CHAPTER 5:

Optimised process for application of the biosensor in organic solvent

5.1 Introduction

One of the major limitations with the AChE based biosensor is that their activities get loss in organic solvents. The application of enzymatic biosensors for pesticide detection in real samples necessitates development of methods for enhanced sustainability of enzymes in the organic media.

The influence of organic solvents on free AChE activity has been reported by different workers.[1, 2] Mionetto et al have studied the influence of as many as 28 organic solvents on AChE activity and found that stability of free AChE strongly decreased in all water miscible solvents but was good to excellent in the alkanes or non polar solvents. Influence of water content in organic solvent on the AChE activity has also been studied by the group.

Wilkins et al[3] have studied the effect on AChE activity of six organic solvents in the range of 0.01-100% and found that the activity of AChE on the electrode surface in mixture of water solution with 0.1%-10% of ethanol was higher than that in pure aqueous solution. Montesinos et al[4] have studied the effect of acetonitrile, ethanol and DMSO in the range 0-30% mixed with phosphate buffer (0.1 M, pH 7). They observed an increase of the recorded current in 5% acetonitrile and 10 % ethanol. They have also found that addition of 0.2% polyethyleneimine to the enzyme preparation, before immobilization, allowed the utilization of 15% acetonitrile without negative effect on the enzyme activity. S.Andreescu. et al [5] have studied the influence of aqueous acetonitrile and methanol in the concentration range 1 to 25% on the catalytic activity of immobilized AChE. They found that presence of more than 20% organic solvent induce a complete and irreversible inactivation of the enzyme, while 80% of the activity is conserved using 1-5% of organic solvents.

Enhancement of stability of AChE in organic solvents through entrapment in hydrophilic media such as PVA-SbQ has been demonstrated by Rouillon et al [6] and subsequently applied for activity analysis in organic phase by different workers.[2, 5]

Ethyl acetate is used as the extraction solvent for many of the OP and OC pesticides. Though water immiscible, it has strong deactivating action on AChE activity and classified as strong distorter of the enzyme with a log P value of 0.68.[7] Biosensing in ethylacetate not possible because 1. It cannot be diluted maintaining sufficient analyte concentration as it is sparingly soluble in water and A 1% v/v solution of ethyl acetate can cause 60% inhibition of the enzyme activity within 10 minutes.[8] Due to its wide application as extraction solvent,

improvement of stability of AChE in ethylacetate may widen the scope of application of AChE biosensors for real sample analysis.

Thus, our aim in this work was to enhance the stability of the enzyme AChE in ethyl acetate and then to combine the method with QuECHERS(named for quick, easy, cheap, effective, rugged and safe)[9-11] the modern and highly used pesticide extraction and clean up method, so that biosensing of pesticide in real sample becomes possible. The linking of the two methods was done through a solvent replacement step involving changing of the extracting solvent from acetonitrile to ethylacetate followed by transforming ethylacetate to a mixture of water soluble components through the use of lipase. Lipases find potential applications in bioprocesses largely due to their availability and stability in organic as well as in aqueous media.[12-14] This enzyme has versatile applications by virtue of its unique properties.[15] Under natural conditions, lipases catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase where the enzyme remains dissolved.[16] So, the reason of choosing lipase was to transform the ethylacetate to water soluble mixture of acetic acid and ethanol. It was known from literature that deactivation of enzyme by the pesticides occurs through the serine residue of the enzyme.[17] So we intended to see whether externally used L-serine may help in protecting the enzyme activity from the secondary, non-target species by sacrificing itself for the cause. Our findings have revealed that indeed the speculation is correct. We are able to increase the stability of the enzyme in the said organic mixture originating from 50% ethylacetate for more than 12 hrs. Recovery of the QuECHERS tandem ethylacetate transformation (QET) method was tested through both GC and biosensor methods. The results not only showed acceptable recovery but has also proved the validation of the biosensor results.

5.2 Experimental

5.2.1 Preparation of the assays

5.2.1.1 Assay 1

1.5 ml of distilled and dried ethylacetate was mixed with 1.5 ml of lipase in water containing 0.0162 g of lipase (0.0108 g/mL of ethylacetate). The solution was kept undisturbed for eight hours. After eight hours a homogeneous solution was obtained with some colloidal mass floating near the top. The solution was filtered twice using Whatman 1 filter paper. The filtrate thus obtained was ethyl acetate transformed mixture (TM_{50}) originating from 50%

ethylacetate. Fig. 5.1 shows the physical state of the lipase treated ethylacetate at two different time interval.

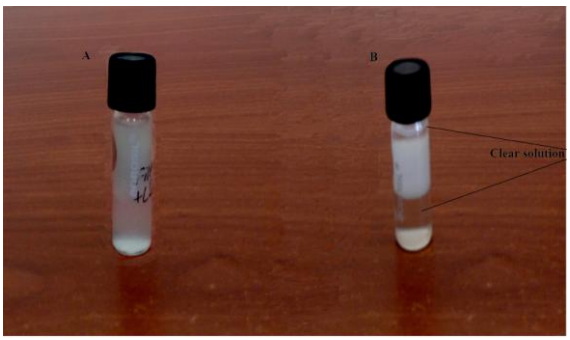

Fig.5.1 Two different states of lipase treated ethyl acetate. A. Snap shot taken immediately after mixing, B. after 8 h.

5.2.1.2 Assay 2

A filtrate was obtained as described above (assay 1). To it, 0.113 g of L-Serine was added. The filtrate thus obtained was ethyl acetate transformed mixture originating from 50% ethylacetate with added L-serin (TM_{50} with L-serine).

5.2.1.3 Assay 3

Above assay 1 was diluted to one third (TM $_{17}$

5.2.1.4 Assay 4

Same assay procedure as above 2, was followed but the filtrate was diluted to one third, that is, 0.5 ml of the filtrate was added to one ml of water, before adding L-serine. The filtrate thus obtained was one third diluted TM_{50} with added L-serine (TM_{17} with L-serine).

5.2.2 FTIR analysis

FTIR analysis was performed to understand the nature of chemical transformation that occurred when ethyl acetate was treated with lipase and L-serine.

