Annexure-IX

FINAL PROJECT COMPLETION REPORT

1. Title of the project:**Development of a process for effective use of acetylcholinesterase biosensors for quantification of Organophosphate and organocarbamate pesticide residues in produce**

2. Principal Investigator(s) and Co-Investigator(s):

   PI: Dr. Panchanan Puzari
   Co PI: Dr. Tapas Medhi

3. Implementing Institution(s) and other collaborating Institution(s): Tezpur University

4. Date of commencement: 1st August 2014

5. Planned date of completion: 31st July 2016

6. Actual date of completion: 31st July 2016

7. Objectives as stated in the project proposal:

   The main aim of the project is to make AChE biosensors for OP and OC pesticides, which work successfully with laboratory test samples prepared in inorganic buffer, more practicable, more efficient, by making them compatible to organic media, so that analysis of real field samples of pesticide residue becomes possible.

   To have this goal, the project work will attempt to fulfill the following objectives:
   1. To find a novel immobilization matrix for AChE which will be very stable in organic solvents?
   2. To have an optimum solvent system in which the AChE activity is least affected and at the same time can be used for efficient extraction of residue from produce.
   3. To evolve a net process/protocol by combining the above two for easy, low cost determination/quantification of residues in produce.

8. Deviation made from original objectives if any, while implementing the project and reasons thereof:

   None

9. Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams & photographs:

Title of invention

Enhancement of stability of Acetylcholinesterase in ethyl acetate through the use of Lipase and L-serine and hence a method for pesticide biosensing in QuEChERS extract.

Abstract

Pretreatment of analytical sample of pesticide solutions is necessary for both chromatographic and biosensing methods. Pretreatment in chromatographic method is essential to eliminate signals from undesired species. The most efficient pretreatment for chromatographic process is the QuEChERS method. On the other hand pretreatment in biosensing method essential for two reasons-1. To eliminate signals from undesired species and 2. To make the solution environment biocompatible, so that, the bio-receptors, mostly the enzymes, can sustain for sufficiently long duration. Due to lack of proper pretreatment procedure for bioanalysis, pesticide biosensors like acetylcholinesterase biosensors, polyphenol oxidase biosensors etc. are not able to give satisfactory results while applied to analysis of field samples, as the later contain organic solvents in which enzymes lose their activities. For the first time we have developed a method for pretreatment of analyte solution of OP and OC pesticides prior to application of bioanalysis through Acetylcholinesterase biosensors. The method involves use of lipase and L-serine in calculated amount. This pretreatment coupled to the QuEChERS pretreatment constitutes the complete pretreatment procedure for applying acetylcholinesterase based biosensors for pesticide analysis. Workability of the method has been demonstrated by taking three sample pesticides, two from the organophosphate class (ethion and temephos) and one from the organocarbamate class (fenbucarb).

PREAMBLE TO THE INVENTION

Acetylcholinesterase(AChE) biosensor is a kind of biosensor that can detect and quantify organophosphate (OP) and organocarbamate(OC) pesticide content in water, phosphate buffer solution or in dilute (5%) alcohols or acetonitrile. It can't be applied in other organic solvents or in methanol and acetonitrile at higher concentration (above 5%) because of enzyme denaturation. This biosensor can't be used in ethyl acetate, the most common solvent for OP and OC pesticides. Due to the restriction in the operating solvents, its application to real field sample analysis still remaining impractical, because most of the OP/OC pesticides residues cannot be extracted with those allowed solvents from the contaminated items (fruits, vegetables etc.) because of their poor solubility in those solvents. Another factor is the dilution factor. Normally, the pesticide residue content in food item is very low (but still above the danger level, with high MRL).

Dilution of such a sample to 5% may lower the sample concentration below the detecting capability of the biosensor (below the LOD).

So, a pretreatment method that can enhance the operating life time of AChE biosensor in organic extracting solvents or its transformed composition will make the use of AChE biosensor more practicable.
We have invented a novel solvent pretreatment method through which the AChE activity can be kept intact for over 12 hours in organic solvent system originating from ethyl acetate. We have shown that the biosensor can operate well in the solvent system which is equivalent to 17% of ethyl acetate. This has made the application of AChE biosensor more practicable because, now the biosensor can be applied to analysis of QuEChERS extract as well as for the analysis of both water soluble and water insoluble OP and OC pesticides.
The method provides lower estimate of the actual residue content. Therefore, to know the actual concentration, a correlation procedure for mapping the analyzed value to the true value has been provided for analysis of known analyte.
We describe here the method, the experimental evidence supporting our claim and examples of application of the method to analyze two OP and one OC pesticide.

(v) Statement of invention

(a).This invention states that thesustainability of the enzyme AChE in ethyl acetate can be achieved up to 12 h or more, when ethyl acetate is treated with calculated amount of lipase and L-serine.
(b).This invention also states that using the above mentioned fact that is, increase in sustainability of AChE in lipase and L-serine treated ethyl acetate, OP and OC pesticides can be detected and quantified in QuEChERS extract using AChE biosensor.

(vi) A summary of invention

General description

A method for sample pretreatment for bioanalysis of OP and OC pesticides after extracting in organic solvents has been developed. The key step of the method is conversion of ethyl acetate to enzyme friendly environment using lipase and L-serine. Combination of QuEChERS technique with the method constitutes complete protocol for sample preparation for bioanalytic detection of OP and OC pesticide content in field samples of pesticides. The method can be described through the following two technical steps.

Technical detail

(a) The Ethyl acetate Transformation (ET) method (Method for transforming ethyl acetate to enzyme (AChE) friendly environment)

To 2.5 ml of ethyl acetate add 2.5 ml of aqueous lipase suspension (containing 0.0108g/ml of porcine pancreatic lipase), shake for 5 minutes in vortex shaker and then keep at room temperature for 8 h until some froth appears at the top. Then filter twice with Whatman 1 filter paper. To 0.5 ml of the filtrate add 1 ml of water and 0.113g (0.0753g/ml) of L-serine and shake for few minutes. The solution mixture thus obtained is enzyme friendly fixture in which AChE activity can sustain for more than 12 hours.

(b)The QET method (QuEChERS tendem ethylacetate transformation method).

Extract the pesticide from produce in 5 mL of acetonitrile or ethyl acetate, apply QuECHERS clean up and then apply above step (a), i.e., reconstitute the residue in dichloromethane (DCM)-ethyl acetate 1:4 mL ratio and evaporate to almost dryness. Then reconstitute in 2.5 mL ethyl acetate, and then add 2.5 mL of lipase solution containing 0.0108 g/ml of lipase water, keep undisturbed for 8 hours until some froth appears at the top, and then filter. Take 0.5 mL of this and add 1 mL water and then add 0.113g of L-serine and shake for few minutes. The sample is now ready for analysis by enzyme based biosensors/bioanalytic methods.

Schematic illustration of the method has been given below (Figure 1)

