# Development of Novel Biosensing Micro Techniques for Analysis of Pesticide Residues and Heavy Metals in Water and Milk

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by

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Under the Supervision of

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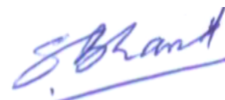
# BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
# PILANI (RAJASTHAN)

## CERTIFICATE

This is to certify that the thesis entitled **"Development of Novel Biosensing Micro Techniques for Analysis of Pesticide Residues and Heavy Metals in Water and Milk"** which is submitted by **RUPESH KUMAR MISHRA** ID No. **2010PHXF437G** for award of Ph.D. degree of the Institute embodies original work done by him under my supervision.



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# ABSTRACT

The effects of human activities on environmental quality are both widespread and varied in the extent to which they disturb the ecosystem. Excessive contamination of various environmental component leads to pollution of the biosphere and eventually reaches humans via the food chain. The ecological balance thus gets disturbed primarily due to accumulation of toxic chemical and carcinogenic compounds. The main classes of pesticides that pose a serious problem are organophosphates (OPs) and carbamate (CM). While pesticides are associated with many adverse health effects, there is a lack of monitoring data on these contaminants. Traditional chromatographic methods are effective for the analysis of pesticides in the environment, but have limitations and prevent adequate monitoring. For immediate control of pollution and to facilitate early warning systems for contaminated samples, biosensor has emerged as choice of analytical method due to its capability for fast, sensitive and cost effective analysis (chapter 1).

Enzymatic methods have been promoted for many years as an alternative method for detection of these pesticides. The main enzymes utilized for pesticide residues analysis have been acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) alkaline phosphatase (ALP), phosphotriesterase (PTE), organophosphate hydrolase (OPH) etc. Special attention is paid to real samples such as water and milk taking into consideration of the aspects such as sample pretreatment, matrix effect and validation of biosensor measurments. Although OPs and CM have a relatively low persistence in the environment, there are a number of ways in which insecticides can reach to milk. Milk is a widely consumed commodity. These insecticides may originate from foodstuffs from plant materials that have been treated with insecticides or the use of insecticides directly on the animal or as hygienic treatments against insects in milk processing factories. To date, among the reported work on food analysis, milk analysis has still remained an analytical problem due to its complex nature. Regular adulteration and effect of pollution has led scientists to develop methods to analyze milk and its constituents on routine basis (chapter 1).

In chapter 2, development of a rapid, high-sensitivity enzyme assay for the determination of OPs residue in milk is presented using chemiluminescence (CL) technique. The assay is based on the inhibition of enzyme BuChE for quantification of OPs residue in milk. BuChE was stabilized

and preloaded in 384 well plates at 30 ºC. The assay permits rapid determination of OPs in milk within 12 min including incubation step. The enzyme assay was tested for individual and mixture of OPs in milk, such as Methyl Paraoxon (MPOx), Methyl Parathion (MP) and Malathion (MT) to evaluate their synergistic effect on BuChE inhibition. Good linearity was obtained in the range 0.005-50 µg $L^{-1}$ for MPOx and 0.5-1000 µg $L^{-1}$ for MP as well as MT in milk. The proposed method can facilitate rapid screening of OPs in milk samples using 384 well plate formats with further miniaturization presented in 1536 well plates.

In chapter 3, a novel micro well chip based biosensing platform was developed for off line analysis of OPs using AChE. The work presented in this chapter is a part of collaborative work between Biosensor lab, BITS, Pilani - K. K. Birla Goa Campus and CARE, IIT Delhi. Chip fabrication was done at IIT Delhi whereas; experimental work for on chip assay development was carried out at Biosensor lab. The determination of potent analytes, MPOx, Ethyl paraoxon (EPOx), MP and carbofuran (CF) were performed on developed biochip. A novel stabilization protocol was developed for immobilization of AChE on chip. The biochip could be stable at room temperature for more than 3 months without significant loss in enzymatic activity. The combinations of chip based devices (biochips) for the detection of OPs have a huge impact on milk analysis and also environmental analysis. The dynamic range studied for all the pesticides were in the range 0.005-200 µg $L^{-1}$. The observed $IC_{50}$ in µg $L^{-1}$ for these four analytes are in the order; MPOx (0.3039) < EPOx (0.6002) < MP (7.9174) < CF (194). For MPOx, the limit of detection (LOD) was found to be 0.001 µg $L^{-1}$ whereas, for EPOx, MP and CF the LODs were found 0.003, 0.01 and 0.0215 µg $L^{-1}$ respectively. The present invention can be applied in dairy industries and milk collection centers as a screening method for point of care testing.