5.2.3 Preparation of the sensor

Enzyme loaded, gelatin-gluteraldehyde-polypyrrole coated platinum electrode (Pt/PPy AChE-Glut-Geltn electrode) was prepared according to the published procedure.[18] AChE was electro entrapped in polypyrrole at 0.7 V from a 0.5 M solution of the pyrrole in phosphate buffer (PBS) pH 7.2 containing 0.02 M KCl and 5 µL (100 U mL^{-1}) of the enzyme. Subsequently gluteraldehyde and gelatin were added in steps and kept the electrode for an

aging period of 5 days in -20 ^{0}C before use. A three electrodes cell set up comprised of platinum working electrode (diameter 3mm), platinum coil auxiliary electrode and Ag/AgCl saturated with 3 M NaCl as the reference electrode were used during film deposition.

5.2.4 Enzyme sustainability study

Sustainability of the free enzyme in the lipase treated ethylacetate in presence and absence of L-serine was studied by mixing the enzyme in TM_{50} and TM_{17} with or without L-serine followed by subjecting it to Ellman test at different time intervals. Sustainability of the immobilized enzyme studied in the same way using the sensor probe

5.2.5 Inhibition study

Inhibition of AChE activity in immobilized state by 100% ethyl acetate, TM_{17} with pesticide, L-serine with TM_{17} in presence and absence of pesticide and by TM_{17} obtained through QET were studied through electrochemical CV and CA methods.

The percent inhibition was calculated by using the formula

$$I\% = \frac{I_1 - I_2}{I_1} \times 100 \qquad (5.1)$$

Where I% is the degree of inhibition, I_1 is the amperometric current of thiocholine oxidation before incubation in inhibitor solution and I_2 is the same obtained after incubation.

CV experiments were performed at a scan rate of 10 mV/s within the potential range from +1V to -1V. Chronoamperometric experiments were performed with initial potential E_1= 0.0 V applied for 60 s and final potential E_2=0.7V for 20 s.

5.2.6 Method recovery and biosensor validation study

Chayote squash samples were fortified with ethion, extracted following standard extraction and clean up procedure (QuECHERS) and the residue finally reconstituted in ethyl acetate, converted to TM_{50} and divided into two parts. One part after drying with anhydrous sodium sulphate subjected to GC analysis. Other part after mixing with L-serine (TM_{17} with L-serine) subjected to biosensor analysis. The recovery was calculated using calibration curves.

5.2.7 Calibration curves

5.2.7.1 Calibration curve 1

A calibration curve for pesticide ethion was obtained by making a series of standard ethion solution in 5% acetonitrile in PB. Percentage inhibition of the biosensor response by these solutions for 1h inhibition was plotted against concentrations. Electrochemical chronoamperometric method was used while evaluating the percent inhibition.

5.2.7.2 Calibration curve 2

For checking the recovery of the GC method, a calibration curve was obtained by using a series of standard ethion solutions (20-200ppb) in dry and distilled ethylacetate and subjecting to GC analysis. Triplicate measurements were made for each point.

5.2.7.3 Calibration curve 3

Another calibration curve was obtained by preparing standard solutions of ethion in acetonitrile and then following the entire steps of QuECHERS and converted to TM_{50}, dried with anhydrous sodium sulphate and then finally subjecting to GC analysis.

5.2.8 Recovery of QET method by GC

10 gram of chopped vegetable (chayote squash) was spiked with 5 mL of standard ethion pesticide solution (prepared in acetonitrile) and then homogenized. 5 mL of acetonitrile was added and shaken in vortex shaker for 5 minutes. Then 4 gram of $MgSO_4.H_2O$ and 1 gram of NaCl was added, shaken for 5 minutes. Then added 1gram of sodium citrate dihydrade and 0.5 g of sodium hydrogen citrate sesquihydrate. The mixture was shaken vigorously for 1minute and then sonicated for 5 minutes followed by centrifugation for 10 minutes at 2000 rpm. The supernatant was taken and treated with 125 mg of PSA(primary secondary amine) and 750 mg of $MgSO_4.H_2O$, shaken for five minutes and then sonicated for 5 minutes and centrifuged again. Then supernatant clean liquid was collected in 50 mL round bottom flask and evaporated to dryness at 40 ^{0}C and 200 mbar in rotavapor. Finally the dried residue was redissolved in mixture of 4 mL ethyl acetate and 1 mL dichloromethane and evaporated again to about 1 mL. The volume was made up to 2.5 mL. Then 2.5 mL of lipase solution (0.027 g in 2.5 mL water or equivalently 0.0108g lipase per mL of ethyl acetate) was added to it and the mixture was kept at room temperature for 8 hours until the froth appeared at the top. Then filtered twice through Whatman 1 filter paper and divided in to two parts. To one part 0.8 g of anhydrous sodium sulphate was added, filtered and subjected to chromatographic analysis

and the pesticide's amount was determined using calibration curve 3. The whole process was repeated with two other fortification levels.

The chromatographic analysis was performed using a Trace GC ultra (Thermo Scientific, USA) equipped with a FID detector. The capillary column used was a TR-5MS (30 m × 0.25 mm i.d. × 0.25µ m film thickness) supplied by Fisher Scientific. The injector and detector temperatures were set at 250 ^{0}C. The temperature program used was: initial 60 ^{0}C for 1 minute followed by 30 ^{0}C per minute up to 150 ^{0}C, hold for 4 minutes followed by 15 ^{0}C per minutes up to 290 ^{0}C and hold for 5 minutes, leading to a total analysis time of 22.33 minutes per sample. Injected volumes were 1 µL each time through auto injection mode.

5.2.9 Recovery of QET method by biosensor

To the second part of the solution obtained through procedure described above (Sec. 5.2.8), 0.08 (g/mL) of L-serine was added after required dilution so as to make it 'TM_{17} with L-serine'. The biosensor was incubated in this solution for one hour. Percent inhibition was determined through CA method. Pesticide's amount was determined from the calibration curve 1 and using the correlation equations. The same was done for the other two solutions of different concentration and the mean % recovery was calculated.

