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Determination of Carbophenothion and Dibrom Pesticides by Liquid Phase Lipase Enzyme by Voltammetric Methods

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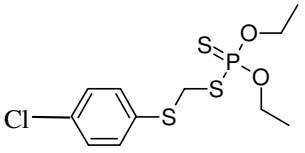
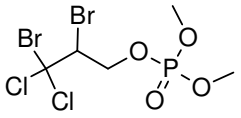
Abstract-Liquid state lipase enzyme sensor was used for the detection of Carbophenothion and Dibrom organophosphorus pesticides and the electrochemical behavior of both the pesticides was studied. The developed enzymatic method is based on in situ generation of *p*-Nitrophenol by lipase enzymatic hydrolysis of *p*-Nitrophenyl Acetate substrate. The electrochemically active *p*-Nitrophenol gives an anodic oxidation peak potential at 0.05 V *vs.* SCE. Based on enzymatic inhibition property of Carbophenothion and Dibrom pesticides are selected for study of lipase enzyme inhibition. The current response is based on the production of *p*-Nitrophenol under the influence of enzyme concentration, substrate concentration, pH and time variation effects were studied. A linear calibration for Carbophenothion and Dibrom was obtained in the various concentration ranges of 10-100 ppb and 100-1000 ppb respectively with a correlation coefficient of 0.947 and 0.936 under the optimized conditions by following the incubation time of 25 min. The electrochemical experiments were performed in 0.1 M phosphate buffer solution (pH 7.0) at room temperature. The limit of detection and limit of quantification values were found to be 37.40 ppb, 124.6 ppb and 371.7 ppb, 1239 ppb for Carbophenothion and Dibrom respectively under optimized conditions. The pesticide carbophenothion is more toxic than the dibrom as evidenced by the electrochemical studies. The developed liquid state lipase enzyme sensor takes less time for the analysis and no preconcentration extraction was needed for the study.

Keywords- Lipase Enzyme, *p*-Nitrophenyl Acetate, *p*-Nitrophenol, Voltammetric Methods

1. INTRODUCTION

The various types of organophosphorus pesticides (OPs) and Carbamates are the most popular insecticides in use today due to their high efficiency for pests and insect elimination, easy synthesis, and low cost. The use of these synthesized pesticides is fairly well recognized as a cost-effective method of controlling pests [1]. However, their wide use has resulted in their widespread distribution in the environment, both surface and ground waters are contaminated by their residues. Because similar compounds have been produced as possible nerve poisons a further area of application is in the military [2]. Many of them are highly toxic, however, and their accumulation in living organisms can cause serious diseases, even if they present a low concentration [3]. Among the existing organophosphorus pesticides the Carbophenothion and Dibrom (Table 1) are mostly used for pests control in agriculture. Therefore, it is very important to carry on the inspection and control of pesticide residues. The use of any technology for detoxification of organophosphorus compounds, performed in laboratories, will need the development of analytical tools of high performance in order to control the concentration of neurotoxics. Recently, there has been bountiful research in rapid detection for organophosphorus pesticide residues [4].

Table 1. The structure and molecular formulae of Carbophenothion, Dibrom

Sl.No	Selected organophosphorus pesticide	Molecular formula	Chemical structure
01	Carbophenothion	$C_{11}H_{16}ClO_2PS_3$	
02	Dibrom	$C_4H_7Br_2Cl_2O_4P$	

The traditional classical methods such as, High Performance Liquid Chromatography [5], Liquid-Liquid Micro Extraction method [6], Gas Chromatography [7], Thick Film technology [8], Gas Chromatography-Mass spectrometry [9] and Liquid-Solid Extraction followed by Liquid Chromatography-diode array detection [10] have been widely used for determination of the organophosphorus pesticide compounds. However these methods are still applicable when high accuracy and differentiation of the individual organophosphorus

pesticides compounds are needed. However, for in situ determination of organophosphorus pesticides, where the overall organophosphorus pesticides quantity is of important, electrochemical biosensors are preferred due to their good sensitivity, selectivity rapid response and portability [11].

