Organic Phase Amperometric Biosensor for Detection of Pesticides

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Abstract

A rapid, simple, and sensitive acetylcholinesterase (AChE) amperometric biosensor for direct measurement of organophosphorus compounds (OPCs) in organic solvent was developed. The biosensors were based on a graphite electrode and on thiocholine-hexacyanoferrate reaction. The enzyme (AChE) was co-immobilized with an electron mediator, Prussian Blue, on the surface of the electrode. The effect of organic solvents on acetylcholinesterase activity was estimated in the presence of polar (hydrophilic) and non-polar (hydrophobic) organic solvents in the range of 0.01-100%. The ability of the AChE biosensors to assay pesticides was demonstrated by quantitative determination of dichlorvos, fenthion and diazinon in ethanol. The assay allows determination of OPCs in sub-micromolar concentration ranges with an overall assay time of 10 minutes. The method allows adapting the biosensor for detection of pesticides in actual water samples, avoiding the procedures of evaporation and resolution.

Keywords: biosensor, amperometric, acetylcholinesterase, pesticides

1. Introduction

Pesticides (herbicides, fungicides, insecticides) are widely used throughout the world, and millions of tons are used each year in agriculture, medicine and industry. Many of them are highly toxic, however, and their accumulation in living organisms can be the cause of serious diseases. Because similar compounds were produced as possible nerve poisons a further area of application is in the military. The destruction of OPC-based chemical weapons mandated by international agreements or as part of routine operations also leads to problems in environmental control and protection. Pollutants of this type are found to be present in many sampled soils, ground and wastewater's streams. One of the most important preventive measures in this case is to rapidly determine the source of the pollutant and the magnitude of the threat using on-site measurements. The mode of action of pesticides is based on irreversible inhibition of acetylcholinesterase and the same principle is utilized for analysis.

The pesticide assay in real samples requires the stage of extraction from water samples and pre-concentration because of relatively low concentration of target analytes and hydrophobic nature of pesticides [1]. This is usually accomplished using organic solvents, such as methanol, acetone, acetonitrile, or hexane or combining them with solid-phase

extraction [2]. In this respect, organic phase acetycholinesterase electrodes capable of measuring directly in the organic extract would be well suited particularly for the rapid analysis of pesticides without further sample processing.

Two basic designs have been used in the construction of organic phase amperometric enzyme electrodes [3]. First, the organic phase may be a gel incorporated into the microenvironment of the immobilized enzyme; the reaction medium is aqueous [4]. The sensitivity, in this case, is determined by the hydrophobic interactions between the gel and the analyte. The second type of organic phase enzyme electrodes is in the form of the reaction medium [5].

Important aspects of biosensors are the immobilization procedure for the enzyme on the electrode surface, and the selection of the solvent and electron communication between the electrode and the enzyme.

The most problematic areas in biosensor design is the interface between the enzyme membrane and the solid phase of an electrode. Different methods have been developed in an attempt to overcome the problem of electron communication between the electrode and the enzyme membrane. One possible approach involves a combination of biosensors with electron mediators that mediate electron transfer between the enzyme and the electrode. This demands a close contact between the enzyme molecules, the mediator and the electrode. In this respect, amperometric detection of the acetyl- or butyrylthiocholine hydrolysis process, catalyzed by AChE on the electrode surface is very attractive [6]. Thiocholine, the product of the enzyme reaction, can be anodically oxidized on an electrode surface. However, the large over voltage (700 mV vs. Ag/AgCl) makes acetyl- or butyrylthiocholine chloride or iodide as a substrate inconvenient for this purpose. This problem may be overcome by the use of chemically modified electrodes [7,8]. Prussian Blue (PB) film immobilized on the electrode surface have found considerable application as redox mediators [9,10].

The most general approach for determination of the organophosphorus compounds is based on their inhibitor properties on the activity of choline esterase enzymes [2]. The presence of low concentrations of inhibitors strongly and specifically affects enzyme activity.

