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Full Paper

Development of Sol–Gel Immobilized Electrochemical Biosensor for the Monitoring of Organophosphorous Pesticides: A Voltammetric Method

Pamula Raghu[1], Matti Mohan Reddy[2], Tukiakula Madhusudana Reddy[1,*], Bahaddurghatta E. Kumara Swamy [3,*] and Kasetty Reddaiah[1]

[1]*Electrochemical Research Laboratory, Department of Chemistry, S.V.U. College of Sciences, Sri Venkateswara University, Tirupati-517502, Andhra Pradesh, India*
[2]*Department of Psychiatry, Sri Devaraj Ur's Acedamy of Higher Education and Research (SDUAHER), Tamaka, Kolar, Karnataka, India*
[3]*Department of P.G. Studies and Research in Industrial Chemistry, Kuvempu University, Shankaraghatta -577 451, Shimoga, Karnataka, India*

*Corresponding Author, Tel.:+91-877-2289303; Fax: +91-877-2248499/2249111
E-Mails: tmsreddysvu@gmail.com, kumaraswamy21@yahoo.com

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Abstract- Acetylcholinesterase (AChE) enzyme was immobilized through the silica sol–gel process onto the surface of carbon paste electrode (CPE). This fabricated monoenzyme biosensor on CPE was used as a working electrode. The enzyme biosensor on reaction with acetylthiocholine chloride (ASChCl or substrate), was found to be enzymatically hydrolyzed to thiocholine and acetic acid, which intern gave a disulfide compound and produced a larger anodic current at 0.63 V. The AChE biosensor was used for determining the two organophosphorous pesticides i.e. quinalphos and malathion in 0.1 M phosphate buffer/0.1 M KCl. The effect of scan rate, pH, enzyme loading and substrate concentration on the biosensor response was studied. Calibration graphs were performed for a concentration range of 20–300 ppb and 0.07–1.3 ppm for quinalphos and malathion respectively by employing the fabricated biosensor electrode. The limit of detection and limit of quantification values was found to be 8 ppb, 0.058 ppm and 26 ppb, 0.194 ppm for quinalphos and malathion respectively.

Keywords- Biosensor, Acetylcholinesterase, Acetylthiocholine Chloride, Sol–Gel Immobilization, Quinalphos and Malathion

1. INTRODUCTION

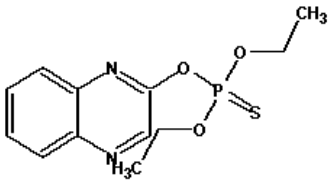
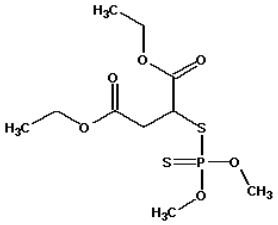
Organophosphorous pesticides are widely used in agriculture because of their insecticidal activity and their relatively low persistence in the environment. Their great success in agricultural applications has led to an increase in the production and spread of pesticide contamination. These pesticides are important pollutants and hazardous to human health and life. In areas where intensive monoculture is practiced, pesticides are used as standard method for pest control. Organophosphorous pesticides are highly effective broad spectrum insecticides. They are increasingly used instead of organochloride pesticides due to their lower environmental persistence. However, they present a high toxicity that may represent in a serious rise of professional exposure and for the equilibrium of acquatic system.

Quinalphos and malathion are organophosphorus insecticides (Table 1) and they are widely used in agricultural due to its acaricidal and insecticidal properties, especially malathion is used in residential landscaping and in public health pest control programs such as mosquito eradication. The misuse of these pesticides results in contamination of fields, crops, water and air. Both the compounds are relatively insoluble in water, poorly soluble in petroleum ether and mineral oils, and readily soluble in most organic solvents. These pesticides represent a source of toxicity towards human beings and vertebrate animals and its mode of action occurs through the contact and intake through food chain. Both the pesticides are more toxic and acts as cholinesterase inhibitors.

Many methods are available for pesticide detection. Chromatographic methods such as high performance liquid chromatography (HPLC) and gas chromatography (GC) are used as reference methods, but they have strong drawbacks, such as complex and time consuming treatments of the samples i.e. extraction of pesticides, extract cleaning, solvent substitution etc. [1,2]. Moreover the analysis usually has to be performed in a specialized laboratory by skilled personnel and is not suitable for in situ application. These issues turnout to be a major problem when rapid and sensitive measurement is needed in order to take the necessary corrective actions in a timely fashion.

To respond to the above issues, the enzymatic methods have been adopted as an alternative to classical methods (GC and HPLC) for faster and simpler detection of some environmental pollutants [3].The cholinesterase based biosensors are one of the best alternatives in the context of this strategy. These biosensors are simple to fabricate and low cost of the equipment also make possible in situ measurement of pesticides by various techniques such as amperometric [4-7], potentiometric [8-10] and conductometric biosensors [11] have been developed using this approach. For amperometric detection of cholinesterase activity the substrate acetylthiocholine chloride has been extensively used [12].

