log [pesticide]
Confidence intervals are calculated at the 95% confidence level

The inhibition effect of carbofuran, carbaryl, dichlorvos, and methylparaoxon on AChE-based biosensors is shown in Fig. 3. Each value is the average of three measurements obtained with three biosensors. All the experiments were performed under the same experimental conditions. Relationships between percentage inhibition and the inhibitory pesticide concentration can usually be characterised by linear, non-linear, logarithmic, or other types of equation. As can be seen in Fig. 3, a linear relationship between inhibition percentage and log [pesticide] was obtained. However these calibration graphs are shifted toward different insecticide concentration ranges depending on the sensitivity of each analysis. There is also a difference in the inhibition percentages between the four pesticides, depending on their specific toxicity towards AChE. As expected from the values of inhibition rate constants, k_i (Table 2), the inhibitory effect of dichlorvos on the electric eel biosensor was weaker than that on the B394 sensor, for example.

Characteristics of each pesticide determination in PBS containing 5% acetonitrile were obtained from each calibration plot (Table 3). The limit of detection was defined as the concentration resulting in inhibition of 10% (I_{10}). As can be seen, the smallest detection limits were obtained when using genetically modified enzymes. For the carbofuran pesticide, the best enzyme was B1 (LOD = 4.5×10^{-9} mol L^{-1}). For inhibition by dichlorvos and methylparaoxon, the best enzyme was B394 (LOD = 9.6×10^{-11} mol L^{-1} and 2.6×10^{-9} mol L^{-1}, respectively). For carbaryl, the magnitude of the LOD obtained was of the same order, irrespective of the enzyme. There is a direct correlation between the LOD and the k_i of the enzymes

Table 4 Comparison between LODs obtained with biosensors incubated in PBS containing 5% acetonitrile and in PBS

Pesticide	Enzyme		
	B394	B1	Electric eel
Incubation in PBS containing 5% acetonitrile			
Dichlorvos	9.6×10^{-11}	1.1×10^{-8}	5.9×10^{-7}
Methylparaoxon	2.6×10^{-9}	4.4×10^{-8}	5.3×10^{-7}
Carbofuran	5.0×10^{-8}	4.5×10^{-9}	3.8×10^{-8}
Carbaryl	1.7×10^{-7}	1.6×10^{-7}	1.6×10^{-7}
Incubation in PBS			
Dichlorvos	6.8×10^{-11}	4.4×10^{-9}	8.4×10^{-8}
Methylparaoxon	6.5×10^{-10}	3.6×10^{-8}	3.5×10^{-7}
Carbofuran	4.5×10^{-9}	1.9×10^{-9}	3.5×10^{-9}
Carbaryl	8.7×10^{-6}	8.4×10^{-6}	8.3×10^{-6}

Table 5 Comparison of LODs for several pesticides using AChE biosensors in our research and in other research

Source	Solvent	Pesticide	LOD (mol L^{-1})	Applied potential	Storage stability	Detection	Year	Ref.
ChO and AChE	Acetonitrile 1%	Carbofuran	1×10^{-8}	700 mV vs. SCE	–	Electro.	1997	[15]
ChO and AChE	Cyclohexane 5%	Paraoxon	5×10^{-7}	700 mV vs. SCE	3–4 weeks	Electro.	1997	[29]
AChE	Ethanol 10%	Dichlorvos	5×10^{-7}	350 mV vs. Ag/AgCl	2 months	Electro.	2000	[16]
		Diazinon	8×10^{-7}					
		Fenthion	1×10^{-6}					
AChE	Acetonitrile 15%	Chlorpyrifos-ethyl-oxon	2.5×10^{-9}	100 mV vs. Ag/AgCl	–	Electro.	2001	[17]
AChE		Paraoxon	1.9×10^{-8}	100 mV vs. Ag/AgCl	3 months	Electro.	2002	[10]
	Acetonitrile 5%	Chlorpyrifos-ethyl-oxon	1.2×10^{-9}					
AChE	Acetonitrile 5%	Paraoxon	8×10^{-8}	100 mV vs. Ag/AgCl	6 weeks	Electro.	2006	[18]
		Dichlorvos	1×10^{-7}					
AChE	Acetonitrile 5%	Methylparaoxon	2.6×10^{-9}	100 mV vs. Ag/AgCl	7 months	Electro.	This research	
		Dichlorvos	9.6×10^{-11}					
		Carbofuran	4.5×10^{-9}					
		Carbaryl	1.6×10^{-7}					