5.3 Results and Discussion

5.3.1 Enzyme sustainability in lipase treated ethylacetate in presence and absence of L-serine

Sustainability of the free enzyme in TM_{50} and TM_{17} in absence of L-serine was studied by mixing 50 µL of the enzyme (AChE) to assay 1and 3 followed by withdrawing 0.5 mL of this at different time intervals and subjecting to Ellman assay (adding to a ready mixture of 150 µL DTNB 0.005 M and 100 µL 0.03 M ATChCl) and monitoring the appearance of yellow color through UV-visible spectrophotometer. Sustainability of immobilized enzyme studied in the same procedure using the sensor probe in place of free enzyme. Sustainability in presence of L-serine was studied by repeating the above experiment with assay 2 and 4 using both free and immobilized enzyme.

In the case when free enzyme was mixed in TM_{50}, prominent yellow color appeared quickly and persisted when the Ellman test was performed at 5 minutes. Ellman test at extended times (10, 20, 30 minutes) produced gradually faded yellow color and no yellow

color was seen at 60 minutes. Same result was obtained with assay 2(TM_{50} with L-serine). This infers that the activity of the free enzyme does not persist more than 5 minutes in TM_{50} and TM_{50} with L-serine. Results of experiment with immobilized enzyme were almost same except slight increase in sustainability. But the result with assay 3 and 4 were different; the yellow color maintained the intensity till the Ellman test performed at 60 minutes. Decrease in intensity of yellow colour was observed thereafter. While performing the same experiment with assay 4, it was found that the yellow colour appeared with same intensity at the end of 12 h. Results with immobilized enzyme also followed the similar trend. The results thus confirm that the activity of the free enzyme remains intact for 1 h in 16.67 % dilution level, i.e. in TM_{17} and over 12 h in TM_{17} with L-serine. In case of immobilized enzyme the corresponding time found to be 2 h and more than 12 h respectively.

5.3.2 FTIR analysis

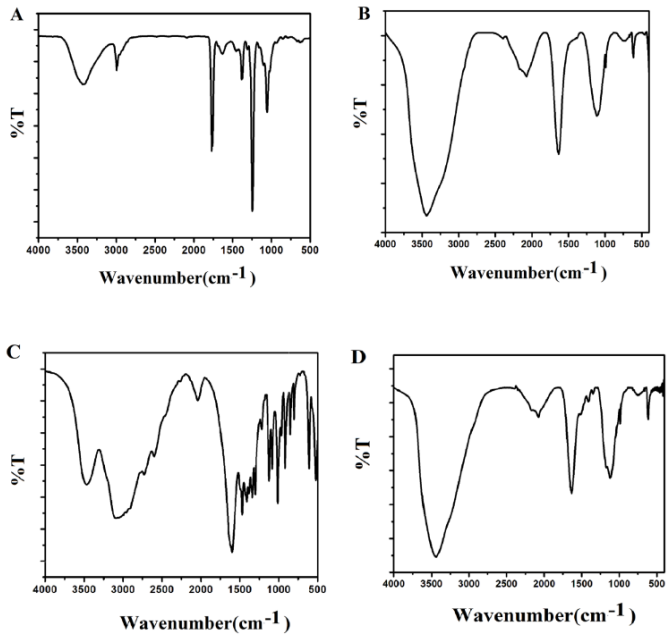

Fig.5.2 FT-IR spectra of pure ethyl acetate(A), anhydrous sodium sulphate treated TM_{17}(B), L-Serine(C) and anhydrous sodium sulphate treated TM_{17} with L-Serine(D).

Fig.5.2 A shows the FT-IR spectra of pure ethyl acetate. peaks observed - at 1055 cm^{-1} and 1250 cm^{-1} due to C-O stretching vibration, at 1752cm^{-1} due to C=O stretching, 2981 cm^{-1} C-H stretching which are well known from literature. The broad band at 3400cm^{-1} is due to OH vibration of moisture present in the sample environment. The spectra of the lipase treated ethyl acetate, $TM_{17,}$ shown in Fig.5.2B. Shifting of peak positions seen and new peak

appeared around 600cm^{-1}. The spectra indicate the formation of new compound(s). Fig.5.2C and 5.2D are respectively the spectra of pure L-serine and of L-serine mixed TM_{17}. Comparison of the two spectra(C and D) does not indicate formation of any new bond. So it is attributed that the interaction between the components of TM_{17} and L-serine occurs through the H- bonding network. However, the actual nature of the chemical reaction is not conclusive at this step and needs further study.

5.3.3 Inhibition study

5.3.3.1 Inhibition of ethyl acetate on immobilized enzyme

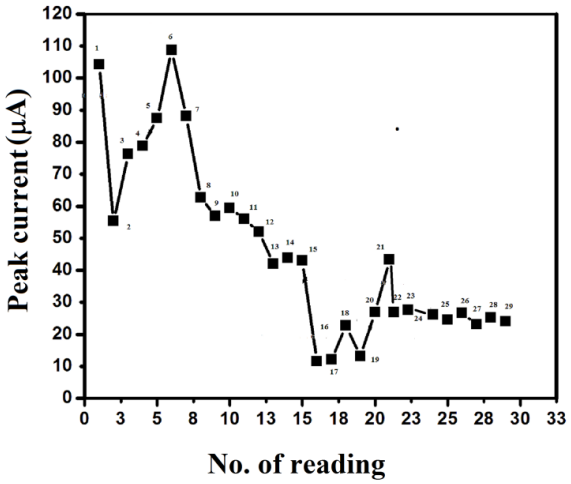

Fig.5.3 Effect of pure ethyl acetate on the CA response of AChE biosensor.