Table 1. The structural and molecular formulae of quinalphos and malathion

Pesticide	Molecular formula	Structure
Quinalphos	$C_{12}H_{15}N_2O_3PS$	
Malathion	$C_{10}H_{19}O_6PS_2$	

Sol–gel immobilization technique can be preferred instead of usual immobilization protocols such as covalent binding, adsorption, encapsulation of sensing agents with in a polymeric matrix etc. Since some of these procedures are tedious, result in poor stability and perturbed function, requiring expensive reagents or environmentally unattractive solvents. So many sol–gel derived enzyme biosensors have been developed at the research level to monitor glucose, lactate, phenols, urea etc. [13-16].

In this paper, we report biosensor derived from TEOS sol–gel system doped with acetylcholinesterase towards quinalphos and malathion detection. Experimental parameters such as the scan rate, pH and enzyme loading have been investigated to evaluate the conditions for the best performance of the biosensor.

2. EXPERIMENTAL

2.1. Materials

Acetylcholinesterase (E.C. 3.1.1.7 type–VI–S/1.5 mg, electric eel source, 500U/1.5 mg) and acetylthicholine chloride were purchased from Sigma–Aldrich chemicals co. USA. Quinalphos and malathion were obtained from Accustandard solutions company, USA. The pesticide stock solution was prepared dissolving in acetone (GR grade) solution. Tetraethylorthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), Triton–X–100 were obtained from Sigma–Aldrich chemicals co. USA. The graphite fine powder was procured from Lobo chemie and silicon oil from Himedia Company. The acetone (GR grade) was obtained from Merk Specialities Pvt. Ltd. Phosphate buffer solution was prepared by mixing 0.1 M sodium dihydrogen phosphate monohydrate and 0.1 M disodium hydrogen phosphate. All the aqueous solutions were prepared with double distilled water. All chemicals were obtained from commercial sources and used without further purification. The enzyme

stock solution was stored at –20^{o}C. All stock and working solutions of chemicals were stored at –4 ^{o}C.

2.2. Apparatus

The electrochemical experiments were carried out using an electrochemical workstation; model CHI–660C (CH instruments). All the experiments were carried out in a conventional three electrode electrochemical cell. The sol–gel immobilized enzyme electrode body was used a working electrode and Pt wire as an auxiliary electrode. To determine the potentials at the surface of working electrode, saturated calomel electrode was used as a reference electrode. All the experiments were carried out at room temperature (25±2 °C).

2.3. Preparation of bare carbon paste electrode

The bare carbon paste electrode was prepared by hand mixing of 70% graphite fine powder and 30% silicon oil in an agate mortar to produce a homogenous carbon paste. The paste was packed into the cavity of homemade PVC (3 mm in diameter) and then smoothed on a weighing paper. The electrical contact was provided by copper wire connected to the paste at the end of the tube [17-19].

2.4. Biosensor construction

2 ml of TEOS, 1 ml of H_2O, 50 µl of 0.1 M HCl and 25 µl of 10% Triton–X–100 were magnetically stirred for 1 hr until obtaining a homogenous TEOS silica sol. The mixture was homogenized before each usage and stored at -20 °C. The mixture was stable for about three months.

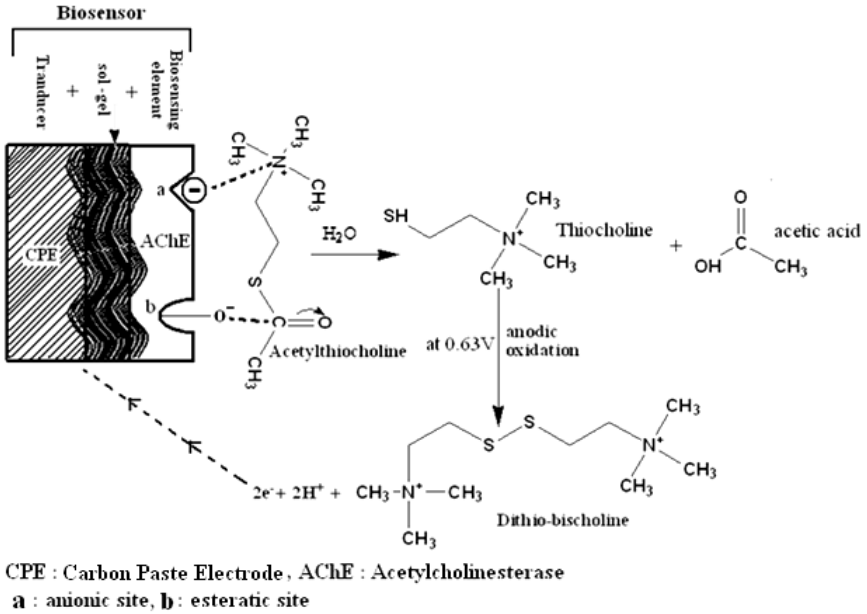
The 5 µl of stock solution of sol–gel was vortexed with 45 µl of phosphate buffer containing 0.5 U of enzyme stock solution. The 5 µl of the enzyme sol was spread on the electrode surface. This film was allowed to polymerize at room temperature for 3–5 min. This electrode was gently washed with phosphate buffer (pH 7.0) and is used for further experimental procedures [20]. The 0.05 U of enzyme was immobilized on the electrode surface.