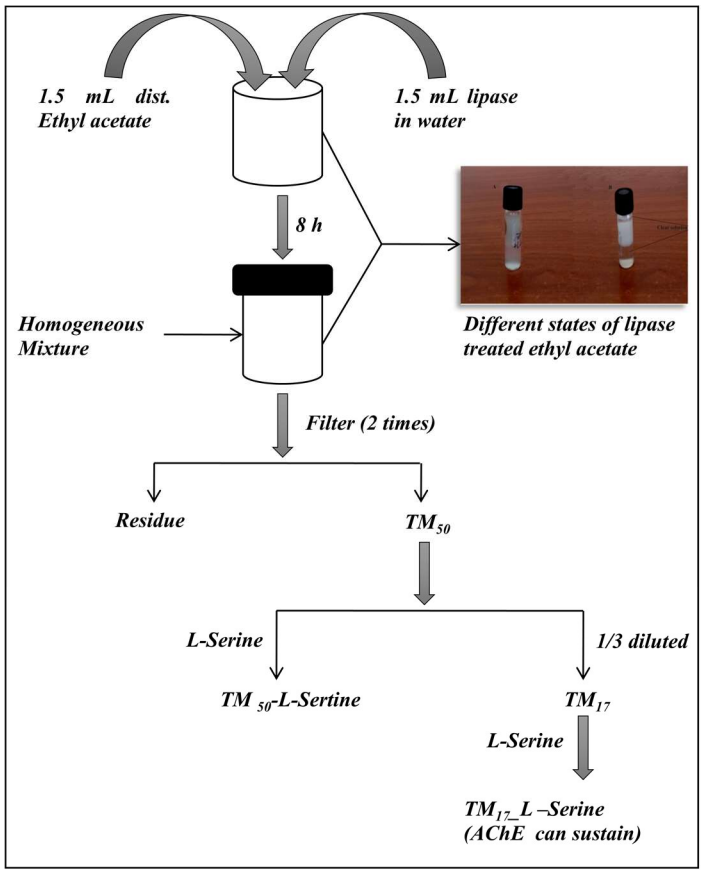


Fig: 1a

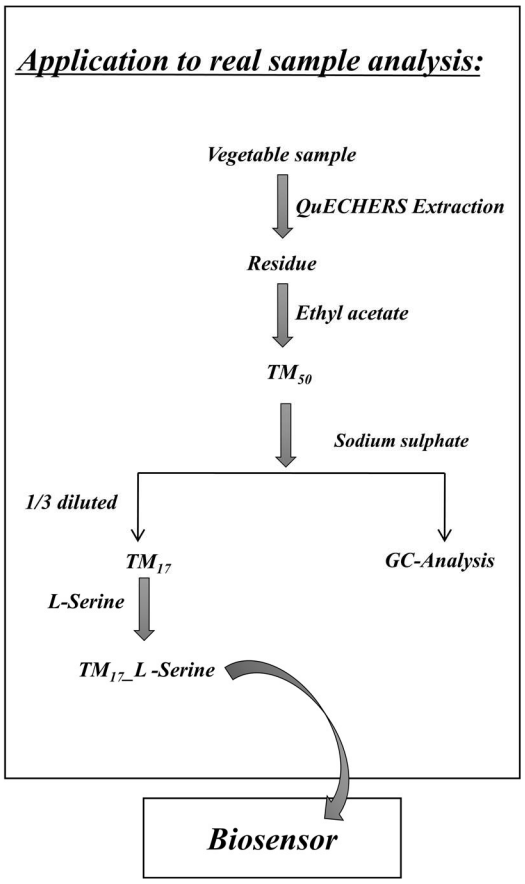


Fig: 1b

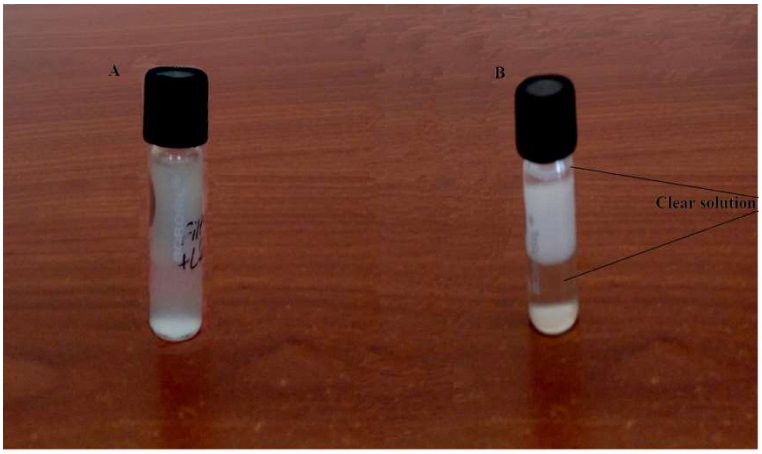


Fig: 1c

Figure 1a showing the complete steps of ethyl acetate transformation ET method of sample preparation. Figure 1b showing the steps for real sample analysis by using QuEChERS coupled ethyl acetate transformation QET method. Figure 1c showing the formation of the froth after 8 hours in the mixture of ethyl acetate and lipase.

(vii) **Novelty of invention**

(a) A novel and efficient method for conversion of ethyl acetate to an enzyme (AChE) friendly environment has been developed. This has rendered it possible to subject QuEChERS extract to bioanalytic protocols for organophosphate and organocarbamate pesticide detection/quantification. Thus, a method for detection and quantification of organophosphate and organocarbamate pesticide residues in QuEChERS extract (and hence in real field samples) has been developed for the first time.
(b) A novel correlation procedure for correlating the results obtained by using AChE biosensor in two different solvent systems has been developed for the first time.

(viii) Detail description of the invention (Experimental evidence of the invention)

Reagents

Acetylcholinesterase (AChE), Type VI-S (500 U/mg) EEL, acetylthiocholinechloride (ATChCl) (99%), were purchased from Sigma Chemicals Co., USA. Pyrrole (98%), gelatin powder (Type A, from porcine skin), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (99%) glutaraldehyde (50 wt % in H_2O), L-Serine (99%) reagent plus, Lipase porcine pancreatic Type III 100-400 U/mg protein were all purchased from Sigma-Aldrich, USA. Ethyl acetate, dichloromethane (DCM) acetonitrile, KH_2PO_4 and K_2HPO_4 were of analytical reagent grade and purchased from Merck chemicals, Ethion (O, O, O′, O′-Tetraethyl S, S′-methylene bis(phosphorodithioate), fenbucarb ((2-butan-2-ylphenyl) N-

methylcarbamate) and temephos **(** (O,OO′, O′-tetramethyl O, O′-thiodi-p-phenylenebis(phosphorothioate)) **)** were of analytical standard and obtained from Pestanal, Sigma-Aldrich. All the chemicals used in dispersive solid phase extraction were purchased from Agilent technology, USA. Phosphate buffer saline (PBS) pH 7.2 was prepared from KH_2PO_4 and K_2HPO_4 in 0.02 M KCl. Doubled distilled water was used throughout in the experiments

Instruments

PAR 273-A Potentiostate/Galvanostate was used for the electrochemical experiments and polypyrrole (PPy) film deposition. Platinum working electrode used was from CH Instrument, USA. The chromatographic analysis was performed using a Trace GC Ultra (Thermo Scientific, USA) equipped with electron capture detector (ECD). The capillary column used was a TR-5MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness) supplied by Fisher Scientific. FT-IR analysis was carried out in Perkin Elmer Frontier FIR-MIR.

Preparation of the assays

Assay 1

1.5 ml of distilled and dried ethyl acetate was mixed with 1.5 ml of lipase in water containing 0.0162 g of lipase (0.01088 g/mL). 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% ethyl acetate. Fig.1c shows the physical state of the lipase treated ethyl acetate at two different time interval.

Assay 2

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

Assay 3

Assay obtained through above procedure 1(assay 1) was diluted one third (TM_{17})

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 1 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).

Preparation of the sensor

Enzyme loaded, gelatin-glutaraldehyde-polypyrrole coated platinum electrode (Pt/PPy AChE-Glut-Geltn electrode) was prepared according to the published procedure [30]. 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 glutaraldehyde 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.

Results

Enzyme sustainability in lipase treated ethyl acetate in presence and absence of L-serine:

Sustainability of free enzyme in absence of L-serine

Sustainability of the free enzyme in TM_{50} (assay 1) and TM_{17}(assay 3) in absence of L-serine was studied by mixing 50 µL of the enzyme (AChE) to the above assay 1 and 3 followed by withdrawing 0.5 mL of this mixture 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 in absence of L-serine

Sustainability of immobilized enzyme studied using the sensor probe in place of free enzyme and immersing it alternatively in transformed mixture and Ellman assay.

Sustainability in presence of L-serine

Sustainability in presence of L-serine was studied by repeating the above experiment with assay 2 and 4 using both free and immobilized enzyme.