Our work has focused on the development of new strategies for the biosensing of OPCs in organic solvents based on an amperometric OPCs assay based on thiocholine-hexacyanoferrate reaction [11]. The following reactions describe the principle of amperometric detection of AChE activity:

$$(CH_3)_3N^+CH_2CH_2SCOCH_3 + H_2O \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH \quad (1)$$

$$2(CH_3)_3N^+CH_2CH_2S^- + 2[Fe(CN)_6]^{3-} \rightarrow (CH_3)_3N^+ CH_2CH_2SSCH_2CH_2N + (CH_3)_3 + 2[Fe(CN)_6]^{4-} \quad (2)$$

$$[Fe(CN)_6]^{4-} - e^- \xrightarrow{electrode} [Fe(CN)_6]^{3-} \quad (3)$$

Thiocholine, produced by enzymatic hydrolysis of acetylthiocholine (ATCh), reacts with hexacyanoferrate (III). Then, the reduced electron mediator is reoxidized at the graphite electrode and the analytical signal is measured amperometrically. Since pesticide inhibits this reaction, the concentration of pesticide may be determined by the decrease in oxidation current. The amperometric AChE biosensor was applied to the determination of fenthion, diazinon and dichlorvos.

Materials and methods

Reagents

Acetylcholinesterase (EC 3.1.1.7) from electric eel, 14.6 IU/mg of solid, acetylthiocholine chloride (ATCh), polyethylenimine and 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) were purchased also Sigma Chem. Co., USA. Fenthion, diazinon and dichlorvos were supplied from Aldrich Chem. Co USA. All other reagents were analytical grade. Stock solutions of pesticides (5×10^{-3} M in ethanol) were prepared fresh every day. Prussian Blue (PB) was a gift from Prof. R.P. Baldwin University of Louisville, Kentucky, USA.

Preparation of Polyethylenimine-Prussian Blue (PEI-PB) modified electrodes

Graphite rods (made from pencil lead, HB 0.9 mm) were cleaned in methanol, rinsed with double-distilled water, and dried. The cellulose acetate film (from a 45% w/v acetone solution) was used for insulating of graphite rods except for 0.1 cm (one end designed as a working electrode surface) and 1 cm (the other end left for current collecting). PEI-PB films were deposited on the exposed 0.1 cm working section of the electrode surface. The working electrode surface was dipped for 15 min in a 0.2 % methanol solution of PEI and air-dried for 8 h. The electrode surface was washed with methanol to remove the excess unbound polymer. A fresh solution of 0.02 M $FeCl_3$ and 0.02 M of $K_3Fe(CN)_6$ was prepared in distilled water. The electrode was dipped in the ferric-ferricianide solution were cathodically polarized for 15 min under galvanostatic conditions with current density of 40 mA/cm^2 [12]. Cyclic voltammetric experiments were performed in a three-electrode cell with counter (Pt) and a commercial Ag/AgCl reference electrodes and BAS CV-50 potentiostate interfaced to a PC computer system with BAS software. The rotation of the graphite electrode was performed with a BAS Instruments rotator provided with a speed controller**.** The electrochemistry of the electrodes covered by the thin film of PB was examined in an acidic 1.0 M KCl solution (pH 4.0).

AChE sensor design

AChE was immobilized covalently on PEI-PB-coated graphite electrodes. PEI-PB modified graphite electrodes were dipped for 4 h in a 2.5% glutaraldehyde solution (pH 7.5). The electrodes were then washed and immersed in a 0.1 M phosphate buffer (pH 7.2) containing AChE (1 mg/ml) and incubated for 12 h at 4^0 C. Unreacted aldehyde groups were blocked by immersion of the electrodes in 0.1 M glycine (pH 7.2). The electrodes were washed and then stored dry in a refrigerator (4^0 C) when not in use. AChE modified graphite electrodes with co-immobilized PEI and PB (AChE-PB-PEI electrodes) were used as sensing elements of the sensor. The enzyme electrodes can be stored dry for 2 months at 4^0 C. All electrochemical measurements were performed in the three-electrode cell (V= 0.3 ml) with a rotating AChE-PB-PEI modified working electrode, a Pt counter and Ag/AgCl reference electrodes. The activity of AChE on the electrode surface was estimated at the initial rate of reduction of hexacyanoferrate (III) and was determined spectrophotometrically at 420 nm [13].

