Chapter 4

Application of GST catalyzed GSH-CDNB reaction in methanol as a new method for detection of selected pesticides belonging to different classes

4.1 Introduction

Development of easy, field deployable and highly sensitive analytical techniques for detection of pesticide content in environmental and food samples is a research area of high demand at the present time owing to the alarming adverse effect of those pesticides on human health and the environment [1-10]. Among the analytical techniques, enzyme inhibition based electrochemical biosensing techniques are extensively used due to the advantages of high sensitivity, reliability, fast response and the feasibility of integrating to a miniaturized sensor device [11-17].

Though many enzyme biosensors have been developed for pesticide detection, there still remains some limitations in their practical utilization. Two obvious limitations are 1. their class specificity and 2. inability to operate in organic solvents. The enzymes used in pesticide biosensors are class specific, e.g., organophosphate hydrolase for organophosphates [18,19], acetylcholinesterase for organophosphates and organocarbamates [20-23], tyrosinase for phenolic classes [24]. Due to the class specificity of enzyme action in one hand and the availability of diverse classes of pesticides in the market on the other hand, it is quite challenging to come up with different enzyme or bio-receptor molecules for each class. Beside this, inhibition based enzyme biosensors cannot operate in organic solvents [25]. Few workers have reported the use of 5% acetonitrile as the solvent but this causes excessive dilution of the sample and also affects the reusability of the biosensor [26]. As an effort towards that direction, attempts have been made to use the cytosolic enzyme glutathione S- transferase (GST) for pesticide biosensing, with the presumption that since it is a detoxification catalyst capable of binding with many hydrophobic compounds [27-33], it may bind with the pesticides also, thus affecting the catalytic action of itself and thereby triggering a biochemical signal. Few workers have developed UV-visible spectroscopic detection protocol using GST enzyme for pesticide detection [34,35]. Some others have used the direct reaction between GSH and pesticides for developing detection protocol for the later [36].

In chapter 3, we have shown that the GST catalyzed reaction between GSH and CDNB as well as the influence of pesticides on the said reaction can be studied through normal, mediator less cyclic voltammetric technique, if 25% methanol is used as the electrolyte

instead of phosphate buffer. We have demonstrated the detection of pyrethroid pesticide, cypermethrin, using the method [37]. In the present chapter we have extended the method for organothiophosphate (OTP) and organocarbamate (OC) classes of pesticides, taking the commonly used organothiophosphate- temephos and dimethoate; and organocarbamate- fenobucarb. The detection method of these pesticides has been validated through analysis of tomato samples spiked with those pesticides. It was found that the method works well in case of those three targeted pesticides.

Fenobucarb is a pesticide of the N-methylcarbamates family, that has a wide range of applications and whose popularity has increased in the time, due to its broad biological activity spectrum as insecticides, miticides, fungicides, nematocides or molluscicides [38-41]. However, their excessive use in pre- and post-harvest treatments to control diseases of fruits and vegetables may cause dramatic impact when discharged recklessly into water, soil and air without any treatment eventually leading to long-term accumulation in ecosystems including humans [42-44]. They emerge as one of the most significant anthropogenic threat to ground and surface water [45].

The organothiophosphate insecticide temephos is among the most widely used insecticide throughout the world for the control of *Aedes aegypti* larvae [46,47]. In India, temephos has been recommended for controlling larval stages in potable water [48] and it is expected to be potentially toxic to nontarget species [49].

Dimethoate (O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate) is one of the highly yielded systemic thio-organophosphorus insecticide. This is used in various countries to control the insect population on wide variety of crops [50]. Even at relatively low levels of residues in food or the environment, dimethoate may be hazardous to human health due to its high toxicity. Its oxon-derivative omethoate is the main oxidized metabolite which displays a higher neurotoxicity than the mother compound [51-53].