When free enzyme was mixed in TM_{50} (assay 1), 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 when withdrawal and mixing was done 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 for more than 5 minutes in TM_{50} or 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(TM_{17}) was different; the yellow color maintained the intensity till the Ellman test performed at 60 minutes. Decrease in intensity of yellow color was observed at 80 minutes. While performing the same experiment with assay 4, it was found that yellow color appeared with same intensity even when the test (withdrawal and mixing) was done at 8 h. The results thus confirm that the activity of the free enzyme remains intact for about 1 h in 17
% dilution level, that is, in TM_{17}, and, over 8 h in TM_{17} with L-serine. In case of immobilized enzyme the corresponding time found to be 1.5 h and over 12 h respectively

in TM_{17} and 'TM_{17} + L-serine'. The sustainability experiment has been illustrated schematically in fig.2

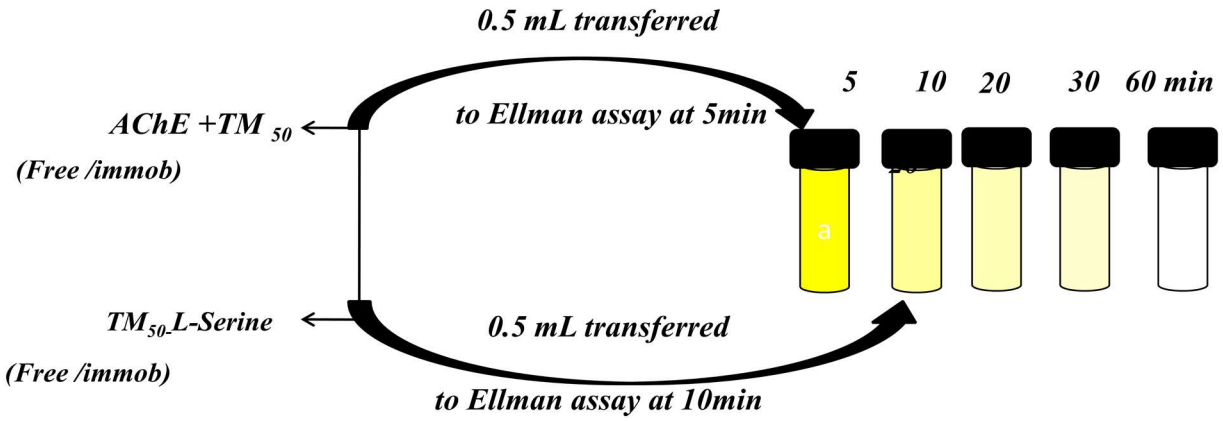


Fig: 2a

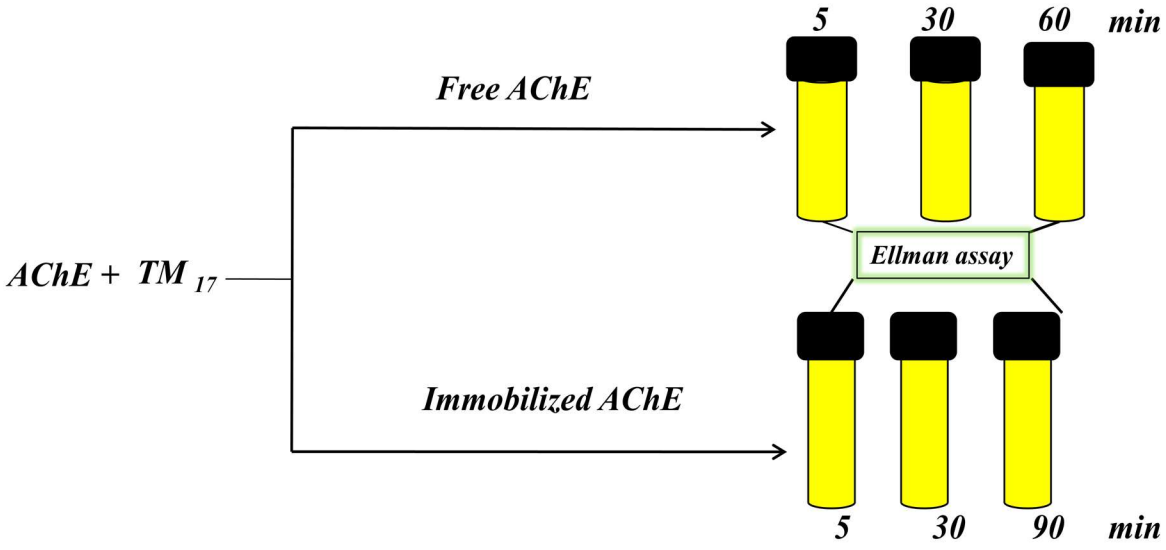


Fig: 2b

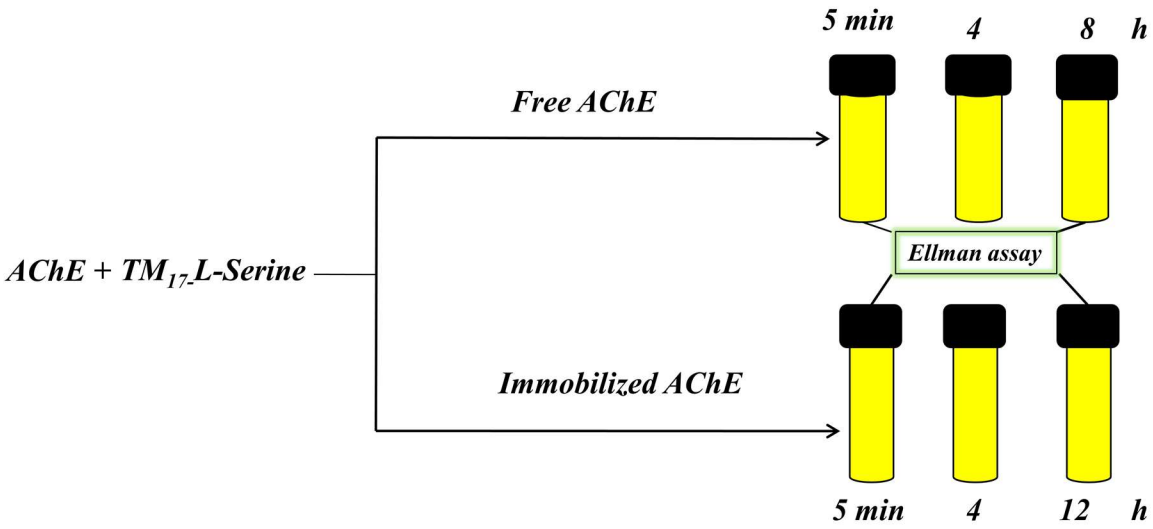


Fig: 2c

Fig.2 (a,b,c): Sustainability of AChE in free and immobilized state in the four assays. The vials show the change of colour of the Ellman assays upon addition of 0.5 ML of the AChE-TM mixture to it at different times.

FTIR analysis

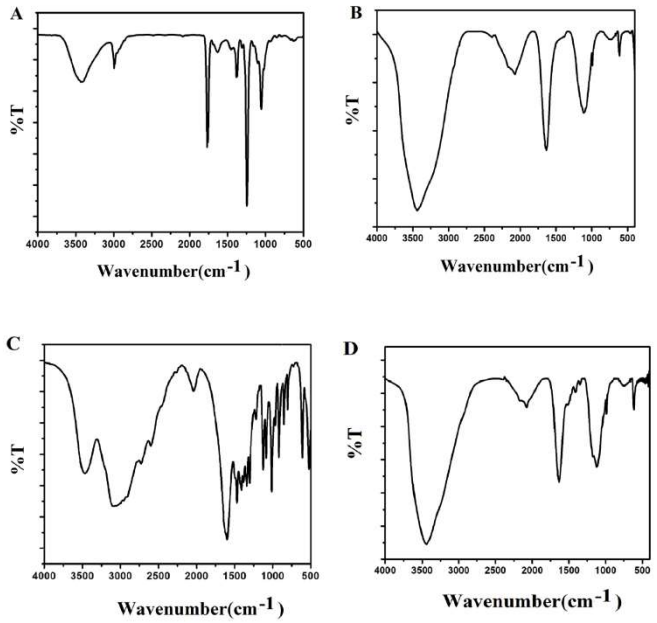


Fig.3. FT-IR spectra of pure ethyl acetate (A), anhydrous sodium sulphate treated TM_{17} (B), L-Serine(C) and anhydrous sodium sulphate treated TM_{17} with L-Serine (D).

Fig. 3 A shows the FT-IR spectra of pure ethyl acetate. Observed peaks are- 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. 3B. Shifting of peak positions seen and a new peak appeared around 600cm^{-1}. The spectra indicate the formation of new compound(s). Fig. 3C and D, 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.