The influence of 100% ethyl acetate on the activity of immobilized AChE was studied by incubating the sensor in dry and distilled ethyl acetate followed by evaluating the sensor response (Fig.5.3). In Fig.5.3, Point 1 represents the initial stable current of the biosensor. Incubation of the biosensor in ethyl acetate for 1 h has reduced the signal to almost 50 %(point 2). Continuous CA analysis (3, 4, and 5) in PBS solution has reactivated slightly beyond the original activity (point 6). Second time inhibition in ethylacetate for another 1 h has brought down the current level almost 20% (point 19 through 29). From the results it is seen that two types of inhibitions were caused by ethyl acetate in this particular immobilized state of the enzyme-one is reversible type occurred after 1 h incubation for the first time and the another irreversible one occurred after the second time incubation for 1 h. It is attributed that the deactivation process involves a particular reorientation of the solvent molecules around the enzyme which either needs longer time or unattainable when the enzyme is in immobilized state. The required orientation is attained quickly when the enzyme is in the free

state and slowly when the enzyme is surrounded by a gel type atmosphere. In the initial incubation step, sufficient time was not attained to get the stable orientation so as to permanently deactivate the enzyme. But in the second time inhibition, due to swelling of the film, a gel type environment was created which helped the solvent molecules to rearrange and cause the permanent deactivation of the enzyme.

5.3.3.2 Inhibition by different constituents

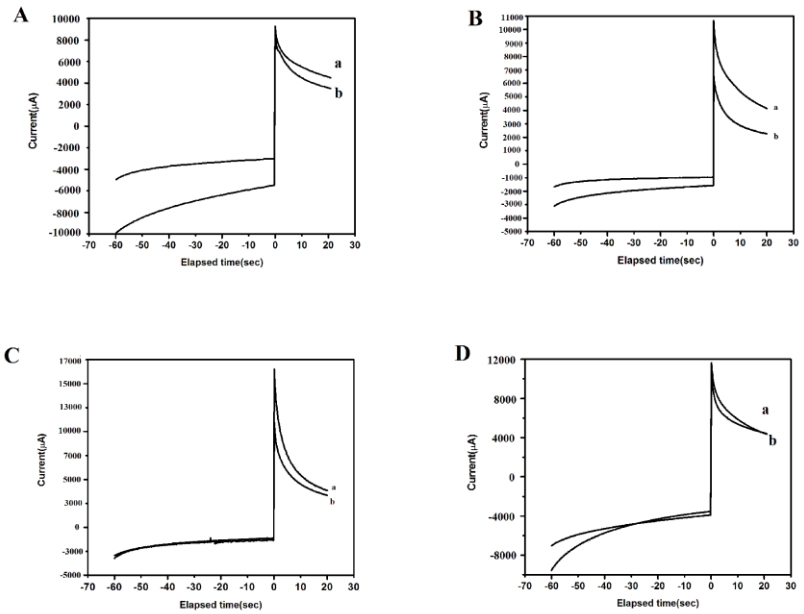

Fig.5.4 Inhibitory effect of different components of the transformed mixture in presence and absence of pesticide on the activity of the immobilized enzyme. A. TM_{17} with L-serine. B. TM_{17} with ethion. C. TM_{17} with L-serine and ethion. D. TM_{17} of QET with added L-serine. In each case, a represent CA response of the sensor to 2.0 milli molar ATChCl before incubation in the solution, b represents the same after 1 h incubation in the same solution.

Inhibitory effect of L-serine and ethion when present individually and together in the transformed mixture TM_{17} towards the immobilized enzyme were studied by CA (Fig.5.4) and CV (Fig.5.5) methods. Fig.5.4A shows the CAs of ATChCl in PBS before (a) and after (b) incubating for 1 h in TM_{17}-L-serine mixture. Fig.5.4B and 5.4C shows the same respectively in pesticide mixed TM_{17} and both pesticide and L-serine mixed TM_{17}. While in, A no inhibition was observed, in B and C the same was found to be present with different extent. With a 120 ppb ethion solution, inhibition in case of B was found to be 41% while that in C was 33.83%. The results indicate that there is no inhibition caused by L-serine, and, inhibition of pesticide mixed TM_{17} is higher than that of pesticide-L-serine mixed TM_{17}.The

observation that inhibition of pesticide mixed TM_{17} is higher than pesticide-L-serine mixed TM_{17} , indicates the possibility of either L-serine –pesticide interaction to some extent, or obstruction of L-serine on pesticide-enzyme interaction. Fig. 5.4D shows the biosensor response before (a) and after (b) incubation in TM_{17} obtained through QET method. It was found that 6% inhibition of the sensor response caused by the QuECHERS chemicals, probably the magnesium ion, in presence of L-serine (Fig.5.4D). This inhibition is 100% reversible and the enzyme gets reactivated when washed with PB. This inhibition will not affect the analytic procedure because, this increment in inhibition along with the decrement in inhibition caused by the solution matrix as a whole, amounts to a definite value of inhibition for each concentration that can be converted to actual concentration using correlation equation.

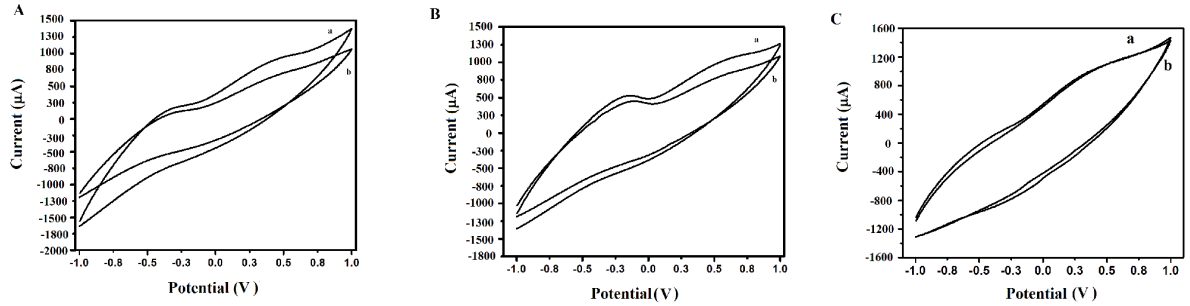

Fig.5.5 Inhibitory effect of different components of the transformed mixture in presence and absence of pesticide on the activity of the immobilized enzyme. A. TM_{17} with ethion. B. TM_{17} with L-serine and ethion. C. TM_{17} with L-serine. In each case, 'a' represent CV obtained in 2.0 milli molar ATChCl PB mixture before incubation of the sensor in the solution concerned, 'b' represents the same after 1 h incubation in the solution.

