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Sensitive amperometric biosensor for dichlorovos quantification: Application to detection of residues on apple skin

Gabriela Valdés-Ramírez [a,c], Didier Fournier [b], Maria Teresa Ramírez-Silva [a], J.-L. Marty [c,*]

[a] *Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa, Área de Química Analítica, San Rafael Atlixco 186, Col. Vicentina, C.P. 09340, Mexico D.F., Mexico*
[b] *IPBS, 117 Route de Narbonne, 31077 Toulouse, France*
[c] *BIOMEM, Centre de Phytopharmacie, UPVD, 52 Avenue Paul Alduy, 66860 Perpignan Cedex, France*

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Abstract

This paper presents the construction of an amperometric biosensor for the highly sensitive detection of the organophosphorus insecticide dichlorvos, based on the inhibition of acetylcholinesterase (AChE). The sensitivity of three AChEs from different sources were tested and compared: AChEs from Electric eel (Ee) and genetically engineered (B394) and wild type (B1) from *Drosophila melanogaster* (*Dm*). The enzymes were immobilized by entrapment in a photocrosslinkable PVA-SbQ polymer on a screen printed graphite electrode. The enzyme activity was estimated amperometrically at 100 mV versus Ag/AgCl by measuring the thiocholine produced by the enzymatic hydrolysis of the acetylthiocholine substrate using cobalt phthalocyanine as electron mediator. The pesticide was measured in the presence of 5% acetonitrile without loss of enzyme activity. The best sensitivity was achieved with the *Dm* mutant B394 with a detection limit of 7×10^{-11} M as compared to 1×10^{-8} M with the B1 *Dm* and 6×10^{-7} M with the Ee. The B394 biosensor was used to quantify dichlorvos in a sample of skin apple after extraction with acetonitrile.

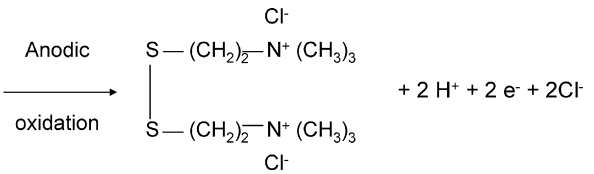
Keywords: Biosensor; Acetylcholinesterase; Dichlorvos; Acetonitrile; Apple

1. Introduction

Organophosphorous pesticides (OPs) are toxic/hazardous compounds that have been used for decades in agriculture, industry and as chemical warfare agents in military applications. The toxic action of OPs is based on their ability to irreversibly modify the catalytic serine residue in acetylcholinesterase (AChE), by phosphorylation the serine of the active site [1,2]. Thus the presence of such pesticides in natural waters and in foodstuffs is a major concern for public health [3,4]. Therefore rapid, simple and sensitive field methods are required to quantify the presence of these compounds [3,5–9]. Traditional analytical technologies used for pesticides quantification are gas chromatography (GC) with different detectors [6] and high performance liquid chromatography with UV detection (HPLC-UV) [6,7]. Nevertheless these methods are known to be expensive and quite laborious.

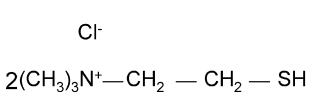
Biosensors are considered an alternative analytical tool for pesticides quantification, due their good selectivity, fast response, miniature size, and reproducible results [9–20].

Amperometric AChE biosensors based on the inhibition of the AChE enzyme by pesticides using screen-printing electrodes have been shown satisfactory results for water samples analysis [9–11]. The activity of the enzyme sensor is measured through the anodic oxidation of the thiocholine (TCh) produced by hydrolysis of the acetylthiocholine (ATCh) substrate (Eq (1)).



(1)

The decrease of the biosensor response is then correlated to the amount of pesticide in the sample following a given incu-



* Corresponding author.
E-mail address: jlmarty@univ-perp.fr (J.-L. Marty).