3. RESULTS AND DISCUSSION

3.1. Cyclic voltammetric characterization of biosensor

The fabricated AChE based biosensor entrapped through sol–gel immobilization method onto the carbon paste electrode (CPE) was found to hydrolyze the substrate (acetylthiocholine chloride), and the mechanism was shown in scheme 1. The active site of acetylcholinesterase consists of two sub sites, namely an anionic site (a) and an esteratic site (b) which contains the crucial serine residue. The anionic site has electrically negative potential which attracts the quaternary ammonium head of acetylthiocholine and helps in orienting the charged part

of the substrate entering the active center. The esteratic site is involved in the actual catalytic process. The activated serine residue undergoes a nucleophilic attack onto the carbonyl group present in the substrate which is present in transition tetrahedral state. The negative charge formed on the carboxylic oxygen of acetylthiocholine was rearranged to give the products as thiocholine and acetylated enzyme. The two molecules of thiocholine undergo oxidation process to give a dimmer with a larger anodic peak current (Ipa) at potential 0.63 V *vs.* saturated calomel electrode. The deacetylation of enzyme takes place in the presence of one water molecule to give acetic acid and native enzyme (serine residue). Fig. 1 shows the cyclic voltammograms of the sensor in the presence and absence of 1mM substrate in phosphate buffer (pH 7.0) at a scan rate of 10 mV s^{-1}. It has been reported that buffer containing substrate electrochemically detected by using AChE [21,22]. The biosensor response varies with addition of concentration of the substrate, first the biosensor response increases with substrate concentration and reaches a plateau level, and it was shown in the Fig. 2.



Scheme. 1. The mechanism of the enzyme catalyzed reaction

The apparent Michaelis–Menton constant (K_m^{app}) was 550 μM, which was calculated from the linear part of the calibration plot using Lineweaver–Burk equation. This data illustrates that the immobilization was successful and AChE maintains its biological activity within sol–gel matrix.

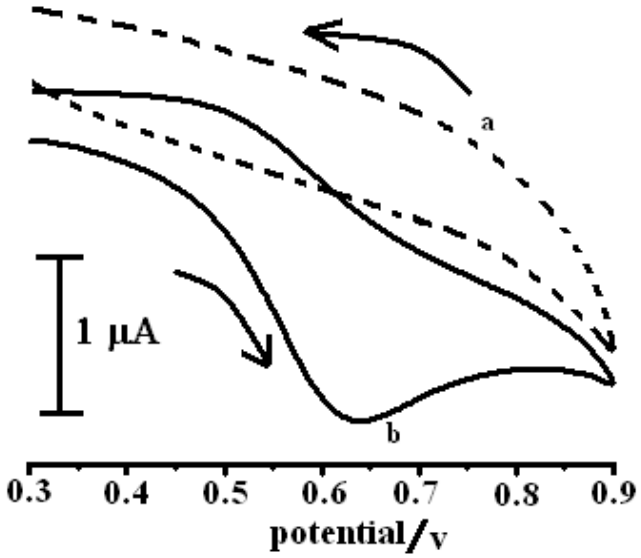


Fig. 1. Cyclic voltammogram of enzyme electrode in 0.1M phosphate buffer, pH 7.0 and 0.1 M KCl (a) without substrate (dashed line) (b) with 1 mM substrate (solid line)