Similar results were obtained through CV experiment (Fig.5.5). From the cyclic voltammograms it is seen that when the electrode is incubated in TM_{17} containing pesticide, there is 28% decrease in current value (Fig.5.5A). When L-serine is present in the mixture, the value is found to be equal to 20% (Fig.5.5B). No change in sensor response was seen when the sensor was incubated in TM_{17} with L-serine (Fig 5.5C).

5.3.4 Calibration curves

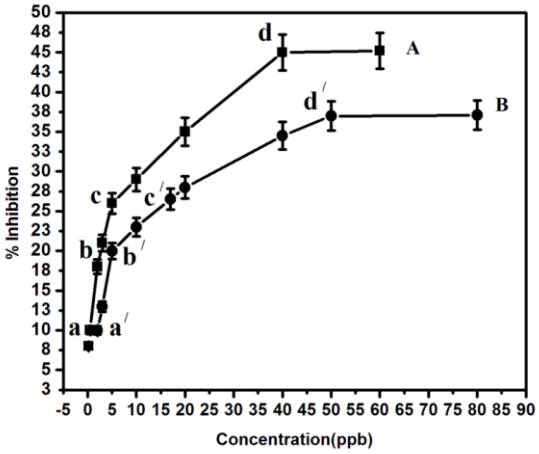

Fig.5.6 Calibration curves of ethion by two different biosensing methods A- ethion standard solutions prepared in 5% acetonitrile. B-ethion standard solution prepared in acetonitrile converted to 'TM_{17} with L-serine' through QET. CA parameters E_0= 0.0 V, t_0= 60.0 sec, E_1= 0.7 V, t_1= 20 sec.

Curve A (Fig.5.6) is the calibration curve for ethion in 5% acetonitrile.The calibration curve for ethion under QET method is curve B (Fig.5.6). Comparison of the two shows that the % inhibition goes almost parallel to that obtained in 5% acetonitrile but with lower magnitude. A correlation between the two curves was obtained after dividing them into three segments based on linearity match. The correlation equations and the corresponding segments are $y = 1.461x + 1.384$ for segments $aa'bb'$ with I% 10 to 20, $y = 4.2x - 5.200$ for segments $bb'cc'$ with I% 20 to 26 and $y = 1.746x + 5.639$ for segments $cc'dd'$ with I% 26 to 46. Any concentration 'x' obtained by the new method but using calibration curve A, will mean an actual concentration given by 'y'. The limit of detection defined as the concentration of pesticide corresponding to 10% inhibition, found to be 2.0 ppb under the QET method and 0.3 ppb by the conventional method (5% acetonitrile).

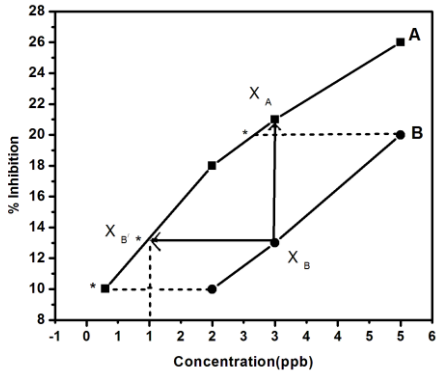

Fig. 5.7 Segment 1of Fig.5.6before regression: a a′b b′ A- 0.3 to 2 ppb, B- 2to 5 ppb, I%= 10.0 to 20

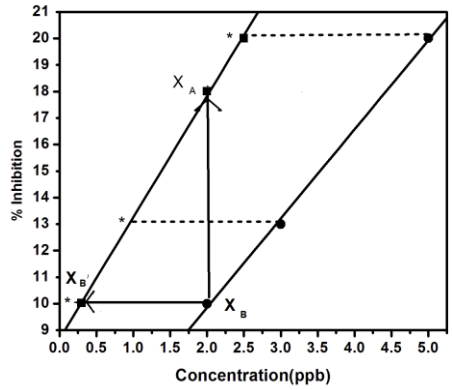

Fig. 5.8 Segment 1of Fig.5.6 after regression.

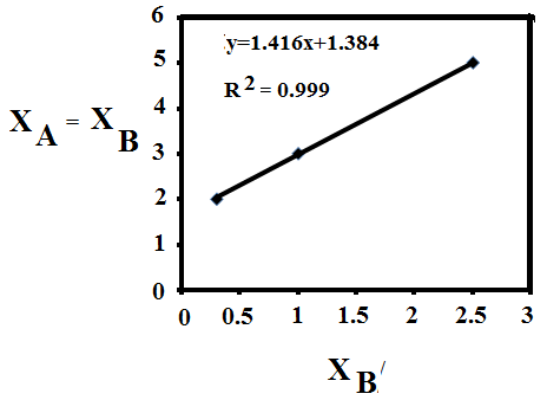

Fig. 5.9 plot of Common Concentration vs. Shifted Concentration of B on curve A for segment 1

Table 5.1 Correlation of concentration in segment 1

Common concentration $X_B = X_A$	Shifted concentration of B on curve A, $X_{B'}$	% Inhibition Common to both A and B
2.0	0.3	10
3.0	1.0	13
5.0	2.5	20

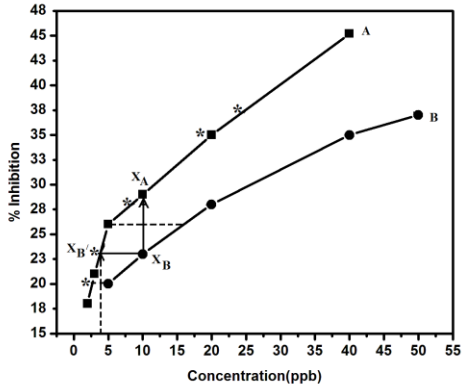

Fig.5.10 Segment 2 of Fig. 5.6 before regression: b b′cc′, A= 2.5to 5ppb, B- 5to 15.6 ppb, I%= 20 to 26