The pesticide analysis in real samples needs extraction and pre-concentration because of relatively low concentration of target analytes and hydrophobic nature of most of the pesticides. This is in practice usually accomplished by using organic solvents, such as ethyl acetate, methanol, acetone, acetonitrile or hexane or combining them with solid-phase extraction. In this regard, enzyme sensors capable of measuring directly in the organic extract would be well suited particularly for the rapid analysis of pesticides

without further sample processing [54]. From this perspective, our method is a promising new tool for pesticide analysis because the detection protocol involved the use of 25% methanol, thus making bioanalysis of real samples more feasible.

4.2 Objectives of this chapter

- To apply the developed method to detect different classes of pesticides.
- To observe the inhibitory effect of different pesticides on GSH-CDNB reaction catalyzed by GST enzyme using CV technique.
- To use UV-Visible spectroscopy for cross verification of obtained results of CV analysis.
- To quantify and then to obtain calibration curves for different pesticides studied and determine limit of detection and linear range for each pesticide.

4.3 Experimental

4.3.1 Materials and reagents

GST (from equine liver), CDNB, GSH, temephos, fenobucarb and dimethoate (analytical standard) were purchased from Sigma–Aldrich. Phosphate buffer (PB) of 0.1 M (pH 6.5) was prepared by mixing KH_2PO_4 and K_2HPO_4 procured from Merck-Germany. Methanol, dichloromethane (DCM) and acetonitrile were of analytical reagent grade and purchased from Merck chemicals. Bondesil-NH_2 and carbon SPE bulk sorbent were purchased from Agilent technologies. The GST solution was prepared in PB containing 0.1 M KCl at pH 6.5 as the supporting electrolyte and stored at - 22 ºC. GSH stock solution was also prepared in PB (pH 6.5). CDNB solution was prepared in 50% aq. methanol so as to maintain the final percentage of methanol 25%. Distilled methanol was diluted to 50% using ultra-pure water from a Millipore Milli-Q system. All the solutions except GST were prepared regularly before experiments. The stock solutions of fenobucarb, temephos and dimethoate were prepared in methanol as both fenobucarb and temephos are water insoluble pesticides. The stock solutions of each pesticide are diluted to the appropriate concentration for further experimental use.

4.3.2 Analysis procedure

4.3.2.1 Cyclic voltammetry measurements

Cyclic voltammetric experiments were conducted using three electrode arrangements. Pt electrode as working, a Pt wire as counter and Ag/AgCl refilled with 0.1 M KCl was used as the reference electrode. The total volume of the working solution in the electrochemical cell was 3 mL and prepared by mixing together 1.5 mL of 2 mM GSH in PB with 1.5 mL of 2 mM CDNB in 50% methanol, unless stated otherwise. CV measurements were done with the potential sweeping from − 0.40 V to 1.00 V at scan rate 20 mV/ s. Inhibition study was carried out by incubating pesticide solutions of different concentrations in the reaction mixture and then subjected to CV analysis.

4.3.2.2 Optimum pesticide incubation time

The effect of pesticide incubation time in the enzymatic reaction was investigated. Figure 4.1 shows percentage inhibition of GST enzyme plotted against pesticide concentration at different incubation times. When the incubation time was longer than 30 min, the curve tended to maintain a stable value, which indicated that the binding interaction with enzyme or enzyme-substrate complex could reach saturation. Thus, the optimum incubation time of 30 min was selected.

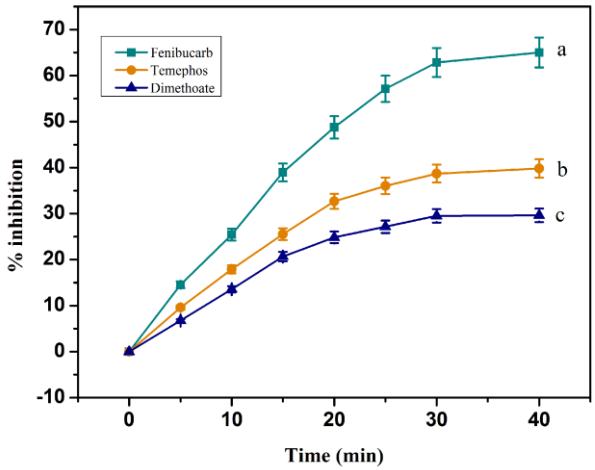

Figure 4.1. Variation of percent inhibition with incubation time determined from cyclic voltammetric response of the sensor towards 2 mM GSH prepared in P.B. and 2 mM CDNB in 50% methanol in presence of 100 µL of 50 ppb each of a. fenobucarb b. temephos and c. dimethoate pesticide.