Inhibition study

Inhibition by different constituents

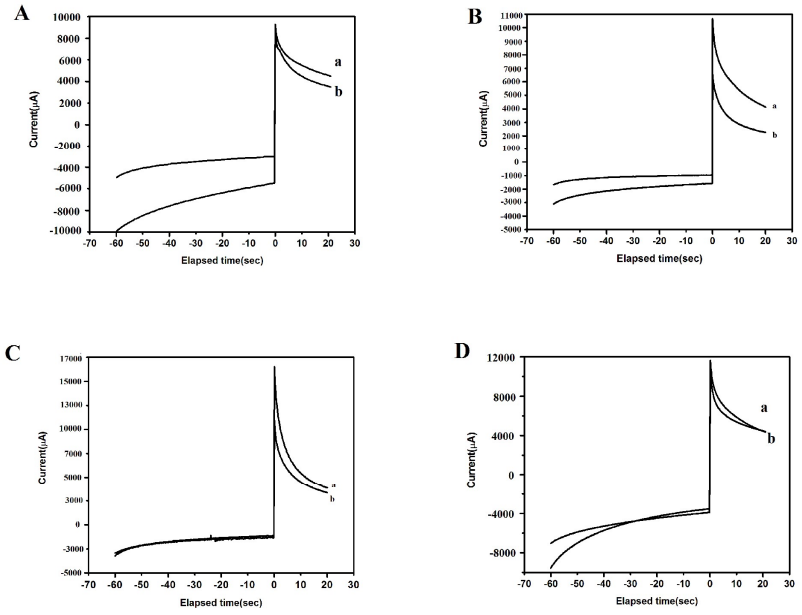


Fig.4. Chronoamperometric study of inhibitory effect of different components of the transformed mixture on the activity of the immobilized enzyme in presence and absence of pesticide. A. TM_{17} with L-serine. B. TM_{17} with ethion. C. TM_{17} with L-serine and ethion. D. QuEChERS coupled TM_{17} with 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 solution.

Inhibitory effects of L-serine and ethion towards the immobilized enzyme, when they present either individually or together in the transformed mixture TM_{17}, were studied by

chonoamperometric (Fig. 4) and cyclic voltammetric (Fig. 5) methods. Fig. 4A shows the CAs of ATChCl in PBS before (a) and after (b) incubating for 1 h in TM_{17} – L-serine mixture. Fig. 4B and 4C shows the same respectively in pesticide mixed TM_{17} and both pesticide and L-serine mixed TM_{17}. While in A, no significant inhibition was observed, in B and C the same was found to be present with different extent. With a 120ppb 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} was higher than that in pesticide and L-serine mixed TM_{17}indicates the possibility of either L-serine -pesticide interaction to some extent or hindrance by L-serine on the pesticide-enzyme interaction. Fig. 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. 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 (e.g. L-serine) as a whole, amounts to a definite average value of inhibition for each concentration. For exact quantification the observed value can be converted to actual concentration by using correlation equation.

Similar results were also obtained through CV experiments (Fig. 5).

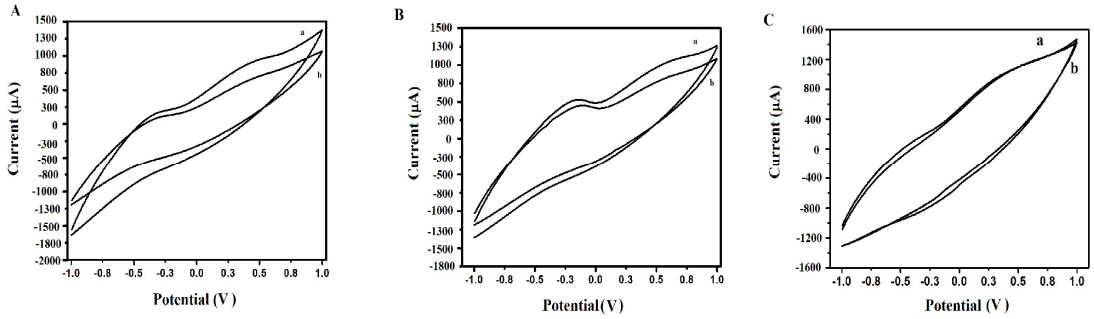


Fig.5. Cyclic voltammetric study of the inhibitory effect of different components of the transformed mixture on the activity of the immobilized enzyme in presence and absence of the pesticide. A. TM_{17} with ethion. B. TM_{17} with L-serine and ethion. C. TM_{17} with L-serine. In each case, a represent CV obtained in 2.0 millimolar ATChCl PB mixture before incubation of the sensor in the solution concerned, b represents the same after 1 h incubation in the solution.

Application to sample analysis (Ethion, fenbucarb and temephose)

The workability of the method has been demonstrated by taking three sample pesticides, two from the organophosphate class (ethion and temephos) and one from the

organocarbamate class (fenbucarb).The calibration curves for those three pesticides obtained through biosensor analysis in QET samples are shown in figure 6.

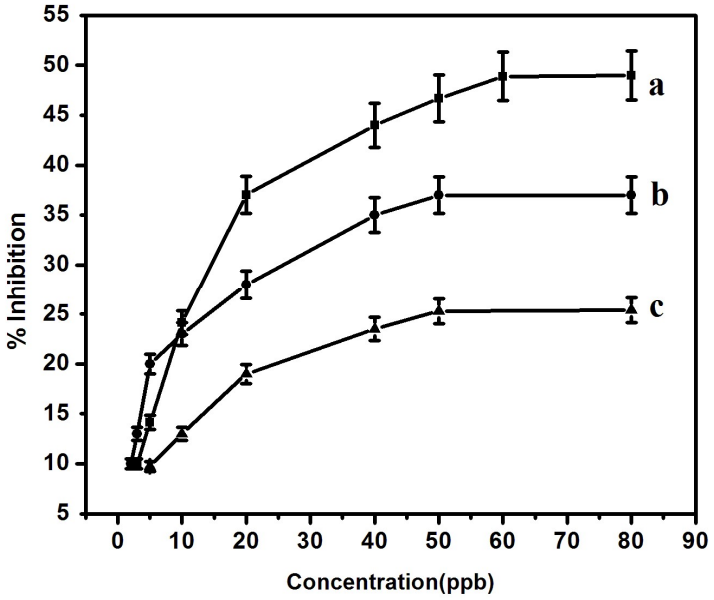


Fig. 6. Calibration curves obtained by applying AChE biosensor in solutions of pesticides prepared by QET method. a. fenbucarb, b. ethion and c. temephose.

Linear ranges and limits of detection for those three pesticides are shown in table 1.

Pesticides	Linear ranges	LOD(ppb)
Fenbucarb	3-20 ppb, 20-60 ppb	3
Ethion	2-5 ppb, 5 to 50 ppb	2
Temephose	5-20 ppb,20-50 ppb	5

Table 1: LODs and the linear ranges of the pesticides analyzed by by AChE biosensor in pesticide solutions prepared by QET method.

The QET biosensing method gives lower estimate of pesticide content than the actual. For known pesticides, a correlation procedure can be applied to know the actual concentration. For unknown pesticides, only an approximate value can be predicted which is almost three times the observed value.

Correlation procedure (taking ethion as an example).

At first calibration curves were obtained both for QET and conventional PB-acetonirile method (Fig.7).

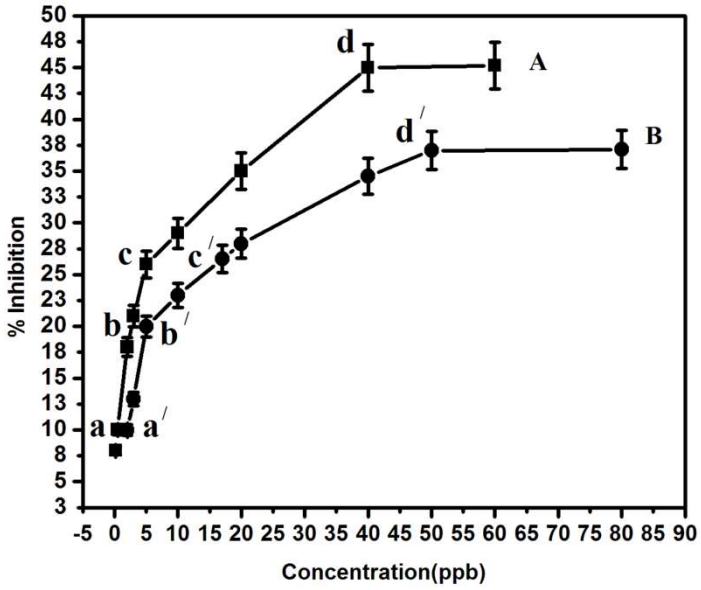


Fig.7. Calibration curves obtained through AChE biosensor application to fortified ethion samples. A. Ethion solution prepared in PB-acetonitrile mixture B. Ethion solution prepared in ethyl acetate subjected to QET method.