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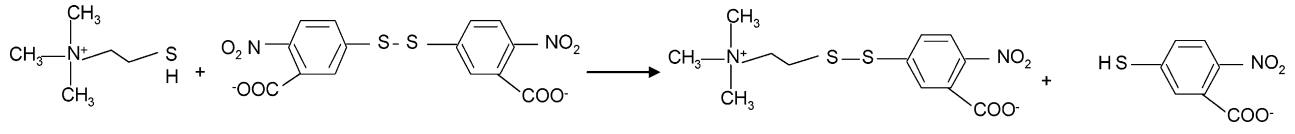
bation time [9–16]. In a biosensor design, electronic mediators such as tetracyanoquinodimetane (TCNQ) and cobalt phthalocyanine (CoPC) have been used to decrease the applied potential from 410 to 100 mV versus Ag/AgCl, hindering the oxidation of other compounds thus reducing the interferences [11–14]. Most biosensors have been developed using commercial Ee AChE. Some research groups, including ours have reported the use of genetically modified AChE from *Dm* [9,15–18]. The majority of AChE biosensors reported to date have been designed for detection of pesticides in water samples [17,19]. Application to other matrices such as food samples (fruits and vegetates) has been restricted due to the problems related to the use of these devices in the presence of organic solvents extracts. Several works reported that enzyme biosensors can function in a mixed aqueous–organic phase in low amounts of organic solvents [20,21]. However, these devices suffered from a low sensitivity and have not been used for the quantification of pesticides in solid sample.

The aim of this work was to develop a fast, simple and sensitive amperometric AChE biosensor for direct measurement of dichlorvos in an aqueous–organic solvent mixture and then, to apply the resulting biosensor for dichlorvos quantification in a fruit sample after a direct sample extraction. The strategy is based on the use of a genetically modified AChE enzyme from *Dm* which was engineered both to exhibit a high sensitivity towards dichlorvos and to increase resistance to denaturation by organic solvents. The performance of this biosensor was compared to those based on wild type *Dm* and commercial Ee AChEs. These enzymes are immobilized on screen-printing electrodes using a PVA-SbQ polymeric membrane. The aqueous–organic solvent ratio was chosen in order to use the highest concentration of organic solvent that has no effect on the enzyme activity.



(2)

2. Experimental

2.1. Chemicals and reagents

The wild (B1) and the genetically modified AChE (B 394) form from *Dm* were supplied from IPBS (Toulouse, France). AChE from Ee (EC 3.1.1.7 type V-S), acetylthiocholine iodide and chloride (ATChI, ATChCl), 5,5′-dithiobis(2-nitro-benzoic acid, DTNB) and albumin bovine (BSA), were supplied from Sigma–Aldrich. Acetonitrile HPLC grade was supplied from Carlo Erba. PVA-SbQ was from Toyo Gosei Company (Tokyo, Japan). Dichlorovos was obtained from Chem Service.

Electrodag (PE-410, 423SS, 6037SS) printing pastes obtained from Acheson (UK) and T15 graphite supplied by Lonza (Switzerland) were deposited on transparent PVC sheets (200 mm × 100 mm × 0.5 mm). Screen printed electrodes were prepared in our laboratory (BIOMEM) according to a published procedure [9].

Other chemical reagents used were analytical grade.

2.2. Apparatus

Spectrometric measurements were performed using a Hewlett Packard diode array 8452 A Spectrophotometer. Amperometric measurements were carried out with a 641 VA potentiostat (Metrohm) connected to a Kipped Zonen Flatbed X-t recorder. Screen-printing electrodes were produced using a Micro-Dek 248 printing machine.

3. Methods

3.1. Screen-printing electrodes manufactured

The screen-printing electrodes (SPEs) were fabricated according to a procedure used in the BIOMEM laboratory and published previously [9]. In this work, the working electrode layer was made using a Co-phthalocyanine carbon based paste.

3.2. Spectrometric measurements

The enzymatic activity of AChE was determined using the Ellman method [22]. The method is based on the quantitative and irreversible reaction of thiocholine, the product of the enzyme catalyzed reaction with DTNB, producing a yellow product 5-thio-2-nitrobenzoato that can be detected spectrophotometrically at 412 nm. The reaction is represented in (Eq. (2)).

The assay was performed by adding 0.700 mL of phosphate buffer (0.1 M at pH 7), 0.09 mL H_2O, 0.1 mL DTNB (3 mg/mL), 0.10 mL substrate (ATChI, 10 mM) and 0.01 mL enzyme. The DTNB solution was prepared in phosphate buffer 0.1 M pH 7.0 solution, and ATChI was prepared in 0.9% NaCl solution. The enzymatic activity calculated from the kinetic data (absorbance versus time) was expressed in U/mL. A blank without enzyme was considered as a control.