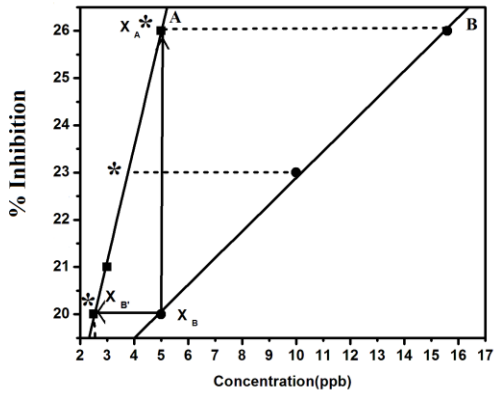

Fig.5.11 Segment 2of Fig.5.6 after regression

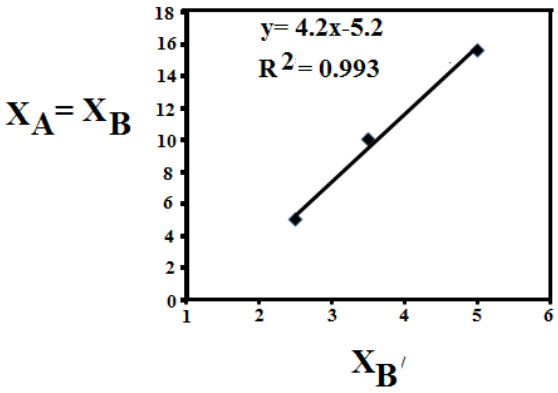

Fig. 5.12 plot of Common Concentration vs Shifted Concentration of B on curve A for segment 2

Table 5.2 Correlation of concentration in segment 2

Common concentration $X_B = X_A$	Shifted concentration of B on curve A, $X_{B'}$	% Inhibition Common to both A and B
5.0	2.5	20
10	3.5	23.0
15.6	5	26

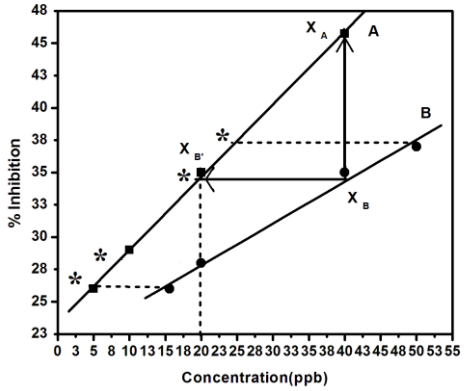

Fig.5.13 Segment 3 of Fig. 5.6 after regression: cc´dd´, A= 5 to 40 ppb B- 15.6 to 50 ppb, I%= 26 to 46

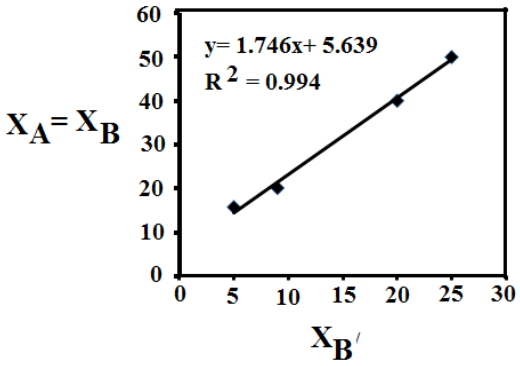

Fig. 5.14 plot of Common Concentration vs. Shifted Concentration of B on curve A for segment 3

Table 5.3 Correlation of concentration in segment 3

Common concentration $X_B = X_A$	Shifted concentration of B on curve A, $X_{B'}$	% Inhibition Common to both A and B
15.6	5	28.0
20.0	9	35.0
40	20	35.0
50	25	37.0

5.3.5 Recovery and validation

Recovery as well as the validation was studied by applying both biosensor analysis and gas chromatographic analysis. The calibration curve for biosensor methods shown in Fig.5.6

Similarly two calibration curves were obtained for GC method. One was obtained by preparing standard solutions of ethion in distilled and dry ethyl acetate in the range 20 to 200 ppb (Calibration curve 2). Another calibration curve (Calibration curve 3) was obtained by preparing ethion standard solutions in acetonitrile followed by QET. The solutions were sufficiently dried with sodium sulphate before subjecting to GC analysis. It is worth mentioning here that it is the matrix-induced response enhancement[19,20] that has necessitated the use of calibration curve 3. While calculating the recovery percentage from fortified samples using calibration curve 2, it was found that the recovery was high (114%) due to the matrix effect. So the calibration curve 3 was used to nullify the error due to matrix effect while checking the recovery. It is worth mentioning that use of internal standard method for

the matrix effect has been avoided considering probable complexity in the biosensing part when applied to the same solution.

Our analytical assay preparation involves two novel steps one is the transformation of organic extract to enzyme friendly composite organic mixture (TM_{17}) and the other is QuECHERS extraction coupled to above transformation. Recovery can be affected by both the steps and hence the recovery study for both the methods is essential. However, since the first step alone does not have any practical utility so we have avoided it and opted for recovery study of the QET method.