In chapter 4, a novel on chip inhibition assay for mercury ($Hg^{2+}$) analysis in water has been developed. Glucose oxidase (GOx) was immobilized on nanostructured aluminium oxide formed on oxidized silicon. Aluminium nano structures have been demonstrated as simple and cost effective platform for $Hg^{2+}$ biosensing. The device facilitates high-throughput analysis of $Hg^{2+}$ in 20 min using CL technique. The device is simple, easy to operate, rugged and can be further extended for analysis of similarly toxic heavy metals (HMs) such as lead ($Pb^{2+}$) and cadmium ($Cd^{2+}$).

In chapter 5, a simple and practical visual detection method has also been developed and described for the quantitative in-field analysis of OPs and CM residues in drinking water. The method utilizes an ALP immobilized silica ($SiO_2$) micro-column. The inhibition is visualized on the micro-column using 0.09 IU ALP and 0.143 mM para nitrophenylphosphate (pNPP) in 60 µL solution. The inhibition caused by OPs and CB was studied in the range 0.01-500 µg $L^{-1}$ and was visually distinct. Inhibition of ALP activity was quantified for MPOx, EPOx, MP and CF in drinking water. The assay was also tested in the presence of metal ions $Hg^{2+}$, $Cd^{2+}$ and $Pb^{2+}$. It was observed that the developed method is free from low level interferences of metal ions. The method can easily quantitate residual pesticide concentration as low as 0.1 µg $L^{-1}$ with relative standard deviation (RSD) of 1.6%, thus meeting the quality criteria set by European Union (EU) and Bureau of Indian standards (BIS) for drinking water. The developed visual detection has been cross validated using approved standard technique such as LC-MS/MS.

In chapter 6, a novel automated flow based biosensor was developed for online monitoring of OPs in milk. This work was carried out in IMAGES, University of Perpignan, France, under the supervision of Prof. J.-L. MARTY. The biosensor employs genetically modified AChE enzymes B394, B4 and wild type B131 to achieve higher sensitivity and stability. These tailor made enzymes can enhance the sensitivity significantly, thus have been adopted. The biosensor was based on a screen printed carbon electrode (SPE) which was integrated into a flow cell and measurments were done using amperometric technique. Enzymes were immobilized on cobalt (II) phthalocyanine (CoPC) modified electrodes by entrapment in a photocrosslinkable polymer (PVA-AWP). The automated flow-based biosensor was successfully used to quantify three OPs, chlorpyriphos-oxon (CPO), EPOx and malaoxon (MAO) in milk samples. The total analysis time was less than 15 min. Initially, the biosensor performance was tested in phosphate buffer solution (PBS) using B394, B131 and B4 biosensors. The best detection limits were obtained with B394; therefore, this biosensor was used to produce calibration data in milk with three OPs in the concentration range of $5\times10^{-6}$ to $5\times10^{-12}$ M. The LOD obtained in milk for CPO, EPOx and MAO were $5\times10^{-12}$ M, $5\times10^{-9}$ M and $5\times10^{-10}$ M, respectively, with a regression coefficient $r^2$=0.9910. The automated flow-based biosensor successfully quantified the OPs in different fat containing milk samples. There were no false positives or false negatives observed for the

analytical figures of merit for the constructed biosensors. This method is inexpensive, sensitive, portable, non-invasive and provides real-time results.

Pesticides have tendency to degrade at natural conditions, therefore, the study was extended for on-line monitoring of OPs detoxification using PTE enzyme. The work presents the development of cost-effective column based biosensor for detoxification of OPs in water and milk. PTE was immobilized on an activated Sepharose 4B via covalent coupling using an Omnifit glass column. Three different OPs, EPOx, MAO and CPO were spiked in water and milk to test the detoxification of OPs. Mixtures of these pesticides were also tested to check the cumulative detoxification in the real samples. The efficiency of detoxification was evaluated using a highly sensitive AChE B394 biosensor based flow system. The column conditions were optimized for the detoxification studied. The method was shown to be promising when we tested real milk samples spiked with OPs. Detoxification obtained in milk was up to 86% whereas in water, 100% detoxification was obtained. This analytical system can provide rapid detection as well as detoxification of highly toxic OPs in water and food matrices such as milk.