3.3. Enzyme immobilization

An enzymatic solution (3 μL) containing 30% enzyme solution (1.12 UI/mL) and 70% PVA-SbQ were deposited manually over the surface of the SPE working electrode. The amount of immobilized enzyme per electrode was calculated to be 1 mIU (apparent activity). The electrodes were dry a room temperature and then, exposed for 3 h under neon light at 4 °C degrees

to allow photopolymerization. Finally, the electrodes were conserved for about three days at 10 °C degrees before use.

3.4. Amperometric measurement

The amperometric measurements were carried out in a cell containing 10 mL PBS, at constant 30 °C under continuous magnetic stirring. A potential of 100 mV versus Ag/AgCl SPE pseudo-reference electrode was applied to the working electrode. After the stabilization of the current, 1 mM ATCh (final concentration in the cell) was injected in the reaction cell and the increase in the current intensity was measured. The stabilization time necessary to reach a plateau was 3 min. This current intensity corresponds to the oxidation of the thiocholine, the product of the enzymatic hydrolysis of the ATCh, and it is proportional to the AChE activity. The procedure was repeated three times to test the operational stability of the sensor; between each measurement the cell was washed with phosphate buffer solution (0.1 M at pH 7).

The determination of pesticide and study of the effect of the organic solvent on the activity of the immobilized enzyme was carried out using a three-step procedure. First, the initial response (I_0) of the biosensor was measured in PBS. Then, the biosensor was incubated for 10 min in a 10 mL mixture of acetonitrile-PBS with or without different concentrations of dichlorvos. After 10 min incubation, the biosensor was washed with PBS and the residual response (I_1) was measured again in PBS. The remaining activity (I%) was determined according to the following formula: $\%I = ((I_0 - I_1)/I_0) \times 100$. I% was then related to the inhibitor concentration. To evaluate the effect of the organic solvent on the enzyme activity, identical tests were carried out in different concentration of acetonitrile, in the absence of dichlorvos.

3.5. Pesticide quantification in apple sample

In order to eliminated pesticides residues over a sample, a middle size apple was washed, dry, and kept under refrigeration 1 week. The apple was cut in two parts. One half was treated with a known amount of dichlorvos. The skin was removed and spiked with a known amount of dichlorvos. After the skin was stirred with 5 mL acetonitrile for 10 min to allow extraction. The solvent was removed and evaporated. Finally, 1 mL acetonitrile was added to the dichlorvos residue, an aliquot of this solution was measured with the AChE biosensor to determine the inhibition percentage. The concentration of dichlorvos is determined using the calibration curve. The concentration of pesticide determined was compared with the results obtained for a blank which was made with the same procedure without apple skin. We have verified that the skin without addition of insecticides did not inhibit the enzyme.

4. Results and discussion

4.1. Selection of solvent concentration

The influence of three different concentrations of acetonitrile on the response of the immobilized AChEs was studied. For the three enzymes, the residual activity of the biosensor was determined (Fig. 1). No significant difference in the biosensor response was observed with 1 and 5% acetonitrile (Fig. 1). In the presence of 10% acetonitrile, the percentage of residual activity decreases 30%. As our goal is to use the highest concentration of acetonitrile which has no effect on the biosensor response, a concentration of 5% (v/v) acetonitrile was selected for further inhibition tests with dichlorvos.

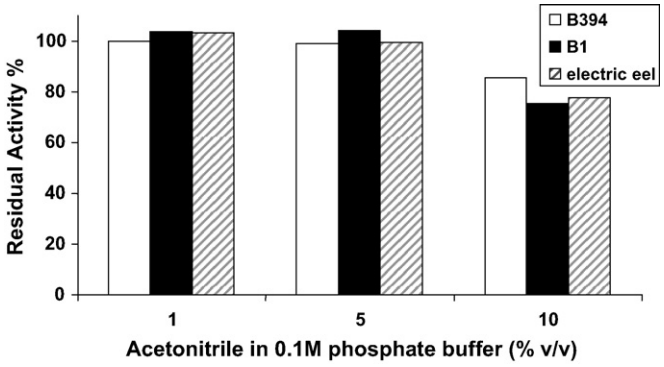


Fig. 1. The effect of acetonitrile on the biosensor responses: B394 *Dm*, B1 *Dm* and Ee. Percentage of relative response after 10 min of incubation in acetonitrile 1, 5 and 10% (v/v). The applied potential was 100 mV vs. the pseudo-reference Ag/AgCl SPE.