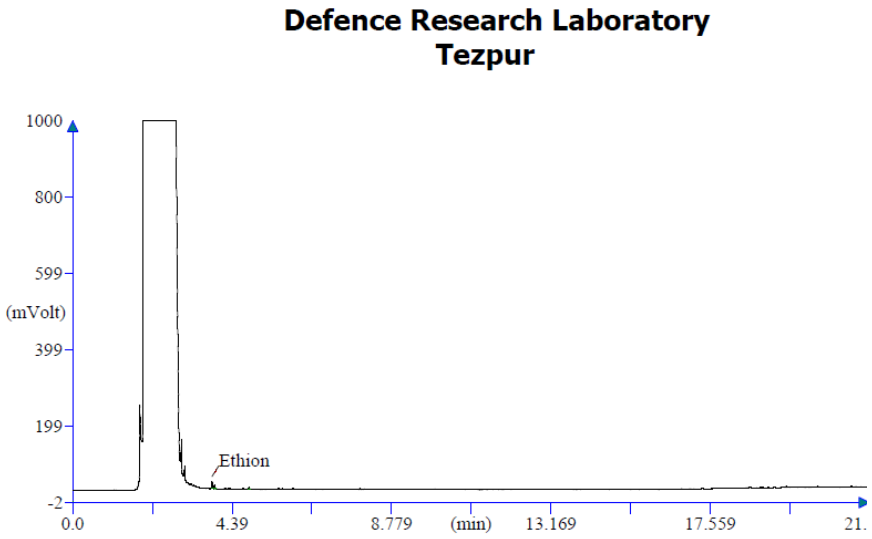

Fig.5.15 Gas Chromatogram of 20ppb ethion

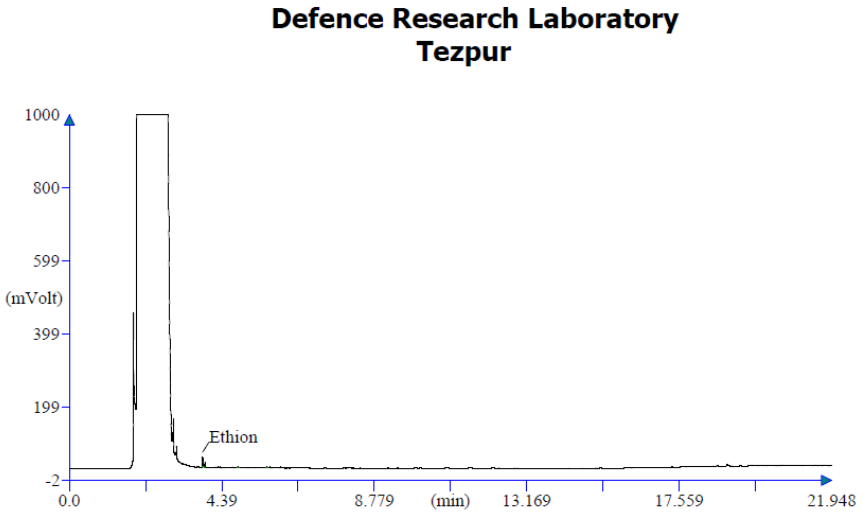

Fig.5.16 Gas Chromatogram of 50ppb ethion

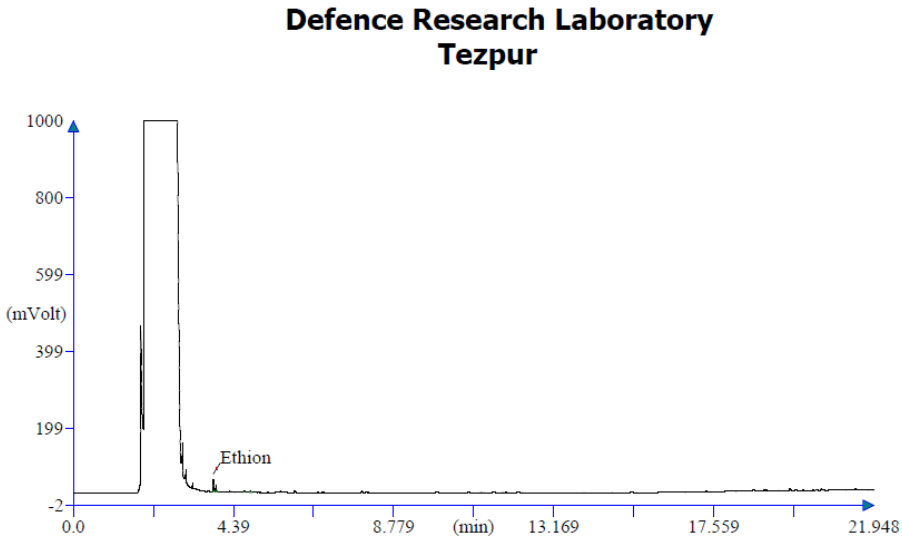

Fig. 5.17 Gas chromatogram of 100 ppb ethion

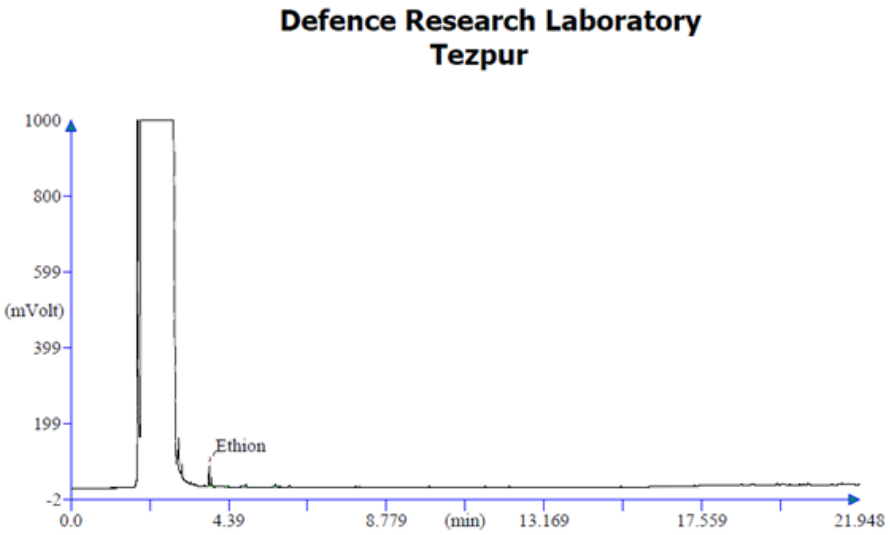

Fig. 5.18 Gas chromatogram of 150 ppb ethion

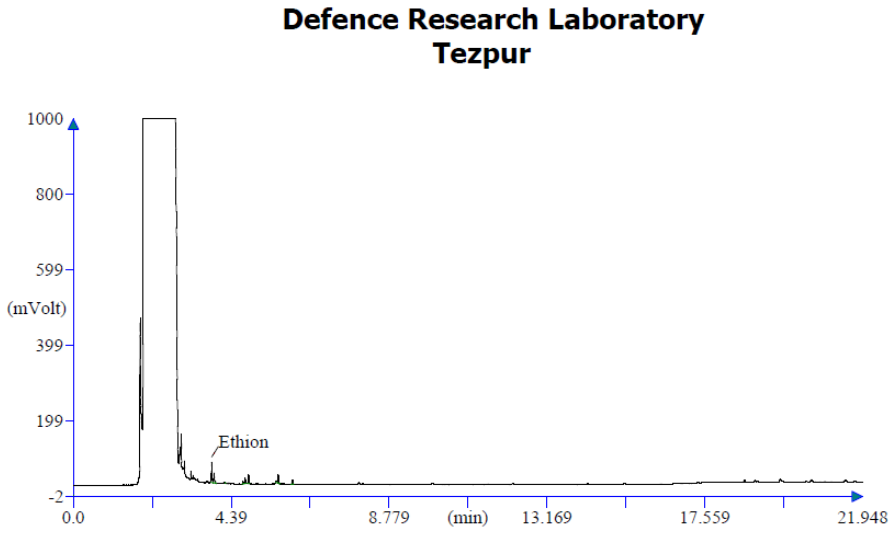

Fig.5.19 Gas chromatogram of 200 ppb ethion

Table 5.4 Recovery of ethion using biosensor in the QET method

Fortification level(ppb)	Observations					Recovery %	Mean recovery %	rsd%
	I%	ppb by calibration curve 1 (x)	Regression equation	ppbfound (y)	ppb expected (1/3rd of fortification)			
10	16	1.30	$y = 1.461x + 1.384$	3.20	3.33	96.10	98.07	1.83
20	21	2.80	$y = 4.2x - 5.200$	6.56	6.66	98.50		
40	24	4.40	$y = 4.2x - 5.200$	13.28	13.33	99.62		

Table 5.5 Comparison of recovery from QET method by biosensor and by GC.

Analytic methods	Fortification level(ppb)	Recovery								
		Using calibration curve 1			Using calibration curve 2			Using calibration curve 3		
		ppb found	Mean recovery %	rsd%	ppb found	Mean recovery%	Rsd%	ppb found	Mean recovery%	rsd%
GC	20	---------	----------		25	114.30	8.13	18	96.20	6.50
	40	---------			44			41		
	100	---------			108			96		
Biosensor	10	3.20	98.07	1.83		--------			----------	
	20	6.56								
	40	13.28								

Though we attempted to compare the GC and biosensor analysis taking same concentrations of the fortified solutions that could not be achieved completely due to the difference of the limit of detection and the linear range of the two methods. In the GC used for the experiment, LOD of this particular pesticide was found to be 15 ppb (S/N=3). On the other hand, the biosensor linear range found to be up to 50 ppb. Therefore two sets of solution concentrations were selected, so as to give maximum overlap subject to another limitation of discriminating capabilities of two close concentrations by the two methods. Thus the GC series taken was from 20 to 100 ppb while the biosensor series from 10 to 40 ppb with common concentrations of 20 and 40 ppb. The results obtained are shown in table 5.4 and 5.5. It is to be mentioned that while preparing the analyte solution through application of QuECHERS, in the

reconstitution(in ethyl acetate) part, total volume was made half of the original concentration(5 mL to 2.5 mL), so that the final concentration after addition of lipase solution(0.027 g in 2.5 mL) remains the same. Thus, the expected ppb of GC analysis was same to that of the fortified level while the same for biosensor method was one third of the original one. Results show that recoveries in the two cases are excellent. It proves the validity of the biosensing method as well as the feasibility of combination of biosensing method with QuECHERS.