To respond these issues enzymatic methods have been adopted as an alternative to classical methods (GC,HPLC,GC-MS,etc.) for faster and simpler detection of some environmental pollutants. Based on enzymatic inhibition method some of the electrochemical biosensors are developed for studies of organophosphorus pesticides. Cholinesterase-based biosensors are considered as one of the best alternatives in the context of this strategy. Such as ferophthalocyanine chemically modified carbon paste electrode by bi-enzymatic immobilized method [12], amperometric Silica Sol-Gel Immobilized Mono Enzymatic Acetylcholinesterase Biosensor [13], amperometric Nano structured polymer membrane containing gold nanoparticles modified acetylcholinesterase biosensor [14], electrochemical sensor based on acetylene black-chitosan composite film modified electrode [15], acetylcholinesterase enzyme modified carbon paste electrode [16], biosensors are applicable for continuous monitoring of various of after types organophosphorus pesticide residues in environmental samplers. However the developments of these acetylcholinesterase enzyme biosensors require costlier enzyme and suitable substrate.

Similarly the lipase enzyme is also inhibited by some of the organophosphorus pesticides like Methyl parathion, Carbophenothion and Dibrom, etc. Due to lack of suitable substrate for direct electrochemical response of the lipase enzyme, various immobilization methods of lipase enzyme are not suitable for construction of lipase based electrochemical biosensor. But according to the chemical properties of lipase enzyme, this enzyme shows an intrinsic capability to catalyze carboxylic ester bonds to the corresponding alcohol and acid. The indirect determination of pesticide concentration by lipase enzyme is based on effective inhibition of catalytic nature of lipase towards esteric bonds. Based on this, some of the lipase enzymatic potentiometric biosensor [17], and surface acoustic wave impedance sensor [18] were developed.

To overcome this problem, an indirect method of mono enzymatic liquid phase electrochemical sensor was developed for the determination of Carbophenothion and Dibrom pesticides. The developed method is purely based on production of alcoholic compound (*p*-Nitrophenol) by *p*-Nitrophenyl Acetate substrate hydrolysis by free lipase enzyme and pesticide effected lipase enzyme.

2. EXPERIMENTAL PART

2.1. Reagents and Solutions

All chemicals were obtained from commercial sources and used without further purification. Lipase from C. Rugosa (EC 3.1.1.3, type VII, ≥ 700/mg) p-Nitrophenyl Acetate, Carbophenothion, Dibrom were purchased from Sigma-Aldrich Chemicals. The pesticide stock solutions were prepared by dissolving in acetone (GR grade solution). The fine graphite powder, Silicon oil, acetone (GR grade) were procured from Himedia chemicals. Phosphate buffer of 0.1 M was prepared by using 0.1 M disodium hydrogen phosphate and 0.1 M Sodium dihydrogen phosphate. All Chemicals were of analytical grade and aqueous solutions were prepared with double distilled water. The enzyme stock solution was preserved at -5^{o}C and all stock and working solutions were stored at 5^{o}C.

2.2. Apparatus

Cyclic voltammetric experiments were performed with a model no CHI model 660c Electrochemical work station with a connection to a personal computer was used for electrochemical measurement and treating of data. A conventional three electrode cell was employed throughout the experiments, with a bare carbon paste electrode (homemade cavity of 3.0 mm diameter) as a working electrode, saturated calomel electrode (SCE) as a reference electrode and a platinum wire as a counter electrode. All the experiments were carried out at room temperature.

2.3. Preparation of bare carbon paste electrode

The bare carbon paste electrode was prepared by hand mixing of 70% fine graphite powder (particle size 50 mm and density is 20 mg/100 ml) 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 [19].

3. RESULTS AND DISCUSSION

3.1. Cyclic voltammetric studies

The electrochemical studies of *p*-Nitrophenol by C.Rugosa lipase enzymatic hydrolysis of *p*-Nitrophenyl Acetate were studied in 0.1 M phosphate buffer, pH 7.0. According to Fig. 1, free substrate has shown only a small back ground current (peak a), but in the presence of 125U of enzyme only a recognized oxidation peak current was observed (peak b) at 50 mV/s scan rate. The electrooxidation of *p*-Nitrophenol was shown in the equation (1) [20].