4.2. Substrate calibration curves of the biosensor

The substrate calibration curves for ATCh with and without 5% acetonitrile are presented in Fig. 2. The results show that the presence of acetonitrile in the reaction medium has no effect on the biosensor response. The apparent Michaelis-Menten constants for the three enzymes are shown in Table 1.These values have the same order of magnitude in the presence/absence of acetonitrile, showing that 5% solvent does not affect the activity of the enzyme. The same saturation concentration of the substrate (1 mM) was found for all the biosensors tested.

4.3. Dichlorvos detection

The majority of acetylcholinesterse sensors are developed using AChE from electric eel. This enzyme has demonstrated good characteristics in term of stability but lacks of sensitivity for the detection of some organophosphorus compounds and carbamates. We obtained engineered cholinesterases with exceptional qualities [23] and we used these enzymes to develop biosensors for the detection of organophosphorus compounds such as metamediphos [18] and carbamates [24]. The detection of chlorpyriphos is a good example of the quality of those enzymes. The

Table 1
Apparent Michaelis-Menten constants (K_m) for the B394 and B1 *Dm* AChE and for the Ee AChE in the presence/absence of 5% acetonitrile

Enzyme	B394	B1	Electric eel
K_m in buffer (mM)	0.21 ± 0.01	0.18 ± 0.02	0.16 ± 0.01
K_m in 5% acetonitrile (mM)	0.20 ± 0.02	0.12 ± 0.01	0.09 ± 0.01

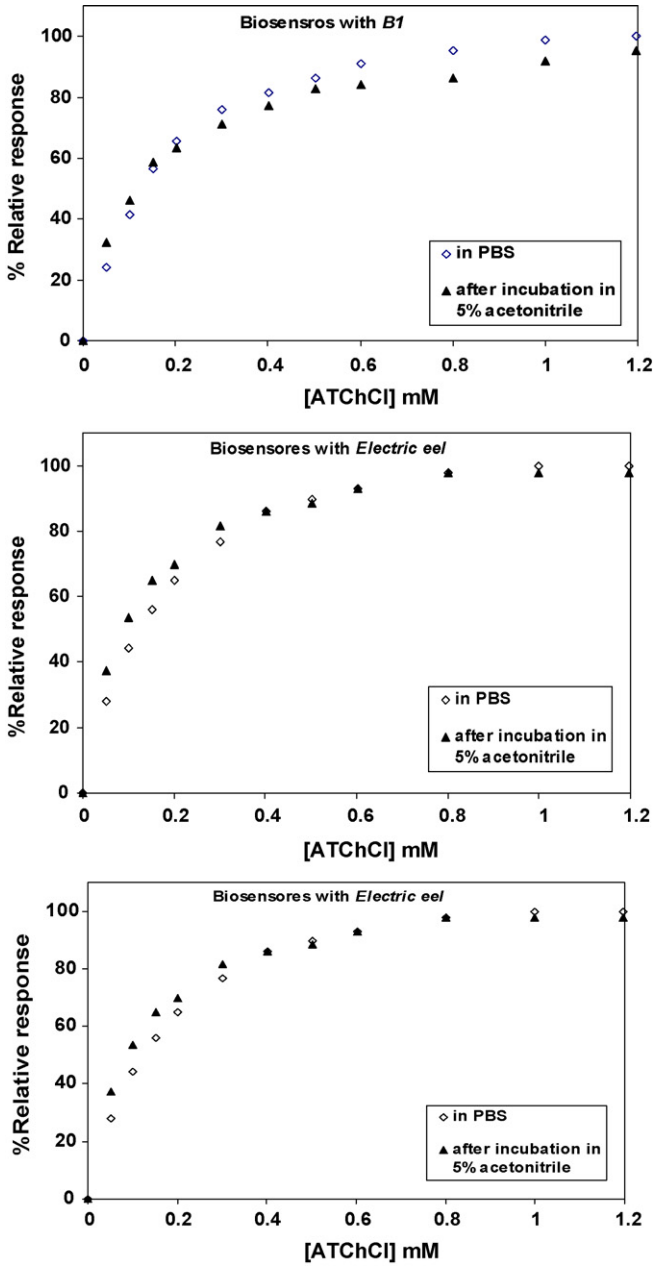


Fig. 2. Substrate calibration curves before and after 10 min incubation in 5% acetonitrile: B394 *Dm* (a), B1 *Dm* (b) and Ee (c) AChE.