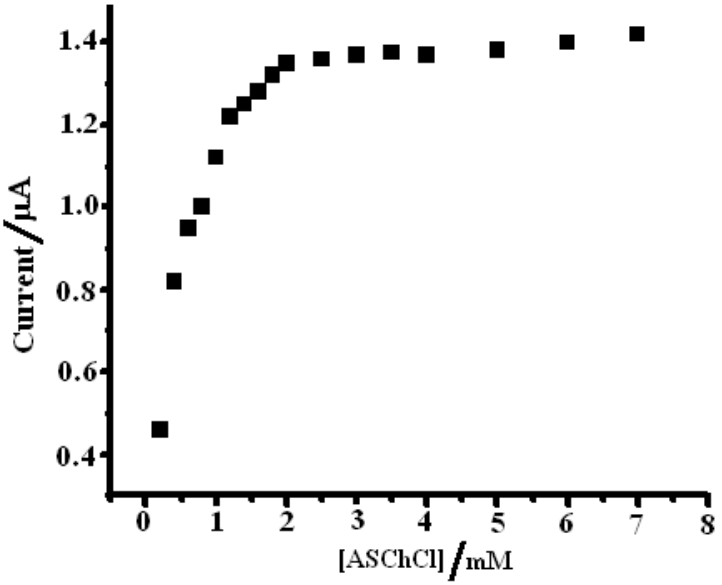


Fig. 2. Calibration graph for acetylthiocholine chloride obtained with AChE immobilized sensor in 0.1 M phosphate buffer / 0.1 M KCl at pH 7.0

3.2. Effect of scan rate and pH

The effect of scan rate for 1 mM acetylthiocholine chloride on the anodic peak current (Ip_a) was studied in 0.1 M phosphate buffer solution/0.1 M KCl solution. The 'Ip_a' increases linearly with increasing scan rate ranging from 5 to 40 mV s^{-1}. The graph between Ip_a *vs.*

square root of scan rate ($\upsilon^{1/2}$) obtained good linearity with a correlation coefficient of 0.9943 (r^2), which was shown in Fig. 3. This indicates the electrode reaction was diffusion controlled process.

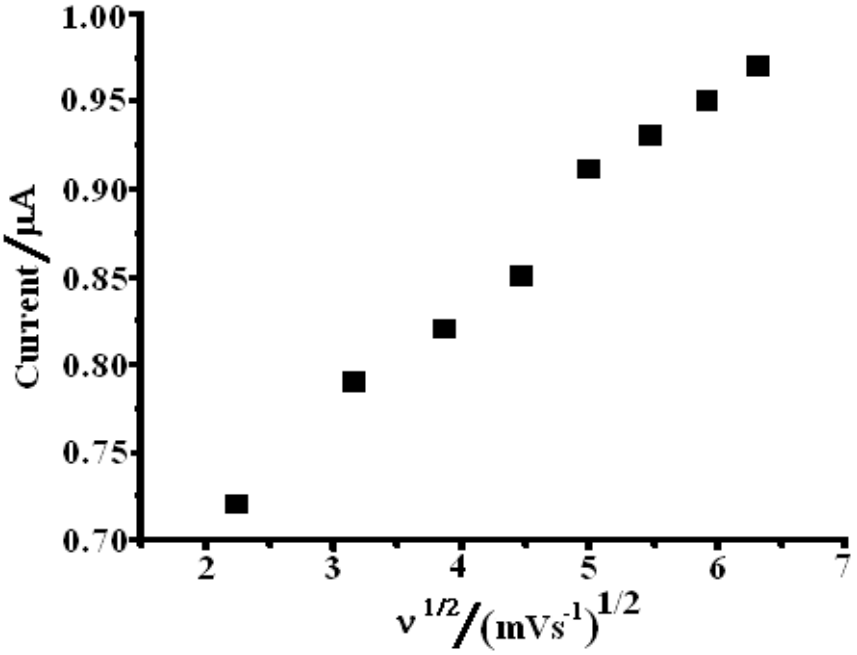


Fig. 3. The enzyme electrode response by varying squareroot of scan rate for 1 mM ASChCl in 0.1 M phosphate buffer (pH 7.0) and 0.1 M KCl

The effect of pH on the biosensor response towards the 1 mM acetylthiocholine chloride over the pH range 5.5 to 8.5 in 0.1 M KCl solution was shown in Fig. 4A. As the pH of the solution increases the response of the biosensor increases until attaining a physiological pH 7.0 and there onwards the response of the biosensor decreases. The enzyme electrode shows maximum sensitivity at pH 7.0. The anodic peak potentials (Ep_a) shifts to less positive side potentials with increasing pH of the buffer solution. The graph of Ep_a *vs.* pH of the solution was shown in Fig. 4B. It shows good linearity with a slope of 53 mV/pH. This behavior nearly obeys the Nernst equation for equal number of electrons and protons transfer reaction [23,24]. All experiments including inhibition studies were carried out at pH 7.0.