An incubation time of 10 min was used in all the studies

used in this study (Table 2). The higher the k_i, the better the theoretically achievable LOD.

In order to compare the LOD obtained when incubating biosensors in PBS solutions containing 5% acetonitrile with that obtained when the incubation was performed in buffer only, the same inhibition experiments were carried out in PBS. The LOD obtained with the two procedures are shown in Table 4. As can be seen, the use of acetonitrile enabled more sensitive detection of carbaryl pesticide. In the other cases, LOD values were lower when acetonitrile was not used. However, results found in this work when quantifying organophosphates and carbamates in acetonitrile were better than those obtained in other research work for the detection of pesticides in organic solvents. Results obtained by different researchers in the detection of pesticides in organic solvents are compiled in Table 5.

Table 6 Quantification of carbofuran, carbaryl, methylparaoxon, and dichlorvos in spiked apple samples

Pesticide	Concentration found in spiked apple samples	Concentration found in blank samples
Carbofuran	5.1×10^{-8} mol L^{-1}	5.4×10^{-8} mol L^{-1}
Carbaryl	4.2×10^{-7} mol L^{-1}	4.5×10^{-7} mol L^{-1}
Methylparaoxon	1.6×10^{-8} mol L^{-1}	1.8×10^{-8} mol L^{-1}
Dichlorvos	2.3×10^{-9} mol L^{-1}	2.6×10^{-9} mol L^{-1}

Analysis of apple samples

Analysis of carbofuran, carbaryl, methylparaoxon and dichlorvos pesticides in apple samples was carried out using the B394 biosensor for the two organophosphate pesticides and the B1 sensor for the two carbamates. For inhibition measurements, the biosensor was incubated for 10 min in a solution containing the pesticide extract in acetonitrile. This solution was prepared from 5% (*v*/*v*) organic sample extract and PBS solution. After incubation, the residual response of the biosensors was measured and compared with the initial response. The concentration of each pesticide was calculated by interpolating the percentage inhibition (*I*%) to the calibration curve obtained with the standard solution of each pesticide. The blanks (samples without apple skin) were tested following the same procedure. The results obtained with apple samples were in good agreement with the blank values, as can be seen in Table 6.

Conclusions

This work shows it is possible to quantify the pesticides carbofuran, carbaryl, methylparaoxon, and dichlorvos in phosphate buffer containing 5% acetonitrile. Amperometric biosensors based on both commercial (electric eel) and genetically modified (B1 and B394) AChE enzymes were

used. The introduction of different mutations in the enzyme structure improved the sensitivity of the biorecognition molecules and consequently of the biosensors. Good operational stability, immobilisation reproducibility, and storage stability were obtained for each biosensor. The lifetime of the developed biosensors was substantially improved by immobilizing the enzymes by entrapment in the PVA-AWP polymer, because this polymer maintained the activity of the enzyme for a long time, enabling mass production of these biosensors.

Pesticide inhibition results showed that the analytical characteristics of the electrodes were very similar when working in either phosphate buffer or phosphate buffer containing 5% acetonitrile. Thus, the developed biosensors seem to be suitable for detection of the pesticides in the presence of small amounts of the organic solvent.

The developed method was applied to the determination of these pesticides in real apple samples. The good results obtained demonstrated their applicability to the quantification of pesticides in real extracted samples.

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