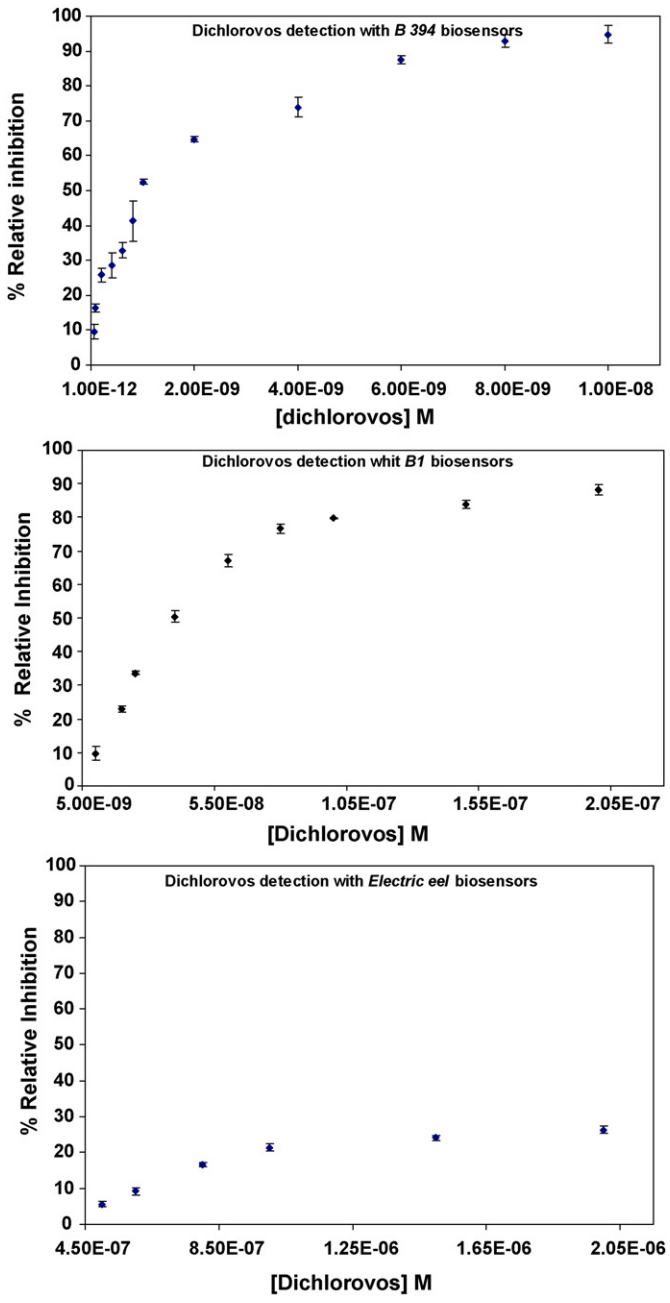


Fig. 3. Inhibition curves of AChE biosensors by dichlorovos. Experimental conditions: 5% acetonitrile, 10 min incubation time, 100 mV applied potential; B394 *Dm* (a), B1 *Dm* (b) and Ee (c) AChE.

inhibition curves of the immobilized AChEs by dichlorvos in the presence of 5% acetonitrile are shown in Fig. 3. Each value represents the average response of measurements obtained with three different electrodes prepared in the same experimental conditions. The detection limit was calculated as 10% inhibition (I_{10}).

Table 2
Inhibition constants K_i for dichlorovos determined by spectrometric analysis with AChE from electric eel, wild type (B1) and recombinant (B394) from *Drosophila melanogaster*

Enzyme	B394	B1	Electric eel
K_i ($\mu M^{-1}\,min^{-1}$)	224	1.9	0.036
LOD (M)	7×10^{-11}	10^{-8}	7×10^{-7}

The K_i of the engineered enzyme (B394) is 117- and 62 222-fold lower than wild type (B1) and electric eel source, respectively (Table 2). The very high sensitivity is confirmed by the results obtained using the same SPE, with the same method of immobilization for the detection of dichlorvos. Table 2 shows a good correlation between K_i and LOD for the detection of dichlorvos. Using biosensor with B394, the LOD obtained is, respectively, 142- and 8570-fold lower than with B1 and electric eel cholinesterase.