Determination of pesticides in organic solvents

The AChE-PB-PEI modified electrodes have been used for measurement of fenthion, diazinon and dichlorvos in ethanol solutions. Stock pesticide solutions were prepared by withdrawing 10 μl of the pesticide and then placing its in a vial and weighting. It was then made up to a final volume of 10.0 ml with a pure ethanol solvent. The standard concentrations of pesticides were prepared daily in ethanol solvent. The kinetic approach was used for determination of pesticides. The AChE-PB-PEI modified electrode was immersed into

electrochemical cell with 0.3 ml 0.1 M phosphate buffer (pH 7.5) containing 0.1M KCl, 0.01 mM $MgCl_2$ and stirred at 300 rpm. The potential of AChE-PB-PEI electrode is set at +350 mV vs. Ag/AgCl. Then 0.01 ml of pesticide in pure ethanol solvent (or only ethanol) is injected and a background signal was recorded. After the current has stabilized (10 min), the 0.2 mM ATCh is added, and a second stationary current state is reached. The percent inhibition was calculated using the formula (1):

$$I\% = (i_1 - i_2) / i_1 \times 100 \quad (1)$$

where I% is the degree of inhibition, i_1 is the steady-state current obtained in the presence of only ethanol and i_2 is the steady-state current obtained in the presence of pesticide in the sample. Steady-state responses were obtained after 10 seconds.

Results and Discussion

Our strategy for pesticide determination in organic solvent was based on the usage of only one enzyme (acetylcholinesterase) in combination with following signal-amplification systems: (1) the co-immobilization of redox mediators (Prussian Blue) and AChE on the electrode surface; (2) the accumulation of the products of enzymatic and electrochemical reactions at the membrane/ electrode interface; and (3) the cyclic regeneration of the redox mediators at the electrode surface.

Prussian Blue (PB) films immobilized on the electrode surface have found considerable application as redox mediators. The reasons for employing Prussian Blue (PB) films include their simple preparation and their potential for electrocatalytic applications in aqueous and non-aqueous media [10]. In addition, this redox system has the advantages of a well- defined electron stoichiometry, insensitivity of the mediation reaction to changes in pH and ionic strength, and a high value of the rate constant for electron transfer between the enzyme and the electrode.

Figure 1 shows the cyclic voltammogram of a PB film on an unmodified graphite electrode, which showed two redox peaks. The reduction peak and reoxidation peak occur at + 0.190 V and + 0.240 V, respectively. The total amount of deposited Prussian Blue was between 4 and 6 nmol/cm^2**.** The PB modified graphite electrodes are very stable and no degradation of the peak height was observed under repeated scanning in pure 0.5 M KCl. The linear dependence of the peak current (i_{pc} and i_{pa}) on the scan rate of the electrode potential was obtained up to 100 mV/sec confirms a surface reaction by the bound ($[Fe(CN)_6]_4/[Fe(CN)_6]_3$) redox groups.

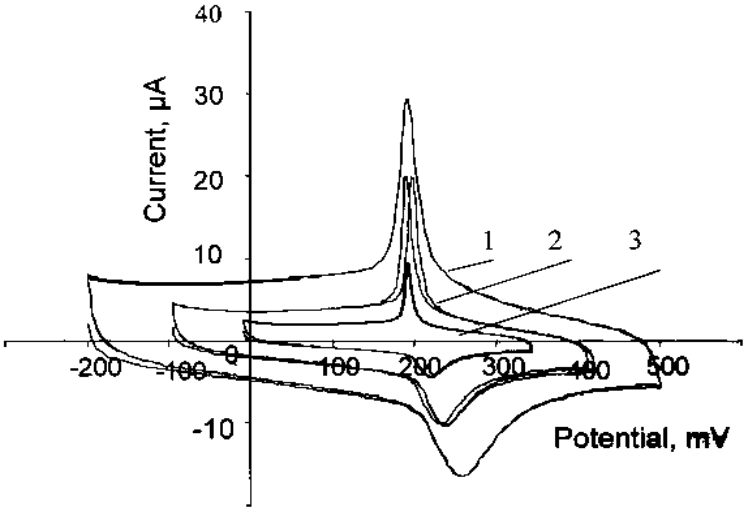

Figure 1. Cyclic voltammograms of Prussian Blue modified carbon electrode in a 1.0M KCl solution, pH 5.0, at different scan rates. Scan rates: (1) 100 mV/s; (2) 50 mV/s; (3) 20 mV/s.