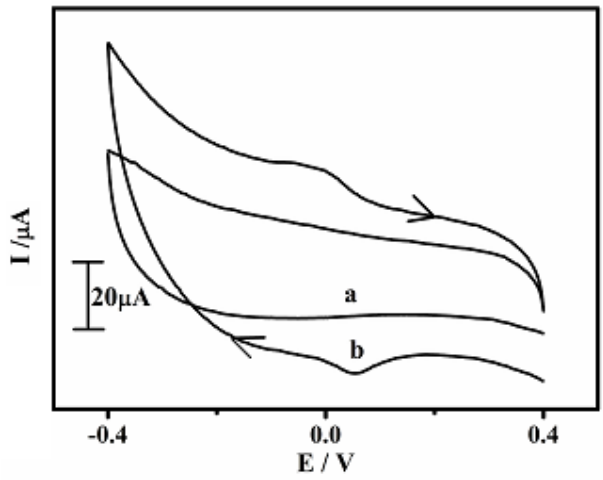


Fig. 1. Cyclic voltammograms of in situ generated p-nitro phenol in 0.1 M phosphate buffer, pH 7.0 a) substrate alone; b) substrate in the presence of 125U of enzyme At 50 mV/s scan rate

$$C_6H_4NO_2OH \longrightarrow C_6H_4NO_2O^{\bullet}+H^+ \quad (1)$$

According to equation (1) the generated nitrophenoxy radical intermediate subsequently undergoes polymerization leading to the formation of a non sticky thin film on the electrode surface. For further studies the non sticky polymeric film electrode surface was removed by physically smoothing against a tissue paper [20]. The anodic peak current was increased with increase of scan rate and the obtained graph was linear (Fig. 2).

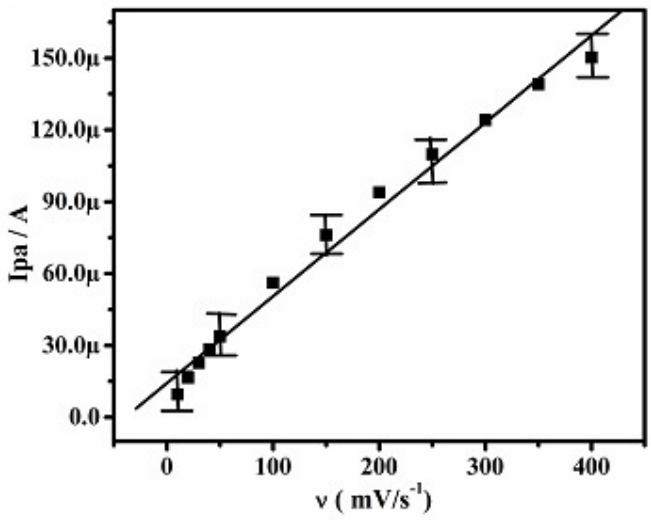


Fig. 2. The effect of scan rate on anodic peak current for *p*- Nitrophenol in 0.1 M phosphate buffer of pH 7.0

3.2. The effect of pH on hydrolysis of *p*-Nitrophenyl Acetate and oxidation of *p*-Nitrophenol

The effect of pH on hydrolysis of *p*-Nitrophenyl Acetate to *p*-Nitrophenol and acetic acid in the presence of C.Rugosa Lipase is studied in (0.1 M) phosphate buffer with pH 6.0-8.0. The graph showed (Fig. 3 (-●-) the maximum hydrolysis of *p*-Nitrophenyl Acetate to *p*-Nitrophenol and acetic acid with liquid state C.Rugosa lipase at pH 7.0 and also the obtained experimental value was similar to the value reported in the literature [21]. The potential diagram was constructed by plotting the graph of anodic peak potential E_{pa} *vs*. pH of the solution and is shown in Fig. 3 (-■-). The pH dependence of oxidation peak potential of in situ generated p-Nitrophenol, reveals that there is a potential shift towards positive, and the E_p=0.5752+0.071 this graph is almost linear with a slope of 71 mV/pH, this behavior was nearly obeying the Nernst equation for equal no of electron and proton transfer reaction [22,23].