Curve A is the calibration curve obtained when ethion standard solutions were prepared in PB-acetonitrile mixture. The calibration curve for ethion under QET method is curve B. Comparison of the two shows that the % inhibition goes almost parallel but with lower magnitude in case of QET method.

The correlation equations and the corresponding segments are $y = 1.358x + 1.612$ 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 method of acquiring these correlation equations has been explained below.

We take the first segment $aa'bb'$ for illustration.

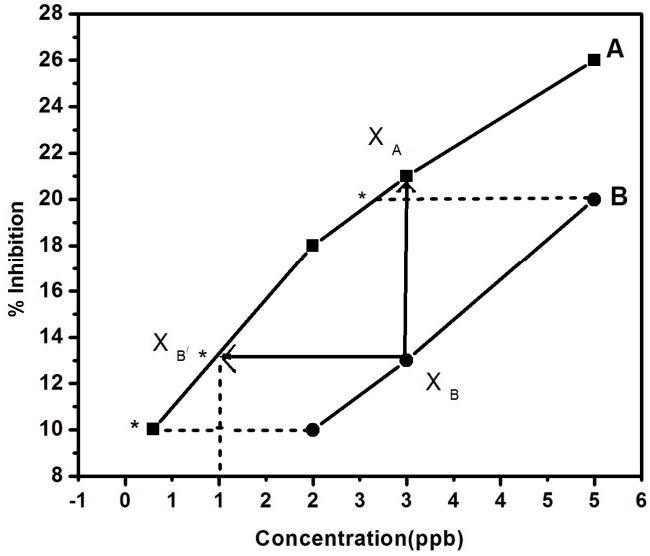


Fig.8. Segment 1of Fig. 7 before regression: a a′b b′ A- 0.3 to 2 ppb, B- 2to 5 ppb, I%= 10.0 to 20. Note: A has been shown in extended form beyond I= 18 %.

For better correlation we take regression of those two curves.

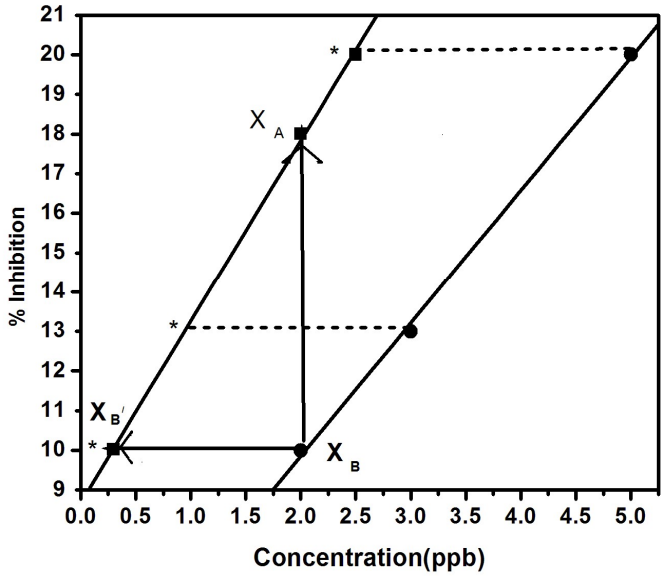


Fig.9. Segment 1of Fig. 7 before regression: a a′b b′ A- 0.3 to 2 ppb, B- 2to 5 ppb, I%= 10.0 to 20. Note: A has been shown in extended form beyond I= 18 %.

For better correlation we take regression of those two curves.

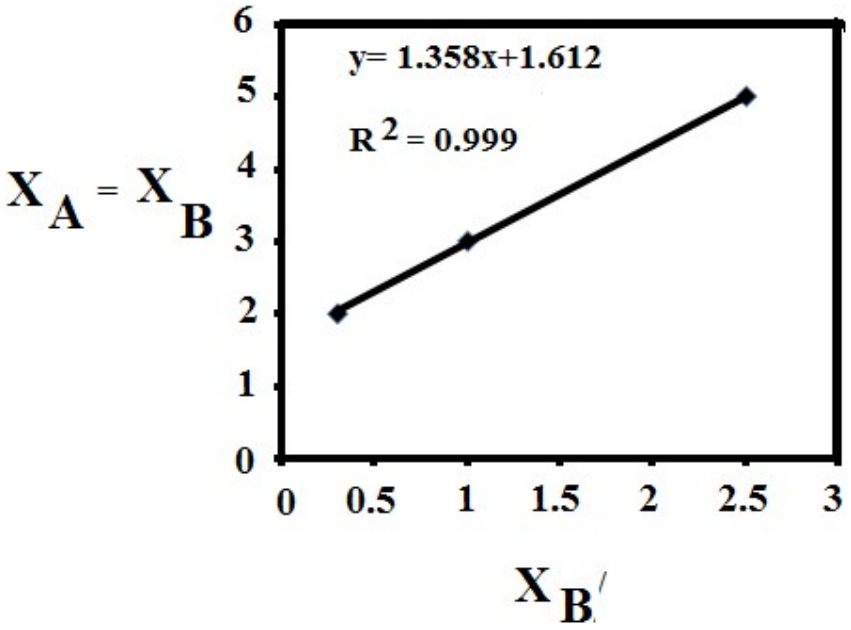


Fig.10. Segment 1of Fig. 7 after regression.Note: A has been shown in extended form beyond I= 18 %.

Then we find out for a particular concentration indicated by curve b, the corresponding concentration on curve a, drawing line parallel to concentration axis. The new point thus obtained is the shifted concentration of a point on curve ato curve b. Then we correlate the moving pattern of the shifted concentration ($X_B^{/}$) relative to the true concentration(X) by plotting the former as abscisa (x-axis) and the later as ordinate (y-axis). The equation thus obtained is the correlation equations.

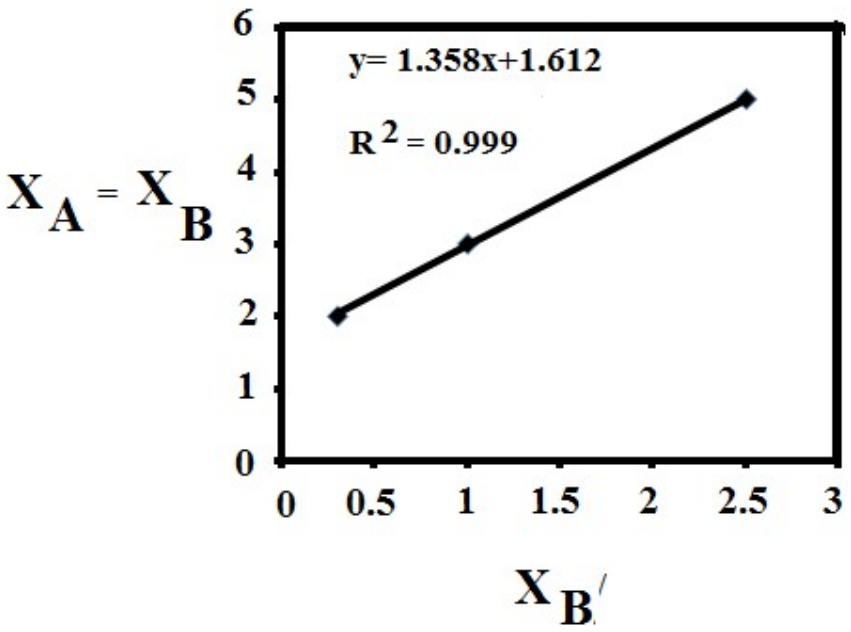


Fig. 11. Plot of common concentration vs. shifted concentration of B on curve A for segment 1