The determinant factors in the development of the fast and sensitive AChE-sensor are the method for the enzyme immobilization and the microenvironment for mediated electron transfer from the enzyme to the electrode surface [10]. A variety of methods for enzyme immobilization, such as crosslinking of the enzyme by bifunctional reagents, covalent binding and entrapment in suitable matrices have been employed [14]. In this work, we have used a bifunctional reagent, glutaraldehyde, as the cross-linking agent for immobilization of AChE on the electrode surface. An advantage of this technique is strong chemical binding of the biomolecules. The polymeric nature of the glutaraldehyde provides attaching the enzyme to the electrode surface, which may permit greater flexibility for protein conformational changes required for enzyme activity. In order to define the optimal conditions for immobilization of AChE on the electrode surface, the relationship between the magnitude of the analytical signal, concentration of polyethylenimine (PEI) and glutaraldehyde (GA) have been studied, optimized and converted into the equation 1, by the BAS-1 software:

$$I = 288 - \{[286\ (A-60)]/30 + [155\ (B-10)]/5\} \qquad (2)$$

where I is the response of the AChE electrode (nA), A and B are the concentration of the PEI (%) and GA (%), consequently. AChE (1 mg/ml) for immobilization and ATCh (1.2 mM) for measurements were used in fixed concentrations.

The effect of a glutaraldehyde (GA) concentration was studied in the range from 0.1% to 5%. We found that for a glutaraldehyde (GA) concentration ranging from 0.5% to 3.0% the activity of immobilized AChE is almost constant. For a GA concentration higher then 3.0%, the activity of AChE decreases. The advantages of glutaraldehyde for AChE immobilization on the PEI-PB electrode surface are simple, rapid and this technique is suitable for manufacturing AChE electrodes with the same enzyme activities.

The ionic, positively charged PEI is used in the biosensor design not only for immobilizing the AChE, but also for accumulating and creating a high local concentration of negative charged products of enzymatic and electrochemical reactions within an electrode surface. Figure 2 illustrates the cyclic voltammograms of a PB film on a bare and a PEI-PB modified graphite electrodes. We found that the rate of electron transfer is significantly enhanced at the PEI-PB modified electrode that on a bare graphite electrode (**Figure 2**).

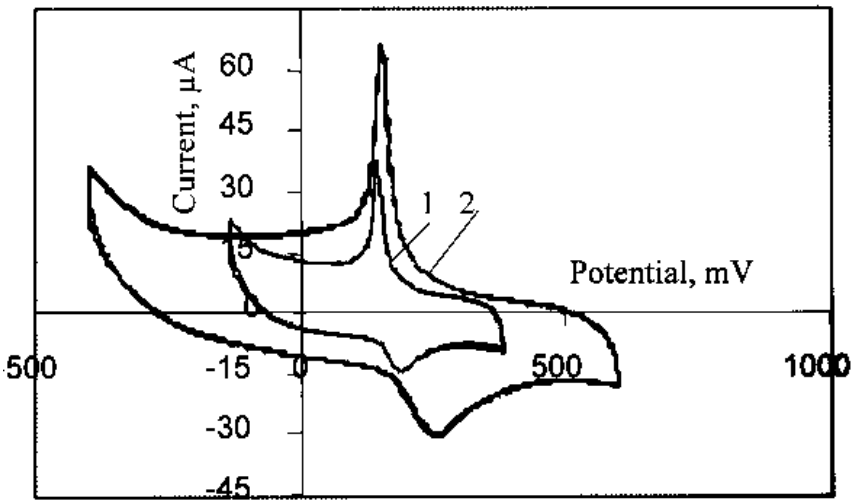

Figure 2. Cyclic voltammograms of (1) PB and (2) PEI-PB modified graphite electrodes in 0.5M KCl solution, 300 rpm, 20^0 C.

Several factors may contribute to this amplification: (i) The co-immobilization of PEI and the redox mediators on the electrode surface; (ii) Positively charged PEI film on the electrode surface is effective not only for immobilizing the AChE, but also for accumulating and creating a high local concentration of products of enzymatic and electrochemical reactions within an electrode surface; (iii) The permanent electrochemical regeneration of hexacyanoferrate (III) ions within the diffusion layer.