4.3.2.3 UV-Visible spectroscopic study

For absorbance measurements of the GSH-CDNB-GST mixture, 0.5 mM of each GSH and CDNB solutions were prepared in a quartz cuvette along with 20 μL GST solution at room temperature 30 ($\pm$ 0.05) ^{0}C.

4.3.2.4 Validation study

Method validation was checked by fortifying tomato samples with known amount of fenobucarb, temephos and dimethoate separately followed by extraction and clean up using solid phase extraction technique and finally transferring to 25% methanol before subjecting to CV analysis.

4.4 Results and Discussion

4.4.1 Cyclic voltammetric study of GSH-CDNB reaction in presence of GST

Figure 4.2 shows the cyclic voltammetric behavior of the GSH-CDNB reaction catalyzed by GST in absence of pesticides. Detail characterization of the various peaks has already been done in chapter 3. However, for completeness of this chapter, those have been introduced briefly here. Peak A at 0.30 V (current 18.51 μA, RSD 3.33%) is the one which is linked to the enzyme activity. This peak showed steady increase in current with consecutive CV run and become stable after 20 min. Peak B appeared from 0.60 V (current 10.86 μA, RSD 1.66%) onward is due to methanol oxidation, the one at 0.30 V is attributed to oxidation of newly formed complex or intermediate. A low intensity oxidation peak C appeared at 0.05 V (RSD 2.59%) in the reverse cycle is attributed to oxidation of :COH produced through dissociation of methanol. Low intensity reduction peak (D) appeared at 0.10 V (RSD 1.00%) is due to adsorption of H_2 at the platinum surface which normally shows up in the potential range from -0.23 to +0.20 V.

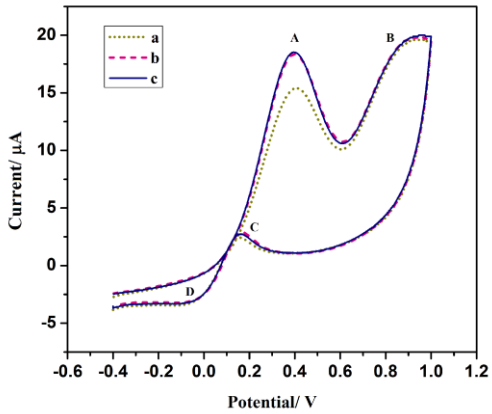

Figure 4.2. Cyclic Voltammograms recorded in a 1:1 volume mixture of 2 mM GSH in PB and 2 mM CDNB in 50% aqueous methanol in presence of 20 µL GST (0.02 mg) at scan rate 20 mV/s. Curve a through c recorded at 15, 20 and 25 minutes respectively.

4.4.2 Pesticides interaction study

Figure 4.3 shows the effect of pesticides on the CV peak at 0.30 V. Curve b in Figure 4.3 shows the CV of the reaction mixture in presence of 100 µL of 25 ppb fenobucarb solution after 30 minutes incubation. With addition of increasing amounts of pesticide solution in the mixture and with an incubation time of 30 minutes, the CV peak currents at 0.30 V get further reduced. Similar results were obtained in case of other two pesticides too. Catalytic activity of GST towards GSH-CDNB conjugation reaction was inhibited after exposure to all the three pesticides.