Table 2. 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

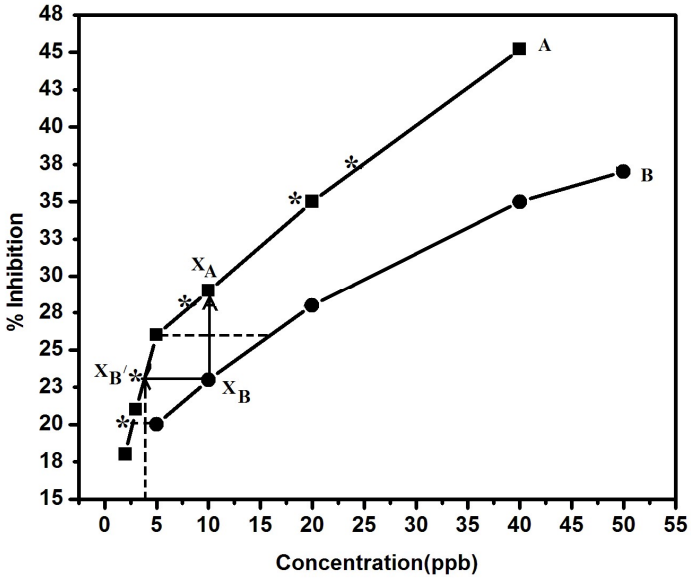


Fig. 12. Segment 2 of Fig. 7 before regression: b b′cc′, A= 2.5to 5ppb, B- 5to 15.6 ppb, I%= 20 to 26

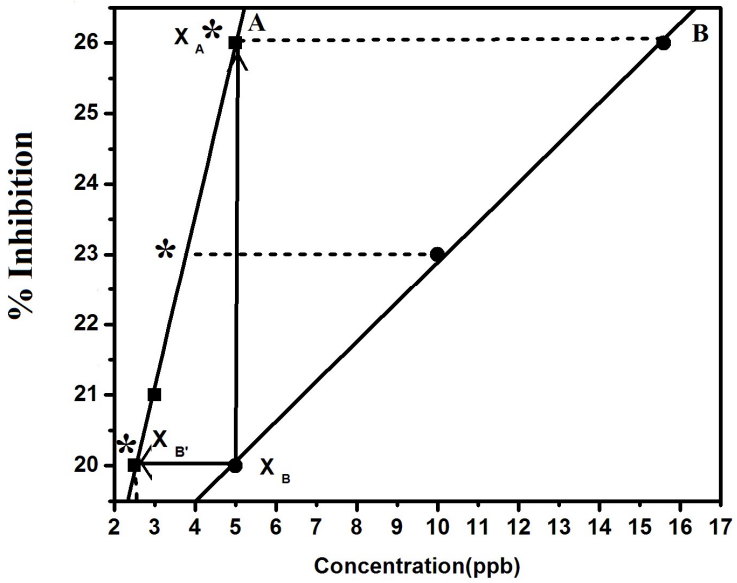


Fig. 13. Segment 2 of Fig. 7 after regression

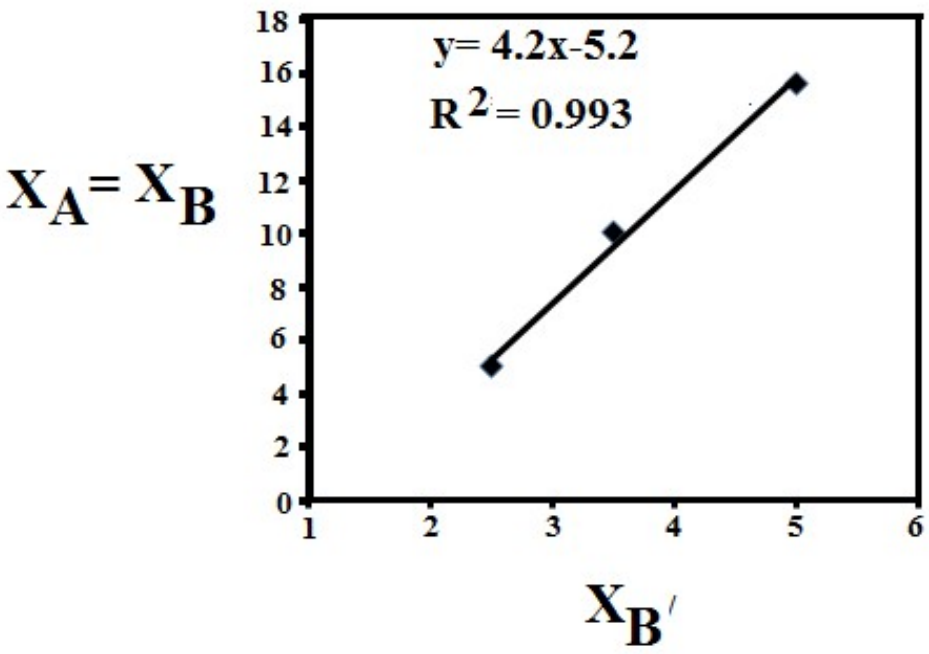


Fig. 14. Plot of common concentration vs shifted concentration of B on curve A for segment 2

Table 3. 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

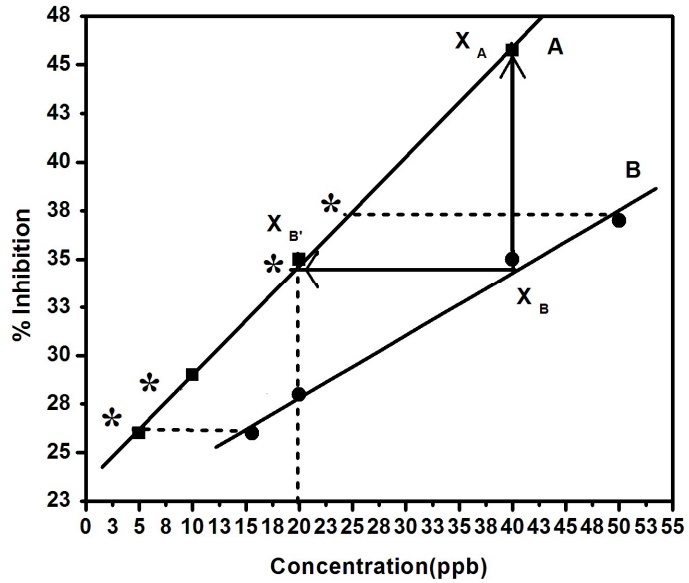


Fig. 15. Segment 3 of Fig. 7 after regression: cc′ dd′, A= 5 to 40 ppb B- 15.6 to 50 ppb, I%= 26 to 46

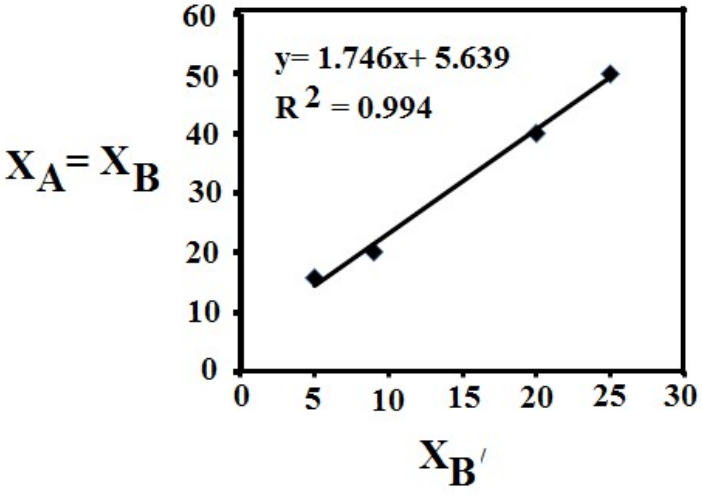


Fig. 16. Plot of common concentration vs. shifted concentration of B on curve A for segment 3

Table 4. 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

Note: For pesticides which are not soluble in PB-acetonitrile mixture, such correlation can be made with GC calibration curve. A model curve is shown in figure 16.

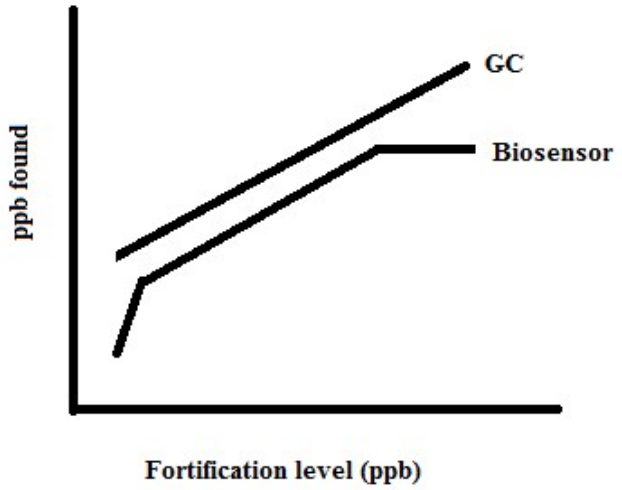


Fig.17. Model curve shows the correlation between gas chromatographic and biosensor calibration curves.