In brief, bioassay and biosensor for OPs and CM have been successfully developed using optical (CL and colorimetry) as well as electrochemical (amperometry) techniques. The major highlights of this work includes development of (i) bioassay and its miniaturization in 1536 well plate using novel stabilization protocol (ii) biochip device for screening of OPs at milk collection centre (iii) affordable visual bioassay for safe drinking water and (iv) highly sensitive assay for OPs using genetically engineered enzymes and SPEs (v) automated flow based biosensor for on line monitoring of OPs detection and detoxification.

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# List of Abbreviation

| Abbreviation | Description |
|---|---|
| AChCl | Acetylcholine Chloride |
| AChE | Acetylcholinesterase |
| ALP | Alkaline Phosphatase |
| ANN | Artificial Neural Network |
| ATChCl | Acetylthiocholine Chloride |
| ATChI | Acetylthiocholine Iodide |
| BDL | Below Detection Limit |
| BIS | Bureau of Indian Standards |
| BuChCl | Butyrylcholine Chloride |
| BuChE | Butyrylcholinesterase |
| BSA | Bovine Serum Albumin |
| CCD | Charge-Coupled Device |
| CFV | Chlorfenvinfos |
| ChCl | Choline Chloride |
| ChE | Cholinesterase |
| ChOx | Choline Oxidase |
| CF | Carbofuran |
| CL | Chemiluminescence |
| CM | Carbamate |
| CoPC | Cobalt (II) phthalocyanine |
| CPO | Chlorpyrifos-Oxon |

| | |
|---|---|
| CV | Coefficient of Variation |
| Dm | *Drosophila melanogaster* |
| DPL | Dip Pen Lithography |
| DTNB | 5,5′-dithio bis (2-nitrobenzoic acid) |
| DW | Drinking Water |
| EB | Electron Beam |
| *E. coli* | *Escherichia coli* |
| EDC | 1-ethyl-3(3/-dimethylaminopropyl) carbodiimide, HCl |
| ELISA | Enzyme-Linked Immuno Sorbent Assay |
| EU | European Union |
| FIA | Flow Injection Analysis |
| FIB | Focused Ion Beam |
| FL | Fluorescence |
| FSSAI | Food Safety and Standards Authority of India |
| GB | Glycine Buffer |
| GOx | Glucose Oxidase |
| GW | Ground Water |
| $H_2O_2$ | Hydrogen Peroxide |
| HEC | Hydroxyethyl Cellulose |
| Hg | Mercury |
| HM | Heavy Metals |
| HRP | Horse Radish Peroxidase |
| IC | Inhibitory Concentration |
| $IC_{50}$ | Half Maximum Inhibitory Concentration |