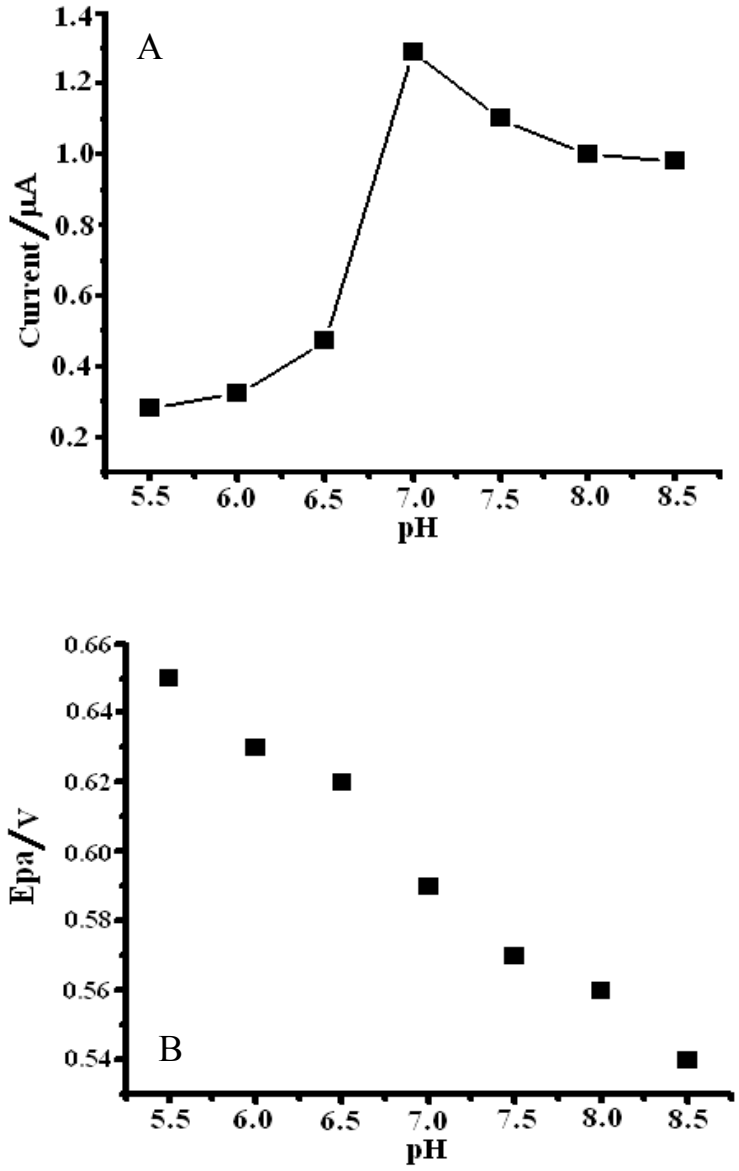


Fig. 4. (A) Effect of the pH on the enzyme electrode response (B) Plot of Ep_a *vs.* pH to 1 mM ASChCl

3.3. Effect of enzyme loading

Fig. 5 shows the study of influence of the amount of enzyme added to the electrode on the current response measured as a consequence of the anodic oxidation of generated thiocholine. Experiments were carried out in 1 mM acetylthiocholine chloride solution prepared in 0.1 M phosphate buffer. The amount of enzyme immobilized onto the CPE was varied between 0.03 to 0.3 U. The biosensor response increases with increasing in the concentration of the enzyme. Accordingly, 0.05 U of acetylcholinesterase was chosen as the optimal enzyme

immobilization solution for the fabricated biosensor due to the fact that it helps in attaining a high percentage of inhibition of pesticides under low concentrations [11].

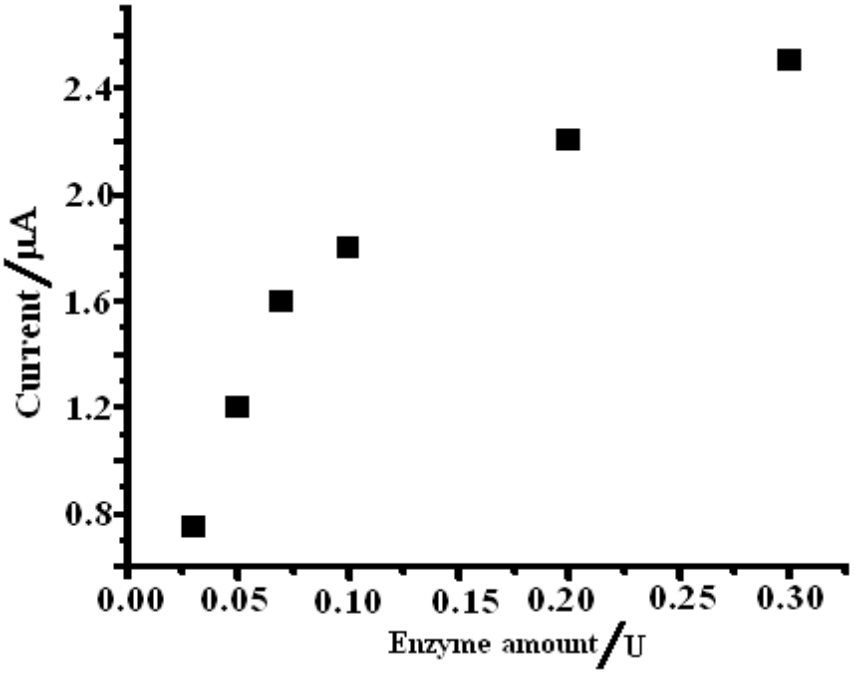


Fig. 5. The effect of enzyme loading into sol–gel matrix on the response of biosensor system