5.4 Conclusions

We have developed a novel method for quantification of OP pesticides in real samples extracted in organic solvents (acetonitrile or ethyl acetate) using AChE biosensor. The method involves two key steps; the first one is the lipase catalyzed transformation of ethylacetate to a mixture of acetic acid and ethanol followed by neutralization of the inhibitory effects of acetic acid ethanol mixture on the enzyme through the use of L-serine. The method can be coupled to the QuECHERS method of extraction and clean up, thus making the biosensing technique more practicable from the view point of application to real sample analysis. The method enables true validation checking of biosensors because the same solution preparation can be subjected to both bio and conventional chromatographic analysis. We have also formulated out a novel method of correlating the results of the new method to the conventional 5% acetonitrile-PB method. The complete steps involved the method and the correlation procedure has been provided in the supporting information. Though we have demonstrated the workability of the method to a typical organothiophosphate pesticide ethion, the same method can be equally applied to organocarbamate pesticides too.

References

1 . Ronzani, N. *Tetrahedron Lett*. **34**, 3867--3870, 1993.

2 . Mionetto, N., et al. *Biosens. Bioelectron*. **9**, 463-470, 1994.

3 . Wilkins, E., et al. *Electro.Comm*. **2**, 786--790, 2000.

4 . Montesinos. T., et al. *Anal. Chim. Acta* **431**, 231--237, 2001.

5 . Andreescu, S. *Talanta* **57**, 169--176, 2002.

6 . Rouillon, R., et al. *Anal. Chim. Acta* **268**, 34350, 1992.

7 . Chalin, M.F., & Bucke, C. *Enzyme Technology*, Cambridge University Press 1990.

8. Palchetti, I., et al. *Anal. Chim. Acta* **337**, 315--321, 1997.

9. Anasstassiadases, M., et al. *J. Chromatogr. A.***1015**, 163--184, 2003.

10. Lehotay, S., et al. *The J. AOAC. I*nt. **88**, 595--614, 2005.

11. Kumar, A., & Kanwar, S. *Dyn. Biochem. Process Biotechnol. Mo.l Biol*. **6**, 13--27, 2012.

12. Kumar, A., & Kanwar, S.S. *Curr. Biotechnol.* **1**, 241--248, 2012.

13. Aulakh, S.S., & Prakash, R. *J. Basic Microbiol*. **50**, 37--42, 2010.

14. Sumanjelin, B., et al. *J. Chem. Biol. Phys. Sci.* **3**, 289--296, 2013.

15. Tembhurkar, V.R., et al. *Sci. Res. Repot.* **2**, 46--50, 2012.

16 . Sharma, R., et al. *Biotechnol. Adv*. **19**, 627--662, 2001.

17 . Anitha, K., et al. *Biosens. Bioelectron*. **20**, 848--856, 2004.

18 . Dutta, R.R., & Puzari, P. *Biosensors. Bioelectrons*. **52**, 166--172, 2014.

19 . Erney, D.R., et al. *J. Chromatogr*. **638**, 57--63, 1993.

20 . Hajslová, J., & Zrostliková, J. *J.Chromatogr. A* **1000**, 181--197, 2003.