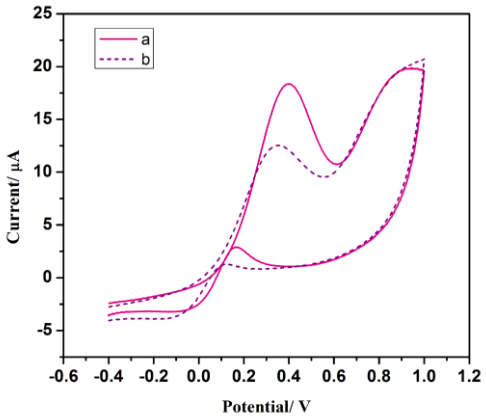

Figure 4.3. Cyclic Voltammograms recorded in a mixture of 2 mM GSH in PB and 2 mM CDNB in methanol (50%) in presence of 20 µL GST at scan rate 20 mV/s. a. in absence of pesticide b. in presence of 100 µL of 25 ppb fenobucarb.

4.4.3 UV-VIS study

UV-VIS spectroscopic method is used to corroborate the results of CV analysis. UV-visible spectra recorded two absorptions, one at the 250 nm and the other at 335 nm (curve a, Figure 4.4). Addition of these pesticides in the initial mixture suppresses the UV-VIS peak at 335 nm to different extent depending on the amount of the pesticide (curve b, Figure 4.4). It was also observed that the amount of inhibition was different for different pesticides.

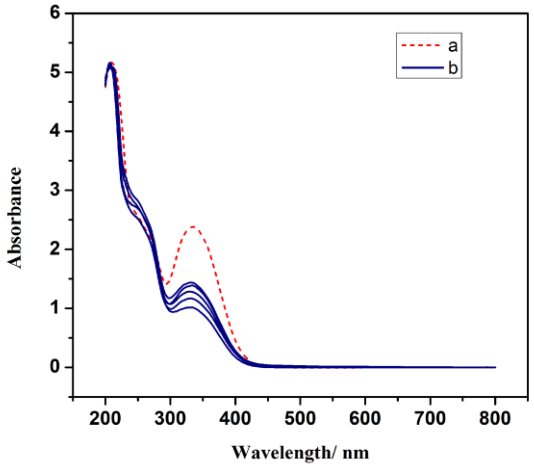

Figure 4.4. UV-visible spectra recorded in a solution mixture of GSH (0.5 mM), CDNB (0.5 mM), GST (20 µL) and PB-MeOH (25%) in absence of temephos (a) and in presence of different concentration of temephos (b).

4.4.4 Optimization of kinetic parameters

To obtain maximum signal from a biosensor, parameters such as the saturated substrate concentration, apparent Michaelis-Menten constant, maximum enzyme loading, maximum inhibition time and best working pH are to be determined. These have been done and detailed in chapter 3.

Saturated substrate concentration determined through Michaelis-Menten plot was found to be 2 mM in respect of both CDNB and GSH [37]. The Michaelis–Menten plots show two different regions of linear dependency. The first one appears at the low concentration region ranging from 0.5 to 2 mM and the second region is from 2 to 4 mM. Apparent Michaelis-Menten constant obtained through the Lineweaver-Burk plots were

0.11 $mmolL^{-1}$ and 0.12 $mmolL^{-1}$ at low concentration and 1.66 $mmolL^{-1}$ and 1.91 $mmolL^{-1}$ at high concentration respectively for GSH and CDNB.

Optimized GST amount was determined to be 0.02 mg/mL and the maximum incubation time of the reaction was found to be 30 minutes. Maximum inhibition time was found to vary with pesticides and their concentrations. With the upper limit of pesticide concentration the inhibition was maximum near zero minutes and the time versus absorbance plot in the UV-VIS spectra runs almost parallel to time axis, while in case of lower concentrations of the pesticides the plot runs below the reference (no pesticide mixture) curve in a divergent manner and becomes parallel to the reference curve at about 30 minutes, that is, at the equilibrium time of the GSH-CDNB reaction (Figure 4.5). This indicates a fast inhibition (near zero minutes) by the pesticides. However, since peak current comparison is to be done at 30 minutes, the incubation time of the reaction, therefore, a time of 30 minutes was considered as the maximum inhibition time in this study. The pH value for the reaction was maintained at 6.5 based on literature data [49].