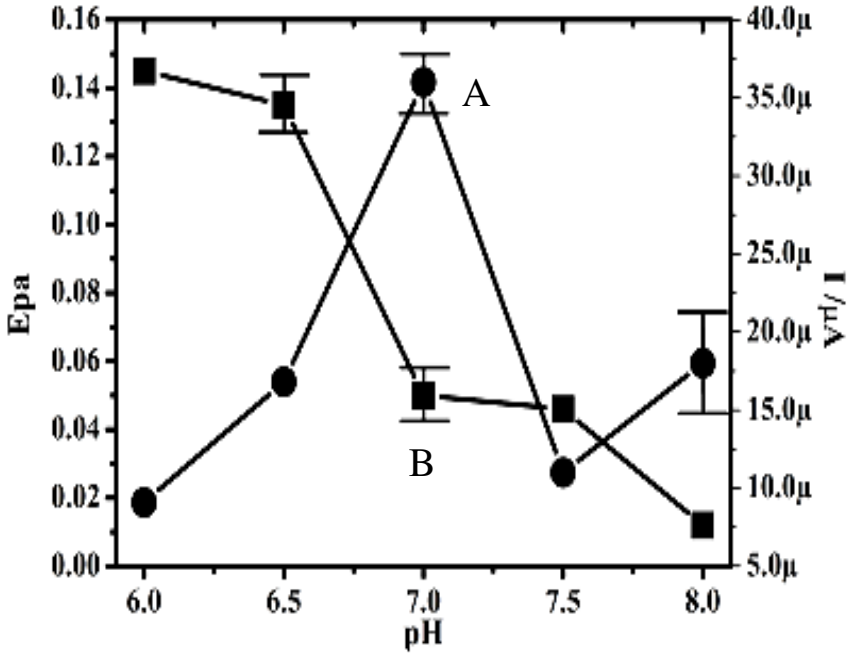


Fig. 3. A) The effect of pH on hydrolysis of 750 µM substrate in 0.1 M phosphate buffer (-●-) **B)** The effect of pH on anodic peak potential (E_{pa}) of *p*-Nitrophenol in 0.1 M phosphate buffer (-■-)

3.3. The effect of substrate concentration on enzyme catalysis behavior and time variation of complete hydrolysis of substrate

The selection of appropriate substrate concentration and hydrolysis time is essential for the study of the enzyme inhibition. The effective hydrolysis of substrate by 125U of C.Rugosa lipase was carried out effectively from 125 µM to 1750 µM. The hydrolysis of *p*-Nitrophenyl Acetate to *p*-Nitrophenol is increased from 125 µM (peak a) to 750 µM (peak b), and after that the hydrolysis was continuously decreased above 750 µM up to 1750 µM (peak c). The corresponding differential pulse voltammograms are shown in (Fig. 4 A) and 750 µM substrate concentration was chosen for further electrochemical studies. The effect of time variation on enzymatic hydrolysis of *p*-Nitrophenyl acetate to *p*-Nitrophenol was studied in 0.1 M phosphate buffer at pH 7.0. The effective hydrolysis of substrate was gradually increased from 5 min (peak a) to 25 min (peak b) of hydrolysis and the increase was continued till a plateau level was reached and after that there is no significant increment in the hydrolysis of substrate up to 40 min. The corresponding differential pulse voltammograms are shown in (Fig. 4B). Based on this experiment 25 min of hydrolysis time was chosen for effective study of C.Rugosa Lipase enzyme activity for pesticide study.

3.4. Pesticides study

The C.Rugosa Lipase enzyme was used to carry out inhibition studies of Carbophenothion and Dibrom pesticides by incubating with each pesticide solution up to 25 min to obtain lower detection limits. Inhibitor was mixed in 1:1 ratio with 125U of enzyme stock solution and incubated at room temperature 25±2 °C. To obtain the inhibition plots for Carbophenothion and Dibrom pesticides the percentage of inhibition method was studied. The detection was based on the measurement of initial (II) steady state current response of complete hydrolysis of liquid phase enzyme towards the selected conc. of 750 µM substrate in a 0.1 M phosphate buffer solution at (pH 7.0). The 1:1 ratio of enzyme and pesticide solution was incubated for 25 min, following the transfer of the inhibited enzyme into the electrochemical cell for the final (I_F) steady state current response towards hydrolysis of 750 µM substrate. The rate of inhibition (%) and residual enzyme activity was determined according to the following equations (2) and (3) [13, 24].