The short diffusion distance on the interface of PEI/PB/electrode permits a rapid diffusion of the electroactive mediators to the electrode surface. Thus, the co-immobilization of the positively charged PEI in the PB layer changes the behavior of the working electrode dramatically. The best results where obtained with 0.2% PEI.

Selection of the organic solvent for biosensing

The effect of organic solvents on activity of AChE immobilized on the electrode surface have been studied in the presence of polar and non-polar organic solvents in the range of 0.01-100%. The response of AChE-PEI-PB electrode was measured in 0.1M phosphate buffer, pH 7.5, and 0.1 M KCl, in the presence of a fixed concentration of ATCh, before and after the working electrode was incubated for 10 min in aqueous-solvent mixtures or pure organic solvent. The percent inhibition was calculated using the formula: $I\% = (I_1 - I_2)/I_1 \times 100$, where I% is the degree of inhibition, I_1 is the steady-state current obtained in buffer solution, and I_2 is the steady-state current obtained after the working electrode was 10 min incubated in organic solvent. Studies of AChE stability on the electrode surface in different organic solvents revealed that exposure of AChE electrodes to benzene or cyclohexanone the catalytic activity of the AChE is markedly decreased (**Figure 3**).

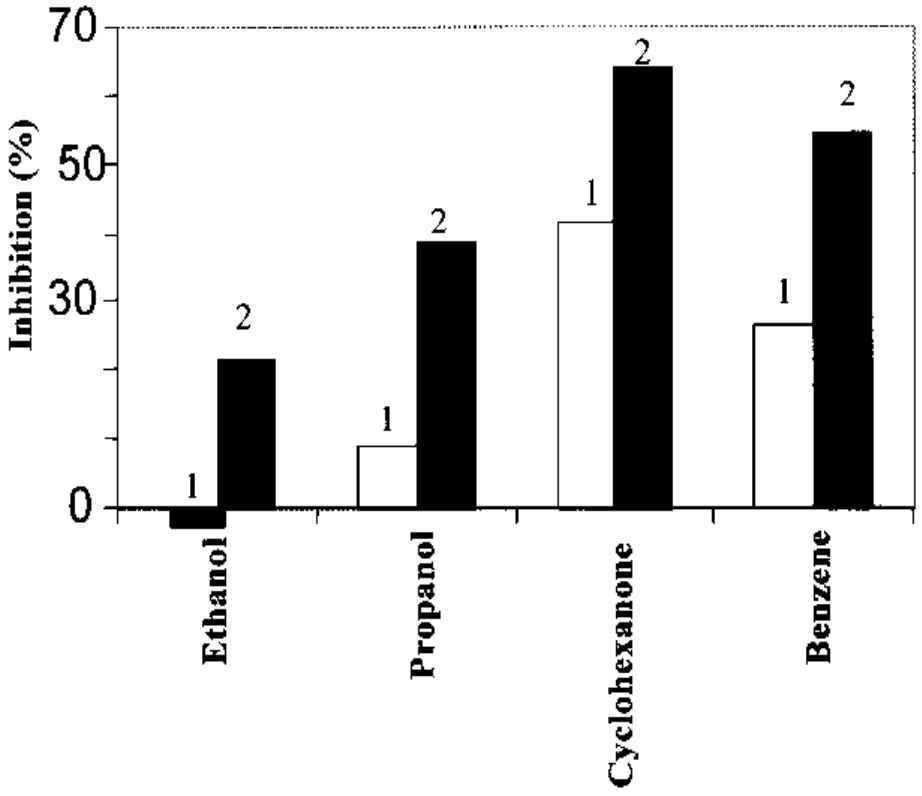

Figure 3. Inhibition of acetilcholinesterase on the electrode surface after a 10 minute incubation in (1)-10% water-organic solvent mixture and (2)-pure organic solvent.

However, the AChE-PEI- PB electrode can operate in the buffer solution in the presence of 0.1%-10% of polar solvents (ethanol or propanol). We found that the activity of AChE on the electrode surface in mixture of the water solution with 0.1%-10% of polar organic solvent, was higher than in pure aqueous solution. The same effect was also described for alcohol dehydrogenase and other enzymes in the water-ethanol mixture [3,9]. With the right amount of water and polar organic solvent, lowering of the dielectric constant of the enzyme active site micro-environment can probably be manipulated to obtain an enhanced sensor response compared to responses in the aqueous phase [3]. However, in water free polar solvent the enzyme on the sensor surface is inactivated. Therefore, for the assay of pesticides in pure ethanol solvent we used a kinetic approach, which was described before.