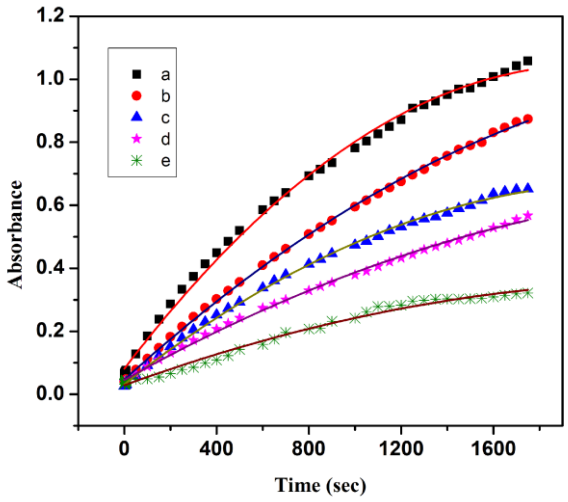

Figure 4.5. UV-VIS study of the inhibition by the three pesticides through time versus absorbance curve. a. Enzymatic reaction mixture in absence of pesticides and b-d. in presence of 25 ppb of dimethoate, temephos and fenobucarb respectively. Curve 'e' shows inhibition by 50 ppb of fenobucarb solution. Reaction mixture was prepared by mixing 0.5 mM GSH in PB (pH 6.5) with 0.5 mM CDNB in 50% MeOH along with 20 µL GST.

Figure 4.5 shows real time UV-VIS spectroscopic monitoring of the inhibition of the catalytic activity of GST by the three pesticides. It is seen that the inhibitory power of the three pesticides follow the order fenobucarb>temephos>dimethoate.

4.4.5 Quantification of fenobucarb, temephos and dimethoate

CV method was used to quantify the three pesticides. It was observed that when 50 ppb, 30 ppb and 25 ppb each of fenobucarb, temephos and dimethoate respectively was mixed initially to the solution mixture and kept for 30 min., the CV peak at 0.30 V almost disappeared. Fenobucarb, temephos and dimethoate solutions of concentration lower than 50 ppb, 30 ppb and 25 ppb respectively when mixed in separate reaction mixtures, found to suppress the peak current up to different extent which was proportional to concentration of the pesticides. Based on these observations, calibration curves were obtained by plotting percentage inhibition i.e., percent reduction in peak current versus pesticide concentration and was found to be linear. For determining the concentration based on peak current reduction, two solution mixtures containing GSH-CDNB-MeOH, PB and GST of exactly same composition were prepared, one of which served as the blank. The other was treated with fixed amount of pesticide and the difference in CV peak current in the two were noted, which was converted to percentage inhibition.

The percentage inhibition of the biocatalyst was calculated using Equation 4.1:

$$Inhibition(\%) = \frac{I_0 - I_i}{I_0} \times 100 \qquad (4.1)$$

Where, I_o and I_i are the CV peak currents obtained from the mixture before and after mixing pesticides. Triplicate measurements were made at each concentration of the three pesticides. Detection limits were determined by the lowest analyte concentration at which a measurable electrochemical change took place. Limit of detection (LOD) is considered as the ppb of the pesticide required for 10% inhibition and found to be 2, 4 and 5 ppb respectively for fenobucarb, temephos and dimethoate (Figure 4.6). The linear ranges for the three pesticides are found to be 2-50 ppb (y = 1.065x + 9.374; regression, R^2 =0.995), 4-30 ppb (y = 1.026x + 6.553; R^2 = 0.984) and 5-25 ppb (y = 0.980 + 5.484; R^2 = 0.993) respectively.