$$\text{Inhibition I}(\%) = [(I_I - I_F) / I_F] \times 100 \quad (2)$$

$$\text{Residual enzyme activity (REA \%)} = [I_F/I_I] \times 100 \quad (3)$$

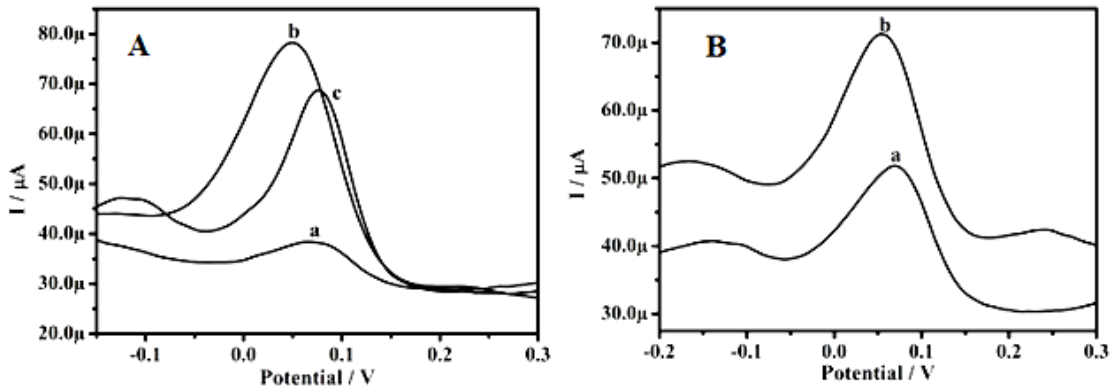


Fig. 4. A) The effect of various substrate concentrations on enzymatic hydrolysis. **B)** Time variation on enzymatic hydrolysis

Quantitative analysis of individual pesticides was carried out according to the above procedure. Carbophenothion and Dibrom pesticides are known to inhibit the catalytic nature of C.Rugosa lipase enzyme towards substrate; therefore a toxic reference to test the lipase enzyme activity was chosen for these studies. The Fig. 5 A, D shows the differential pulse voltammograms of Carbophenothion and Dibrom pesticides study. In the (Fig. 5 A, D peak c) indicates the presence of little amount of *p*-Nitrophenol, a starting substance for synthesis of *p*-Nitrophenyl Acetate substrate. The (Fig. 5 A, D peak b) indicates enzyme inhibition in the presence of pesticide and catalysis towards substrate inhibition was completely observed and is evidenced by the reduction in current response. In the (Fig. 5 A, D Peak a) represents complete hydrolysis of substrate in the presence of enzyme.

Table 2. The various parameters determined for Carbophenothion, Dibrom pesticides

Sl.No	Parameters	Carbophenothion	Dibrom
1	Incubation time (min)	25	25
2	Response time (min)	5	5
3	Linear range (ppb)	10-100	100-1000
4	Correlation coefficient	0.947	0.936
5	Standard deviation	8.435	12.39
6	Detection limit (DL) (ppb)	37.40	371.7
7	Quantification limit (QL) (ppb)	124.61	1239