Validation study through spiked sample analysis (taking ethion as example)
Sample preparation by applying QET

Two sets of solutions were prepared, one set for GC analysis and the other set for Biosensor analysis. GC series taken was from 20 to 100 ppb while the biosensor series from 10 to 40 ppb with two common concentrations of 20 and 40 ppb (same set of concentration for both could not be taken because of the difference in limit of detection of the two process/instruments). The results obtained are shown in table 5 and 6.

Biosensor analysis

Three fortified samples were treated by QET method and analyzed by biosensor. The results were compared with calibration curve A. The results are shown in table 5.

Table 5. Recovery of ethion using biosensor in the QET method

Fortification level(ppb)	Observations					Recovery %	Mean recovery %	rsd%
	I%	ppb by calibration curve A (x)	Regression equation	ppb found (y)	ppb expected ($1/3^{rd}$ 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		

Cross verification by GC analysis

Two calibration curves were obtained, one by preparing a series of standard ethion solution in acetonitrile followed by QET treatment(calibration curve C, not shown here) and the other with direct solution of ethion in ethyl acetate(calibration curve D, not shown here). Note that solutions prepared through QET method(calibration curve, C) rather than direct solutions of ethion in ethyl acetate (calibration curve D) was used for result comparison because, in the later case, matrix enhancement of GC results does not get nullified, and thus, the recovery becomes more than 100%.

Table 6. Recovery by GC method.

Analytic methods	Fortification level(ppb)	Recovery					
		Using calibration curve D (direct solution of ethion in ethyl acetate)			Using calibration curve C Ethion solution obtained through QET method)		
		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		

Table 7. Comparison of recovery from QET method by biosensor and by GC

Analytic methods	Fortification level(ppb)	Recovery								
		Using calibration curve A			Using calibration curve D			Using calibration curve C		
		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								

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 biosensing (QET) method was one third of the original one. Results show that recoveries in the two cases are excellent. It proves the validity of the biosensor analysis in the described novel procedure.

As a side work we have developed a method for detection of a pyrethroid pesticide Cypermethrin using an another biochemical reaction catalyzed by the enzyme Glutathion transferase(otherthan acetylcholinesterase). The work has been published in Electrochimica Acta, 205, 198-206, 2016 (copy of the paper attached herewith).

10. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject:

The new knowledge gained:
Lipase-L-serine combination can protect the activity of the enzyme AChE in ethyl acetate.
Inhibition of the enzyme acetylcholinesterase in the transformed ethyl acetate solution by pesticide in absence of L-serine is higher than the same when L-serine is present. It indicates that there is pesticide L-serine interaction to some extent. So, a new strategy seems to be possible for determination of pesticides using L-serine. Further study on this observation will enhance the knowledge on enzyme (AChE) pesticide interaction.

We have found that the reaction between reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene(CDNB) which is catalyzed by GST and which is most extensively studied one among all GST catalyzed detoxification reaction, is more prominent in 25% methanol then in phosphate buffer(PB). Spectroscopic study reveals that this happens due to formation of hydrogen bonds between GSH and PB. This will provide a strong thrust in the study of GST catalyzed detoxification reactions and will help in better understanding of the cause of pesticide tolerance developed in certain mosquitoeswith time.

11. Conclusions summarizing the achievements and indication of scope for future work

The method we have developed through this project work has rendered it possible to apply biosensor to field sample analysis which was a major hurdle earlier. The concept of application of biosensor for pesticide analysis in QuEChER extract is novel one and no group has reported its possibility earlier. We have demonstrated that it is possible. Now, OP and OC pesticides in food and agricultural samples can be detected in easy way through electrochemical biosensing as well as UV VIS method instead of going for the cumbersome and costly GCMS or LCMS method
.
Most importantly, it has opened up the possibility of developing hand held biosensor kits for OP/OC pesticides. The kits can be either immunosensor kits based on ELISA test or colorimetric test sensor kits based on colorimetric assay like the Ellman test (becausenow

we have got a means to maintain the enzyme in active state in the organic mixture/extract containing the pesticide). **The method will get commercial importance once such kits are prepared.Preliminary work towards that goal is going on. Some extra funding and extra time will be required.**

On the other work related to GSH-CDNB reaction cum cypermetrhin detection,we have published a paper in a peer reviewed international Journal with impact factor 4.5.

12. S&T benefits accrued:

i. List of Research publications

S No	Authors	Title of paper	Name of the Journal	Volume	Pages	Year
01	H. Borah, R.R.Dutta, S. Gogoi, P. Puzari	Influence of methanol, ethanol and cypermethrin on the Glutathione S-tranferase catalyzed reaction of Glutathione with 1-chloro-2,4-dinitrobenzene: A method for detection and quantification of cypermethrin	Electro chimica Acta	205	198-206	2016
02	Borah H, Dutta R R, Gogoi S, Medhi T & Puzari P.	'Glutathion-S-transferase catalyzed reaction of glutathion for electrochemical biosensing of Temephos, Fenbucarb and Dimethoate',	Analytical Methods.	9	4044-4051	2017

ii. Manpower trained on the project

a) Research Scientists or Research Associates: Nil

b) No. of Ph.D. produced: 01

c) Other Technical Personnel trained: Nil

iii. Patents taken, if any: One patent application filed. Application No. **201631008813**
Ref. No. E-2/481/2016-KOL

Title : 'Enhancement of stability of acetylcholinesterase in ethyl acetate through the use of lipase and L-serine and hence a method for pesticide bio-sensing in QuEChERS extract', Indian Patent

13. Financial Position:

No	Financial Position/ Budget Head	Originally sanctioned budget	Revised budget*	Expenditure	% of Total cost
I	Salaries/ Manpower costs	4,22,400/-	5,20,267/-	5,20,267/-	89%
II	Equipment	1,36,400/-	1,02,247/-	1,02,247/-	75%
III	Supplies & Materials	4,15,600/-	4,71,698/-	4,71,698/-	137%
IV	Contingencies	Nil	Nil	Nil	
V	Travel	60,000/-	82,474/-	82,474/-	76%
VI	Overhead Expenses	1,03,440/-	1,03,045/-	1,03,045/-	80%
VII	Others, if any		Nil		
	Total	**11,37,840/-**	12,79,731/-	12,79,731/-	**99.90%**

*Diary No. SERB/F/8368/2017-18 dated 10/01/2018.

14. Procurement/ Usage of Equipment

a)

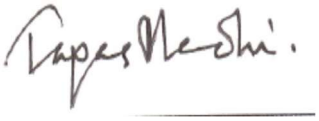
S No	Name of Equipment	Make/Model	Cost (FE/ Rs)	Date of Installation	Utilisation Rate (%)	Remarks regarding maintenance/ breakdown
01	**Deep Freezer-200 ltrs -18-20^{0}C**	**HAIER/ HDF-385H**	**19700**	**3/06/15**	**100%**	
02	**Platinum working electrode**	**CHI 102**	**26565**	**15/02/15**	**100%**	
03	**Magnetic stirrer**	**REMI/2ML**	**5450**	**28/11/15**	**100%**	
04	**Vortex shaker**	**TARSONS /3022**	**5611**	**8/4/15**	**100%**	
05	**Ultrasonic cleaner**	**WENSER**	**13740**	**12/3/15**	**100%**	
06	**Steam bath with 6 holes**	**DYNAMIC SCIENTIFIC WORKS**	**6870**	**19/3/15**	**100%**	

b) Plans for utilizing the equipment facilities in future

The equipment are in regular use for research purpose by the research scholars of the Department as well as by the research group of the PI.

PI Co-PI


09.09.2016



a._____
(Principal Investigator)

09.09.16

b._____
(Co-Investigator)

Updated on : 04.10.2020

To

Dr. Ramesh Vijayan,

Scientist,"C"

Science and Engineering Research Board(SERB), New Delhi

Date: 12.02.19

Sub: Utilization Certificate and statement of expenditure of project (2017-18).

Ref: Project No. SERB/MOFPI/0005/2014.

Sir,

Please find submitted herewith the Utilization Certificate (2017-18) and final consolidated Statement of Expenditure of the above referred project for your needful.