3.4. Pesticide detection studies

The monoenzyme biosensor was used for the measurement of quinalphos and malathion. The sensor was used to carry out inhibition studies by incubating with pesticide solution up to 4 min to obtain lower detection limits. Sol–gel immobilization method could provide wider concentration range of pesticide detection sensitive enough up to ppb level. The biosensor entrapped through sol–gel immobilization technique onto the CPE was dipped into the electrolytic cell containing 5 ml of 0.1 M phosphate buffer solution and 1 mM acetylthiocholine chloride (substrate), the signal originated from the enzyme–substrate reaction was recorded (I_i). The enzyme inhibition study was carried out by adding the pesticide samples of various concentrations into the electrolytic cell. The electrochemical measurement was recorded for every addition of pesticide sample and the corresponding pesticide inhibition was recorded (I_F). The enzyme inhibition was found to be proportional to the concentration of pesticide solution. The enzyme inhibition percentage and residual enzyme activity percentage was calculated using the following equations [25].

$$\text{Inhibition \%} \quad (I\%) = [\,(I_i - I_F) / I_i\,] \text{ X } 100 \tag{1}$$

$$\text{Residual enzyme activity \%} \quad (\text{REA \%}) = [\,I_F / I_i\,] \text{ X } 100 \tag{2}$$

The organophosphorous compounds inhibit the enzymatic hydrolysis reaction which in turn decreases the concentration of thiocholine and leads to the decreases in anodic peak current with increasing inhibition. The detection of individual pesticides was carried out according to the above procedure. Calibration plots based on the dependence of the % inhibition *vs*. concentration was linear and the same was shown in Fig. 6A&B for quinalphos and malathion respectively. The behavior of enzyme activity within the concentration range of 20–300 ppb and 0.07–1.3 ppm at different incubation times was shown in Fig.7A&B for quinalphos and malathion respectively. It reveals that the level of inhibition of enzyme increases with increase in incubation time and as well as increase in concentration of pesticides. A complete inhibition was observed at shortest incubation time of 4 min for quinalphos and malathion of concentrations 300 ppb&1.3 ppm respectively. Fig. 8 shows the differential pulse voltammograms of substrate alone and pesticide solution, where complete inhibition occurred. As the concentration of pesticides increases the residual enzyme activity of the enzyme decreases with respect to time and same was shown in the Fig. 9A&B for quinalphos and malathion respectively. The determination of LOD and LOQ was carried out by using the following expression [17,26-28].

$$\text{LOD} = 3S_b/S \tag{3}$$

$$\text{LOQ} = 10S_b/S \tag{4}$$

Where 'S_b' is standard deviation of the mean values for ten voltammograms of the blank solution, 'S' is the slope of the working curve, LOD is the limit of detection and LOQ is the limit of quantification. The limit of detection (LOD) and limit of quantification (LOQ) values was found to be 8 ppb, 0.058 ppm and 26 ppb, 0.194 ppm for quinalphos and malathion respectively. The lowest LOD value was found for quinalphos, which is more toxic than malathion. Thus these results clearly indicate that the proposed electrochemical method of analysis is reliable for the determination of individual pesticides. Table. 2 shows the various parameters determined for quinalphos and malathion.