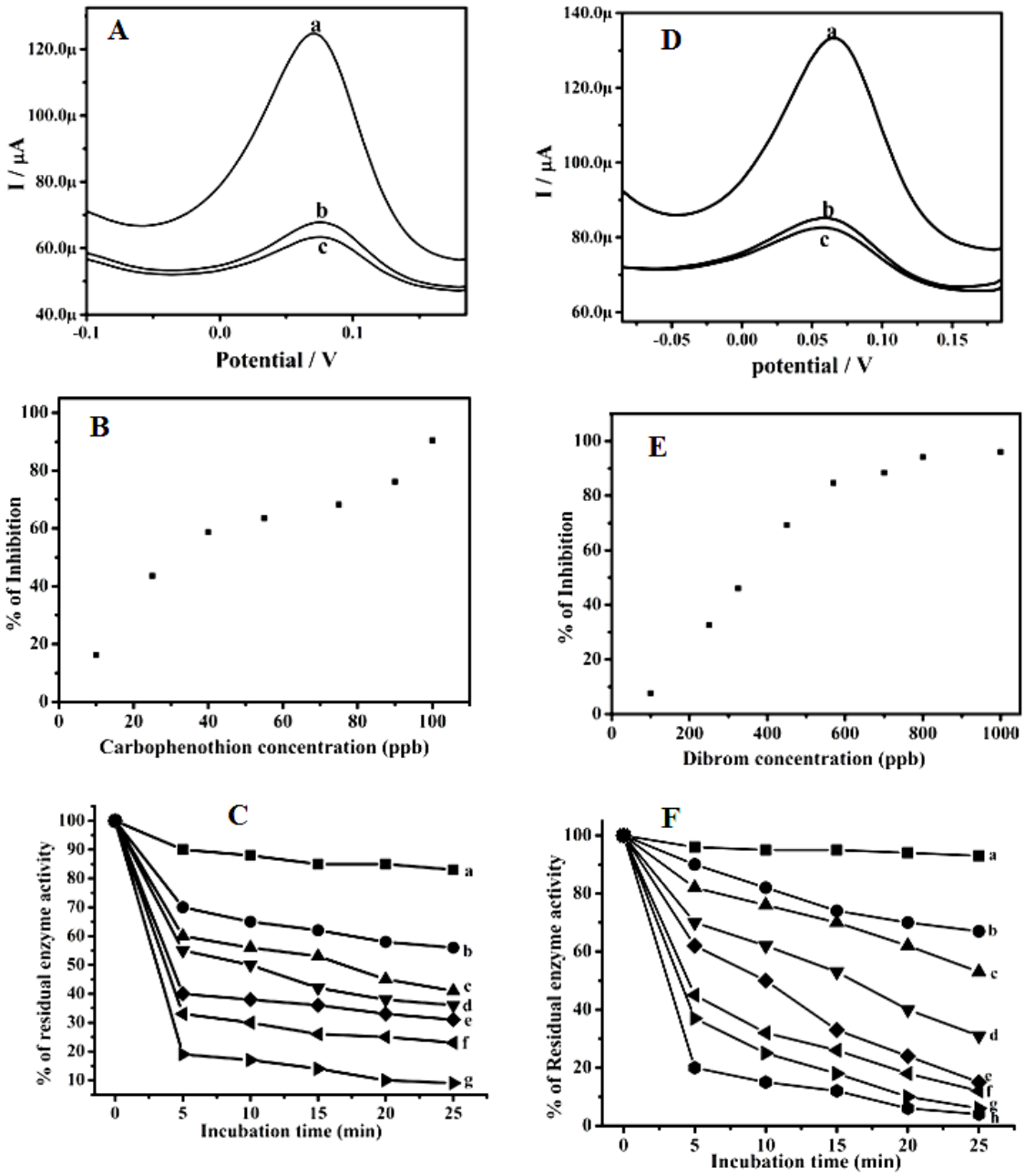


Fig. 5. (A), (D) Differential pulse voltammograms of Carbophenothion, Dibrom pesticides in 0.1 M phosphate buffer, pH 7.0 and 750 µM substrate measurement conditions, **(B), (E)** Inhibition plots of C. Rugosa Lipase enzyme by Carbophenothion, Dibrom pesticides after 25 min of incubation time, **(C), (F)** The effect of incubation time at various inhibitor concentration on the activity of liquid phase C. Rugosa Lipase enzyme in for Carbophenothion (a) 10 ppb ,(b) 25 ppb, (c) 40 ppb, (d) 55 ppb, (e) 75 ppb, (f) 90 ppb, (g) 100 ppb; and Dibrom (a) 100 ppb, (b) 250 ppb, (c) 325 ppb, (d) 450 ppb, (e) 570 ppb, (f) 700 ppb, (g) 800 ppb, (f) 100 ppb

4. CONCLUSION

In the present study, the preparation of lipase based liquid phase sensor within the electrochemical cell to determine the various concentration levels of Carbophenothion and Dibrom pesticides was studied. Electroanalytical investigation of Carbophenothion is achieved down to 10 ppb (correlation coefficient=0.947 and slope=0.6765) and Dibrom is achieved down to 100 ppb (correlation coefficient=0.9362 and slope=0.101). The detection limit and quantification limit values of Carbophenothion and Dibrom are 37.40 ppb, 124.61 ppb and 371.7 ppb, 1239 ppb respectively. From the developed method it is can also be concluded that the toxicity of the substance can also be tested. From this study it is known that the Carbophenothion is more toxic than the Dibrom as evidenced by the decrease in their current responses. The proposed electrochemical voltammetric detection method was simple, cost effective, less tedious, eco friendly and can be used for determination of organophosphorus pesticide residues in environmental samples.

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