Thanking you,

With regards,

12/02/19

(Panchanan Puzari)
PI & Assistant Professor
Department of Chemical Sciences,
Tezpur University, Assam.

FINAL STATEMENT OF EXPENDITURE

1. SERB Sanction Order No and date : SERB/MOFPI/0005/2014 ; Dt. 27.06.2014, 03.10.2015 and 11.01.2018
2. Name of the PI : Dr. Panchanan Puzari
3. Total Project Cost : 11,37,840/-
4. Revised Project Cost : 12,79,731/-
5. Date of Commencement : 01.08.2014
6. Statement of Expenditure :
   (Month wise expenditure incurred during current financial year)

Month & year	Expenditure incurred/ committed
February, 2018	Nil
March, 2018	144000

1. Grant received in each year:
   a. 1stYear : 6,37,120/-
   b. 2nd Year : 4,00,000/-
   c. 3rdYear : 2,42,611/-
   d. Interest, if any : 7267/-
   e. Total (a + b + c + d) : 12,86,998/-

Final Statement of Expenditure

(For the financial year01-08-2014 to 31-03-2015, 01-04-2015 to 31-03-2016 and 01-04-2016 to 31-07-2016 and 2017-18)

Sr No	Sanctioned Heads	Total Funds Allocated (Revised)	Total Released Amount	Expenditure Incurred				Total Expenditure	Balance as on 1st April 2018	Requirement of Funds	Remarks (if any)
				1st Year (01-08-2014 to 31-03-2015)	2nd Year (01-04-2015 to 31-03-2016)	3rd Year (01-04-2016 to 31-03-2017)	4th year 01-04-2017 To 31.03.2018				
(I)	(II)	(III)	(IV)	(V)	(VI)	(VII)	(VIII)	(V + VI + VII + VIII) = (IX)	(X)= (IV-IX)		
1.	Manpower costs	5,20,267/- revised	5,20,267/-	1,28,000/-	184267/-	64000/-	144000/-	5,20,267/-	Nil	Nil	
2.	Consumables	4,71,698/- revised	4,71,698/-	2,50,825/-	46,261/-	174612/-	Nil	4,71698/-	Nil	Nil	
3.	Travel	82,474/- revised	82,474/-	23,344/-	52,287/-	6843/-		82,474/-	Nil	Nil	
4.	Others, if any	-	-	-	-	-		-	-		
5.	Equipment	1,02,247/- revised	1,02,247/-	77,936/-	24,311/-	-		1,02,247/-	Nil	Nil	
6.	Interest, if any	Nil	7267/-	Nil	Nil	Nil		Nil	7267/-	Nil	
7.	Overhead expenses	1,03,045/- revised	1,03,045/-	32,325/-	32,325/-	38395/-	Nil	1,03,045/-	Nil	Nil	
8.	Total	12,79,731/-	12,86998/-	5,12,430/-	339,451/-	283850/-	144000/-	1279731/-	7267/-	Nil	

Amount to be refunded: Rs. 7267/- (Rupees seven thousand two hundred and sixty seven only).

(Panchanan Pujari)
Name and Signature of Principal Investigator:

Date: 29.01.2019

Seal:

Signature of Competent financial authority:

Date:

Seal: Finance Officer
Tezpur University

UC for Recurring Grants

UTILISATION CERTIFICATE
FOR THE FINANCIAL YEAR – **(April 2017-March 2018)**

UC pertains to
✓ *appropriate box*

First Release	Second Release	Third Release	Fourth Release	Final Release√

Is the UC provisional : No

1. Title of the Project/ Scheme : "Development of a process for effective use ofinacetylcholinesterase biosensors for quantification of Organo Phosphate and Organo Carbamate pesticide residues in produce"

2. Name of Principal Investigator : Dr. Panchanan Puzari

3. Implementing Institution : Tezpur University

4. SERB sanction order No & date : SERB/MOFPI/0005/2014, Dated 27.06.2014 & SERB/F/8368/2017-18, Dt. 10.01.2018

5. Amount brought forward from the previous financial year quoting SERB letter number and date in which the authority to carry forward the said amount was given
   : Amount (-) Rs. 98611/-
   : Letter/Order No Nil
   : Date Nil

6a. Amount received during the financial year (Please give SERB letter/order no and date for the amount)
   : Amount: Rs. 2,42,611/-
   : Letter/Order No. SERB/F/8368/2017-18
   : Date: 10.01.18

6b. Interest earned, if any : Rs. 7267/-

7. Total amount that was available for expenditure *Rs.* (excluding commitments) during the financial year (Sr. No. 5+6a+6b) : Rs. 151267/-

8. Actual Expenditure (excluding commitments) incurred during the financial year (up to 31st March) : Rs. 144000/-

9. Balance amount available at the end of the financial year (8-7): OR/ Negative balance (If expenditure incurred is more than the funds released) : Rs. 7267/-

10. Unspent balance, if any, refunded to SERB (give details of cheque/DD No etc.) : DD. No. 53332690/dt-13.11.2018

11. Amount to be carried forward to the next financial year (if any) : Nil

Finance Officer
Tezpur University

UTILISATION CERTIFICATE

Certified that out of Rs 242611/-/ of Recurring grants-in-aid sanctioned during the year 2017-18 in favour of **The Registrar, Tezpur University** under SERB letter/ order No SERB/F/8368/2017-18 dated 10.01.18 and (-)Rs. 98611 on account of committed expenditure of the previous year plus the interest amount of Rs.7267 earned as on 31.03.2018, a sum of Rs.144000 has been utilised for the purpose for which it was sanctioned and the balance amount of Rs. 7267/- remaining unutilised at the end of the year has been refunded to SERB vide DD No.. 53332690 dated. 13.11.2018.

Certified that we have satisfied ourselves that the conditions on which the grants-in-aid was sanctioned have been fulfilled and that we have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Cash Book
2. Ledger Book
3. Internal Audit

Signature of PI
Date 29.01.19

Signature of Registrar/Account Officer
Date
Finance Officer
Tezpur University

Signature of Head of Institution
Date
Registrar
Tezpur University

Science and Engineering Research Board

UC accepted has been accepted by

Signature _____
Name of the SERB officer _____
Designation _____

जारी करने वाली स्टेट बैंक
Issuing SBI TEZPUR UNIVERSITY
कोड क्र /CODE No: 14259
Tel No. 03712-267285

मांगपत्र
DEMAND DRAFT

Key: SUHHUT
Sr. No: 510586

1	3	1	1	2	0	1	8
D	D	M	M	Y	Y	Y	Y

मांगे जानेपर FUND FOR SCIENCE & ENGINEERIN RESEARCH**********
ON DEMAND PAY

या उनके आदेश पर
OR ORDER

Seven Thousand Two Hundred and Sixty Seven Only

रुपये RUPEES

अदा करें ₹ 7267.00

IOI 000471532690 Key: SUHHUT Sr. No: 510586 AMOUNT BELOW 7268(7/4) मूल्य प्राप्त / VALUE RECEIVED
Name of Applicant TEZPUR UNIVERSITY R& D

भारतीय स्टेट बैंक
STATE BANK OF INDIA
अदत्तकर्ता शाखा / DRAWEE BRANCH:NEW DELHI MAIN BRANCH
कोड क्र . /CODE No: 00691

प्राधिकृत हस्ताक्षरकर्ता
AUTHORISED SIGNATORY

शाखा प्रबंधक
BRANCH MANAGER

Hiraj Kalita
हिराज कलिता
K- 13886 के १३८८६

₹ 1,50,000/- एवं अधिक के लिए दो [illegible]
PAYMENTS FOR ₹ 1,50,000/- & ABOVE [illegible]

कम्प्यूटर द्वारा मुद्रित होने पर ही वैध
VALID ONLY IF COMPUTER PRINTED

केवल 3 महीने के लिए वैध
VALID FOR 3 MONTHS ONLY

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"532690" 000002000: 0004?6" 16

Sent on 24/12/18:

To Dr. Ramesh Vijayan
Scientist C
SERB
SERB, lower ground floor
Vasant Square mall, Sector B, Pocket 5 Vasant Kunj N. Delhi

ES827058251N IVR:[illegible]

SP NAPAAM S.O <78402>

Counter No:1,24/12/2018,11:14

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PIN:110070, Vasant Kunj SO

From:DR. PANCHANAN PUZARI,T.U

Wt:48gms

Amt:41.30(Cash)Tax:6.30

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