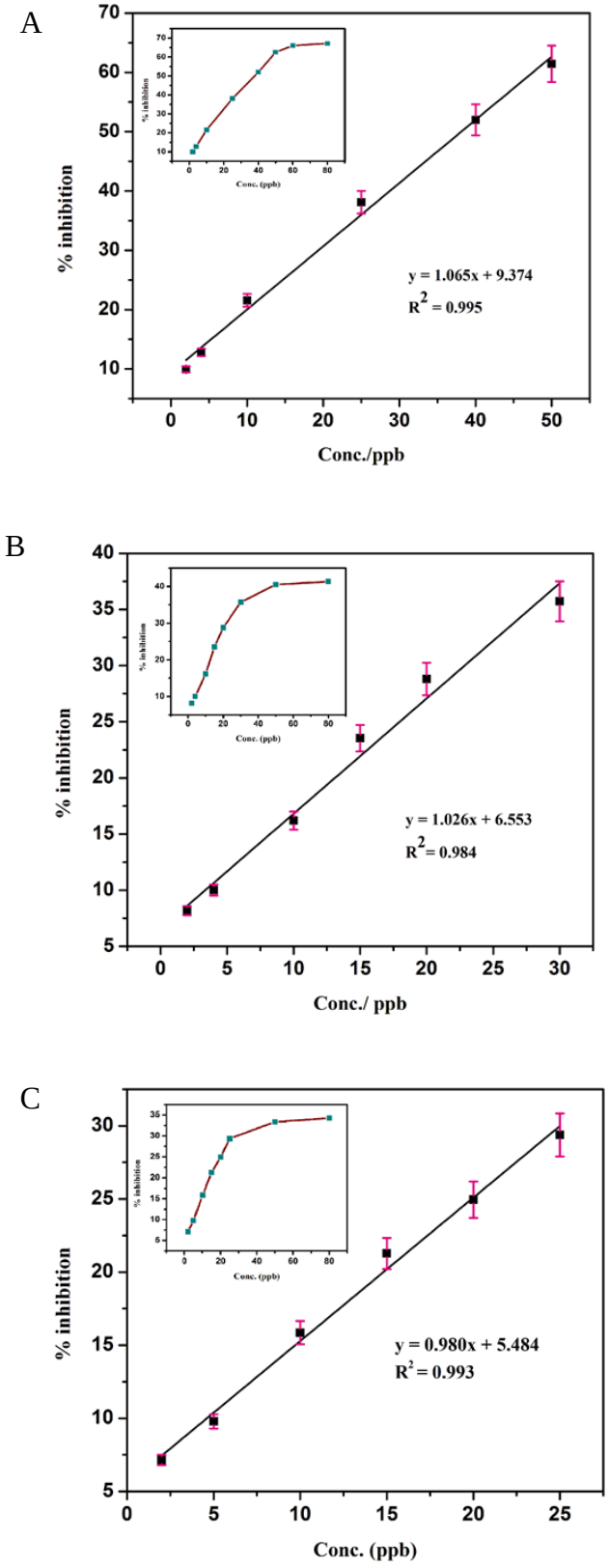

Figure 4.6. Calibration curves for the three pesticides studied. A. fenobucarb. B. temephos and C. dimethoate.

4.4.6 Method validation

Method validation was done by solid phase extraction method.

10 grams of chopped vegetable (tomato) was spiked with 5 mL of 50 ppb fenobucarb solution (prepared in acetonitrile) and then homogenized. 5 mL of acetonitrile was added and shaken in vortex shaker for 5 minutes, sonicated for 5 minutes and then centrifuged for 10 minutes at 2000 rpm. 5 mL of the supernatant was separated out and passed through a pre-conditioned (acetonitrile-hexane mixture in a 3:1 ratio) column of size 14 mm x 160 mm and packed with 5 g each of bondesil-NH_2 and carbon SPE bulk sorbent. Solution passing through the column was collected in 50 mL round bottom flask and evaporated to dryness at 40 ^{0}C and 200 mbar in rotary evaporator. The dry residue was reconstituted in mixture of 4 mL methanol and 1 mL dichloromethane and evaporated again to about 1 mL and then diluted to 5 mL by adding extra methanol. To 1 mL of this solution was added 1 mL each of of 3 mM GSH and CDNB and 20 μL GST. Percentage inhibition in peak current calculated and pesticide amount determined with the help of the calibration curve. The whole process was repeated thrice to get triplicate results. The recovery was found to be 97% (RSD 2.76%). Same procedure for validation study was applied for temephos and dimehoate also. Recovery for temephos and dimethoate were found to be 90% (RSD 1.88%) and 83% (RSD 4.80%) respectively.