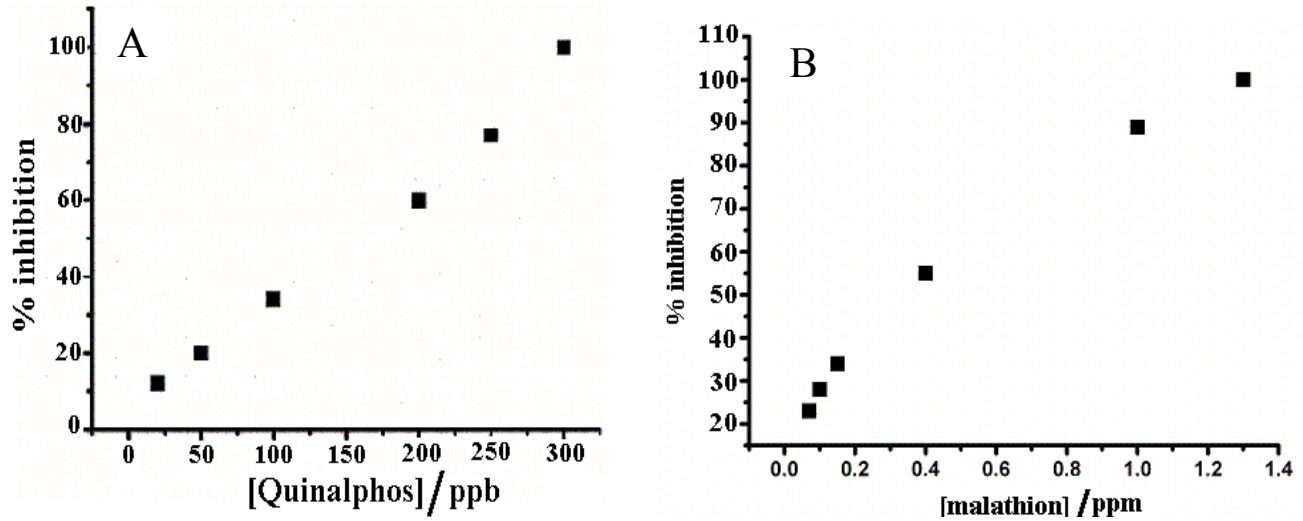


Fig. 6. Inhibition plots of (A) Quinalphos after 4 min incubation time (B) Malathion after 4 min incubation time in 0.1 M phosphate buffer (pH 7.0)/0.1 M KCl

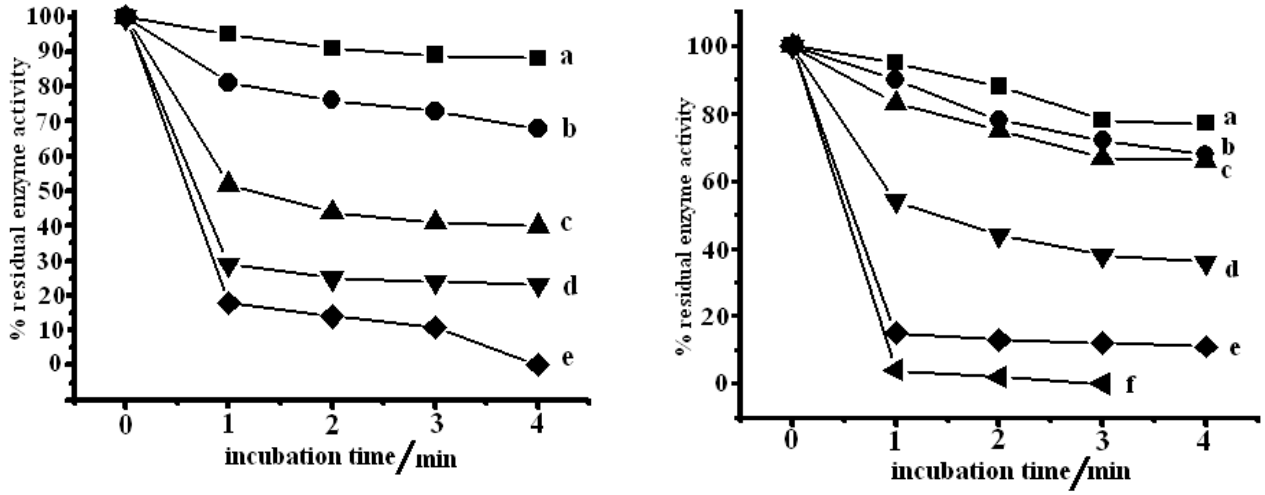


Fig. 7. Effect of incubation time on residual enzyme activity for various inhibitor concentrations in 0.1M phosphate buffer / KCl pH 7.0 (A) For Quinalphos (a) 20 ppb (b) 50 ppb (c) 200 ppb (d) 250 ppb (e) 300 ppb (B) For Malathion (a) 0.07 ppm (b) 0.1 ppm (c) 0.15 ppm (d) 0.40 ppm (e) 1.0 ppm (f) 1.3 ppm