The calibration curve for ATCh obtained with AChE-PEI-PB modified electrodes in 10% ethanol-phosphate buffer solution is presented in **Figure 4**.

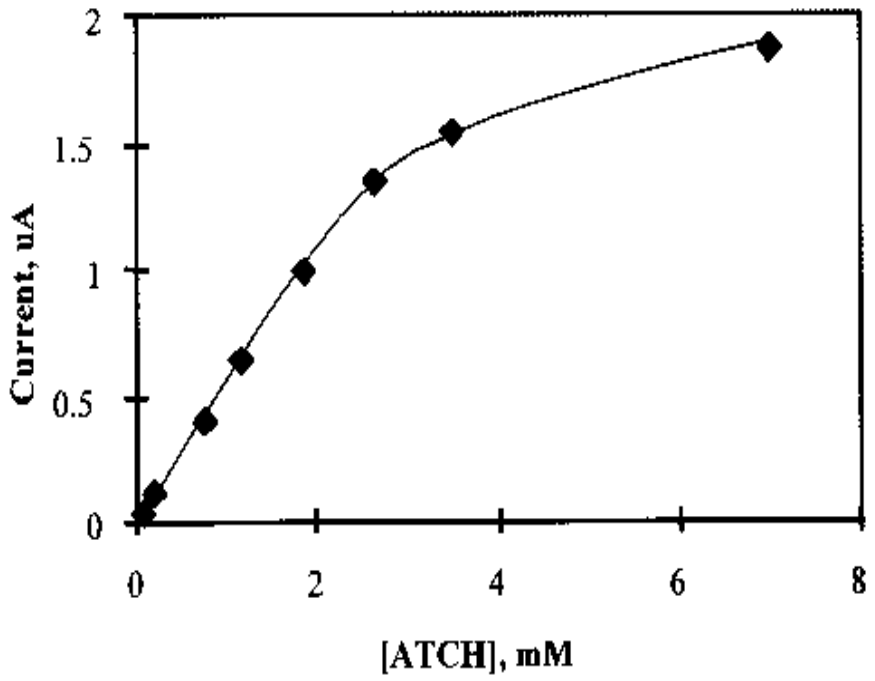

Figure 4. Calibration plot for the ATCh in 10% ethanol-phosphate buffer solution, pH 7.2.

The optimum pH (7.0-8.0) for the sensor was chosen according to results previously published [15]. This pH profile is related to both the kinetics of the enzymatic hydrolysis of ATCh and the kinetics of the reduction of hexacyanoferrate (III) by thiocholine. Responses of the AChE-PEI-PB electrode to ATCh chloride were measured over the concentration range 0 - 7 mM, at an applied potential of 350 mV vs. Ag/AgCl. The relationship between the AChE-PEI-PB electrode response and the value of ATCh concentration is linear in the range 0 - 900 mM ATCh. The response time of the AChE sensor is one min.

Unlike potentiometric the AChE sensors based on pH measurement, the response of the AChE sensor increases with increasing of the buffer concentration. AChE electrodes have a long shelf life. The electrodes were stable over at least 60 days at 4^0 C when stored under dry conditions. After 60 days the retained activity was 65% of the original activity.

Determination of pesticides in organic solvent

Ethanol was used as an extraction organic solvent for the determination of pesticides. The advantage of using ethanol as organic solvent lies in a good solubility of ATCh and pesticides in the solvent and also in causing the need to avoid the inactivation effect of the solvent on biosensor response.

Figure 5 shows the result of a quantitative determination of fenthion, diazinon and dichlorvos in ethanol obtained by the kinetic mode as described in the experimental section. Data are recorded in terms of percentage inhibition as a function of the inhibitor concentration. Standard deviation is represented as an error bar. Each point represents the average of at least five measurements. The lowest detection limits for dichlorvos, diazinon and fenthion are 0.5 x 10^{-6} M, 2.2 x 10^{-6} M and 1.0 x 10^{-6} M (signal-to-noise ration = 4), respectively. They are close to the lowest values found in the literature [2], but the assay of pesticide in ethanol solvent can be done in a relatively short time, not more than 10 min. The coefficient of variation of the blank measurements and inhibition measurements were found to be 5.8% and 6.3%, respectively.