4.4.7 Comparison of the method with other methods

Table 4.1 shows a comparison of our method with the other methods available in literature. The table reveals that the detection limits of all the four pesticides obtained by our method is within the range of those reported methods. It is evident from the table that for the two pesticides fenobucarb and temephos no reliable biosensing method other than our one available in literature. It is the most promising aspect of the present method.

Table 4.1: Comparison of the present method with other methods:

Sl. No.	Pesticide	Method of detection	LOD	Linear range	Ref.
1.	Fenobucarb	Flow Amperometry	2 ppb	-	[55]
2.	–do-	Liquid chromatography–tandem mass spectrometry	1.5 ppb	-	[56]
3.	–do-	Gas chromatography/Mass spectrometry	4 ppb	-	[57]
4.	-do-	GST biosensor	2 ppb	2-50 ppb	This work
5.	Temephos	Liquid Chromatography/Diode Array	9 ppb	-	[58]
6.	–do-	-do-	0.02-0.2 ppb	-	[59]
7.	–do-	Liquid chromatography–ion trap-mass spectrometry	-	-	[60]
8.	–do-	Thermospray liquid chromatography-mass spectrometry	0.038 ppb	-	[61]
9.	-do-	GST biosensor	4 ppb	4-30 ppb	This work
10.	Dimethoate	Acetylcholinesterase biosensor	0.4 ppb	2.29-5.5x10^2 ppb	[62]
11.	–do-	-do-	2.29 ppb	11.45-22 ppb	[63]
12.	-do-	-do-	389 ppb	-	[64]
13.	-do-	GST biosensor	5 ppb	5-25 ppb	This work
14. *	Cypermethrin	Surface plasmon resonance	0.5 ppb	-	[65]
15.	–do-	Nucleic acid sensor	2.5 ppb	2.5–2x10^3 ppb	[66]
16.	–do-	Enzyme-linked immunosorbent assay	0.5 ppb	-	[67]
17.	–do-	DNA biosensor	2.5 ppb	-	[68]
18.	-do-	*GST biosensor	2 ppb	2-25 ppb	[37]

*Cypermethrin was analyzed by the same method summarized in chapter 3(Reference 37)

4.5 Conclusion

In this work we have applied the newly developed [37] bio-electrochemical method for detection and quantification of organocarbamate and organothiophosphate pesticides taking fenobucarb, temephos and dimethoate as typical examples. The method uses the GST catalyzed *in vitro* detoxification reaction between glutathione and 1-chloro-2,4-dinitrobenzene as the benchmark reaction and methanol as the electrolyte. The work has proved that the newly developed method is effective for detection and quantification of three classes of pesticides, the pyrethroid, the organocarbamate and the organothiocarbamate class. The effectiveness of the method to the former class was demonstrated in our previous chapter [37], while the effectiveness of the later classes is evident from the present work. Both fenobucarb and temephos are water insoluble pesticides. Successful application of the method to detect these two water insoluble pesticides as well as its applicability to different classes of pesticides has proved the versatility of the method. Moreover, since methanol is used as the medium during analysis, the method become more practicable, because, now the bioanalysis of pesticides no longer restricted only to the laboratory test samples prepared in buffer medium or to very diluted field samples. Detection limits of the selected pesticides obtained by the present method are within the range of other reported methods (Table 4.1).

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