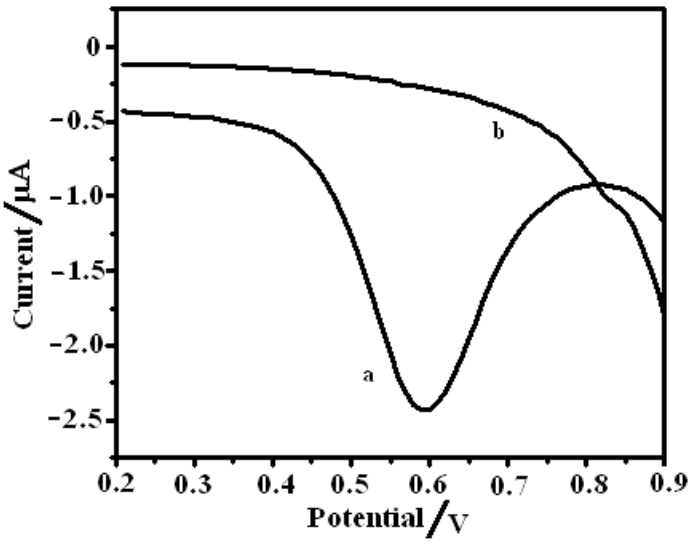


Fig. 8. Differential pulse voltammograms of (a) substrate alone (b) with 300 ppb of quinalphos/1.3 ppm of malathion pesticide solution

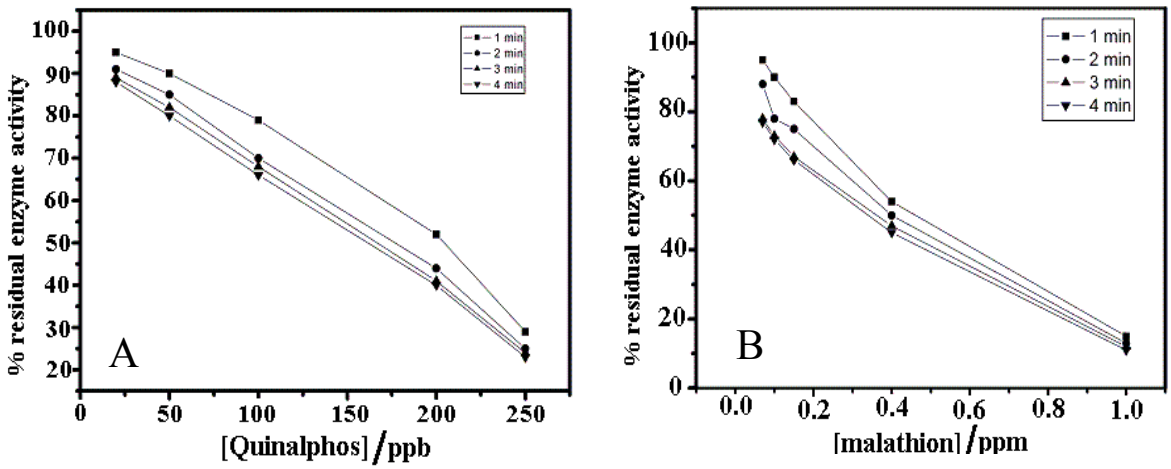


Fig. 9. The variation of residual enzyme activity with different inhibitor concentrations with respect to time in 0.1 M phosphate buffer / 0.1 M KCl, pH 7.0. (A) For quinalphos, (B) For malathion

The robustness of the developed method was evaluated by studying the concept of repeatability (new electrode, same standard solution, same day, same analyst, 'n' is number of assays) and reproducibility (new electrode, new standard solution, different days, different analyst, 'n' is number of assays) of the biosensor towards the inhibition of pesticides [26,27]. To study this experiment the chosen concentration of the stock solutions of quinalphos and malathion were 100 ppb and 0.5 ppm. The results obtained for the developed procedure towards the inhibition of both the pesticides was reproducible, because there was no

significant difference between the RSD values of the both the pesticides. The results were shown in Table 2. The long term stability of the AChE biosensor was investigated under the storage conditions (at 4°C), it was noticed that the activity of immobilized AChE was stable up one month.

Table 2. The various parameters determined for quinalphos and malathion

Parameters	**Quinalphos**	**Malathion**
Response time (min)	1	1
Incubation time (min)	4	4
Linear range	20 – 300 ppb	0.07 – 1.3 ppm
Intercept of calibration curve	4.1902	23.5756
Slope of calibration curve	0.3020	62.1014
Correlation coefficient	0.9949	0.9905
Standard deviation of calibration curve	3.8482	5.0397
Limit of detection (LOD)	8 ppb	0.058 ppm
Limit of quantification (LOQ)	26 ppb	0.194 ppm
Repeatability (%RSD) (n=3)	2.54	3.72
Reproducibility (%RSD) (n=3)	3.11	4.29

(n = number of assays)

4. CONCLUSION

The present investigation demonstrates the electrochemical detection of pesticides by using acetylthiocholine chloride as a substrate. The thiocholine can be electrochemically detected at sol – gel immobilized carbon paste electrode, through direct oxidation of these analytes at slightly more anodic potential 0.63 V vs. saturated calomel electrode. The immobilization of enzyme is much simpler and generates good results. The fabricated electrochemical biosensor is of fast response, adequate reproducibility, large pesticide working ranges and sensitive to the determination of organophosphorous pesticides.

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