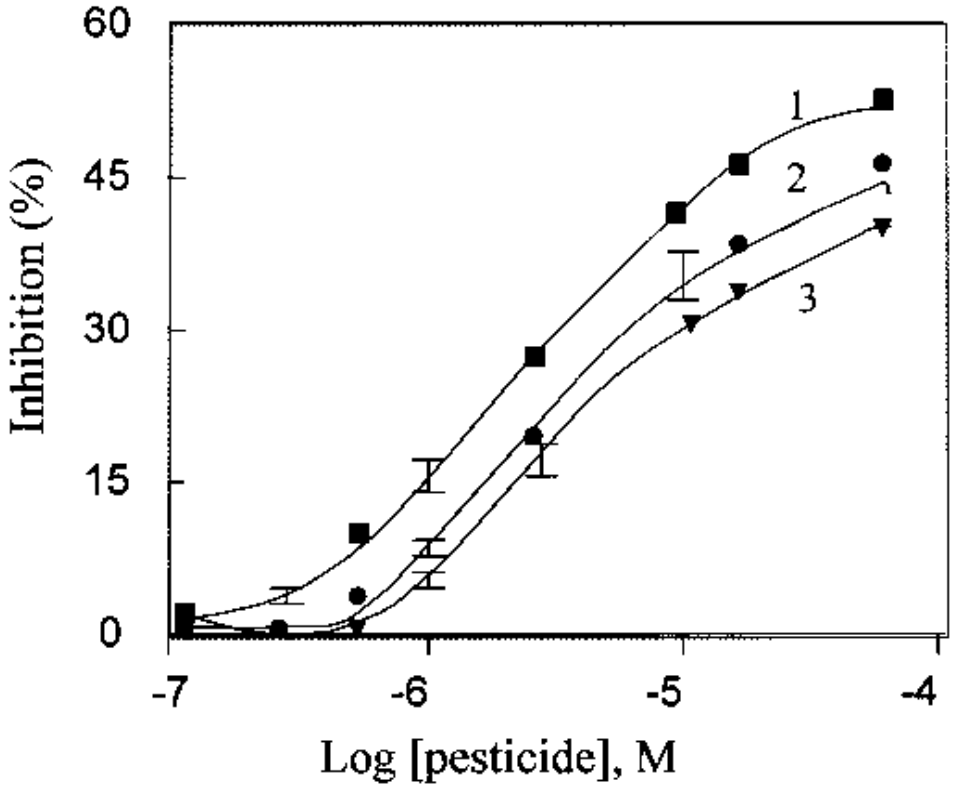

Figure 5. *Determination of pesticides with the amperometric AChE-PB modified sensor (0.1M phosphate buffer, pH 7.2, containing 0.05M KCl and 0.1 mM ATCh, at 25^0 C). 1-Dichlorvos; 2- Diazinon; 3-Fenthion.*

Conclusions

The research described in this paper resulted in amperometric method for direct OPC detection in organic solvents. Various properties of the biosensors were investigated and the ability of the AChE biosensors to assay pesticides was demonstrated by quantitative determination of dichlorvos, fenthion, and diazinon in organic solvents.

The technique of measurement is simple and the complete assay is carried out in 10 min. The detection level of the AChE sensor is directly related to the capacity of the pesticide to inhibit AChE. The amperometric AChE biosensor can be stored in a dry state for more than 2 months without any loss of the activity. The new sensors are of single use and can be mass-produced at low cost. The amperometric biosensor may be easily transformed into a multi-channel analytical device and it may be extremely useful in screening tests in field conditions. The AChE biosensor possesses distinct advantages, including monitoring of hydrophobic substrates, elimination of microbial contamination, and relative ease of enzyme immobilization.

Potential application of the AChE-PEI-PB sensor may be in a clinical laboratory, food industry and for environmental monitoring where the analytes of interest have poor water solubility or where existing methods lack sensitivity. The biosensors are also useful in military situations when the use of chemical warfare agents is expected.

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