Table 3 is a compilation of the results obtained by different researchers for the detection of dichlorvos. Some laboratories developed biosensors using tyrosinase or acetylcholinesterase,

Table 3
Detection limits for dichlorvos using the AChE biosensors in our research and detection limits obtained in other researches

Source	LOD (M)	Origin	Transducer	Detection	Year	Ref.
AChE Ee	23×10^{-6}	SIGMA	Single bead reactor	Electro.	1992	[25]
AChE Ee	0.5×10^{-6}	SIGMA	Graphite	Electro.	2002	[26]
AChE Ee	0.5×10^{-6}	SIGMA	SPE	Electro.	2002	[27]
AChE *Dm*	3.6×10^{-8}	SIGMA	Cho Ox SPE	Electro.	2006	[28]
AChE Ee	1.8×10^{-10}	IPBS				
AChE *Dm*	1.5×10^{-9}	IPBS	SPE	Electro.	2006	[29]
AChE *Dm*	4×10^{-9}	IPBS	SPE	Electro.	2004	[10]
AChE Ee	10×10^{-13}	SIGMA	Nanoporous carbon FIA	Electro.	2005	[30]
AChE *Dm*	10×10^{-18}	IPBS	SPE (FIA)	Electro.	2005	[31]
AChE Ee	10×10^{-13}	SIGMA				
AChE *Dm*	10×10^{-18}	IPBS	SPE	Electro.	2005	[11]
Tyrosinase	0.08×10^{-6}	SIGMA	Coupled with HRP	Electro.	2005	[32]
Tyrosinase	0.06×10^{-6}	SIGMA	Glassy carbon/composite	Electro.	2006	[33]
OPH	50×10^{-6}	Unknown	Lithography FIA	Electro.	2003	[34]
AChE Ee	2.2×10^{-6}	SIGMA	Membrane	Optical	2006	[35]
AChE Ee	0.2×10^{-9}	SIGMA	Membrane	Optical	2007	[36]
AChE Ee	0.6×10^{-6}	SIGMA	SPE	Electro.	This work	
AChE *Dm*	70×10^{-12}	IPBS				

electric eel or genetically engineered enzymes. Usually genetic engineered enzymes show better sensitivity than enzyme from natural sources, varying from two [28] to five [31] order of magnitude. Some authors reported exceptional results with LODs as low as 10^{-17} M [11,31], This high performance is due to the association of a good transducer and the genetically engineered enzyme used in this study. However, such a high sensitivity has its drawback, it was found to be associated with a low repeatability.

4.4. Apple sample analysis

Analysis of dichlorvos in the apple sample was carried out using the B394 biosensor. For inhibition measurements, the biosensor was incubated for 10 min in a solution containing the dichlorvos extract in acetonitrile. This solution was prepared 5% (v/v) of organic sample extract and PBS solution. After incubation, the residual response of the biosensor was measured and compared with the initial response. The concentration of dichlorvos was calculated by interpolation of the inhibition percentage (*I*%) to the calibration curve obtained with standard solution of dichlorvos, the reference was tested following the same procedure (Fig. 3).

The results obtained with the apple sample show a 63% inhibition which is in good agreement with the 65% inhibition percentage obtained with the reference sample (without apple). These results are shown in Table 4.

Table 4
Dichlorovos quantification in apple sample

	Apple simple spiked with dichlorovos	Dichlorovos blank
%Inhibition	63	65
Dichlorovos concentration	2.3×10^{-9} M	2.6×10^{-9} M

5. Conclusions

This work shows the possibility to quantify dichlorvos in solid matrices using biosensors based on genetically modified *Dm* AChEs. The results demonstrate that the use of genetically modified AChE decreased considerably the detection limit (with four orders of magnitude) as compared to biosensors based on wild type *Dm* and Ee AChEs. In addition, the biosensor was able to work in the presence of 5% acetonitrile, which was necessary for the extraction of pesticide from the sample. The use of a disposable biosensor offers some additional advantages such as mass production, possibility for miniaturization and low cost.

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