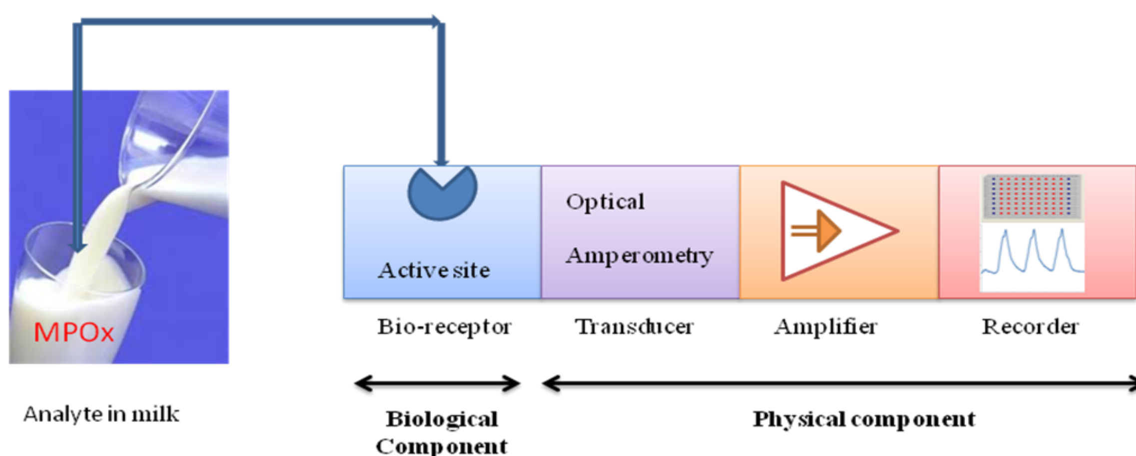
| | |
|---|---|
| I% | Inhibition Percentage |
| IPA | Indophenyl Acetate |
| IUPAC | International Union of Pure and Applied Chemistry |
| IU | International Unit |
| $K_M$ | Michaelis Menten Constant |
| LC-MS | Liquid Chromatography–Mass Spectrometry |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| MIP | Molecularly Imprinted Polymer |
| MAO | Malaoxon |
| MCP | Micro Contact Printing |
| $MgCl_2$ | Magnesium Chloride |
| MP | Methyl Parathion |
| MPOx | Methyl Paraoxon |
| MRL | Maximum Residual Limit |
| MT | Malathion |
| MUA | 11-Mercaptoundecanoic Acid |
| NHS | N-Hydroxysuccinimide |
| OPH | Organophosphate Hydrolase |
| OP | Organophosphate |
| P | Product |
| PB | Phosphate Buffer |
| PMT | Photomultiplier Tube |
| pNPP | *p*-Nitro Phenyl Phosphate |

| | |
|---|---|
| POx | Paraoxon |
| PTE | Phosphotriesterase |
| PVA | Poly Vinyl Alcohol |
| RSD | Relative Standard Deviation |
| S | Substrate |
| SAM | Self Assembled Monolayer |
| SD | Standard Deviation |
| SEM | Scanning Electron Micrograph |
| $SiO_2$ | Silicon di Oxide |
| SPE | Screen Printed Electrodes |
| SPL | Scanning Probe Lithography |
| SPR | Surface Plasmon Resonance |
| TCh | Thiocholine |
| TCNQ | Tetracyanoquinodimethane |
| TW | Tap Water |
| UV-Vis | Ultra Violet Visible Spectroscopy |
| WHO | World Health Organization |

# CHAPTER 1

# Introduction

**Thesis overview:** In this thesis, we have adopted novel strategy for highly sensitive detection of OPs in milk using microwell plate (highthroughput analysis) and microfabricated biochips. The biochip is fabricated using microfabrication technology and used as a biosensing platform. For highthroughput measurments in micro well plates, a novel stabilization protocol was developed for BuChE enzyme using a novel combination of sugars. Genetically engineered enzymes are more stable as the modifications are made to achieve more sensitivity and stability. We have used genetically engineered AChE from *Drosophila melanogaster (Dm),* which provided ultra sensitivity for OPs screening in milk. The genetically engineered variant of Dm showed 10 times differences in LOD and $IC_{50}$ values as against wild type enzyme. By using the genetically engineered enzymes, we could overcome the poor sensitivity and stability provided by native enzymes. The research work in this thesis has been carried out using first (bioassay using microplate and biochip) and second (automated flow based biosensor using amperometry) generation of biosensor.




Schematic representation of milk analysis using optical and amperometric techniques

## 1.1 Introduction to Biosensor

A rapid proliferation of biosensors and their diversity has led to a lack of rigour in defining their performance criteria. Although each biosensor can only truly be evaluated for a particular application, it is still useful to examine how standard protocols for performance criteria may be defined in accordance with standard IUPAC (International Union of Pure and Applied Chemistry) protocols or definitions.

In accord with IUPAC definition "a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals (Compendium of Chemical Terminology, 2nd edition (the "Gold Book")". The transducer converts the biochemical signal, into a quantifiable electronic signal which is proportional to the concentration of a specific analyte or group of analytes present. Biosensors are characterized by a high level of specificity generated by the biocomponents, which specifically reacts with a given analyte or substrate. The combination of this specificity, with a sensitive transducer, gives to biosensors their unique and unrivalled characteristics for the detection of a variety of analytes, even when they occur in complex matrices. The different components of a biosensor are shown in Figure 1.1.

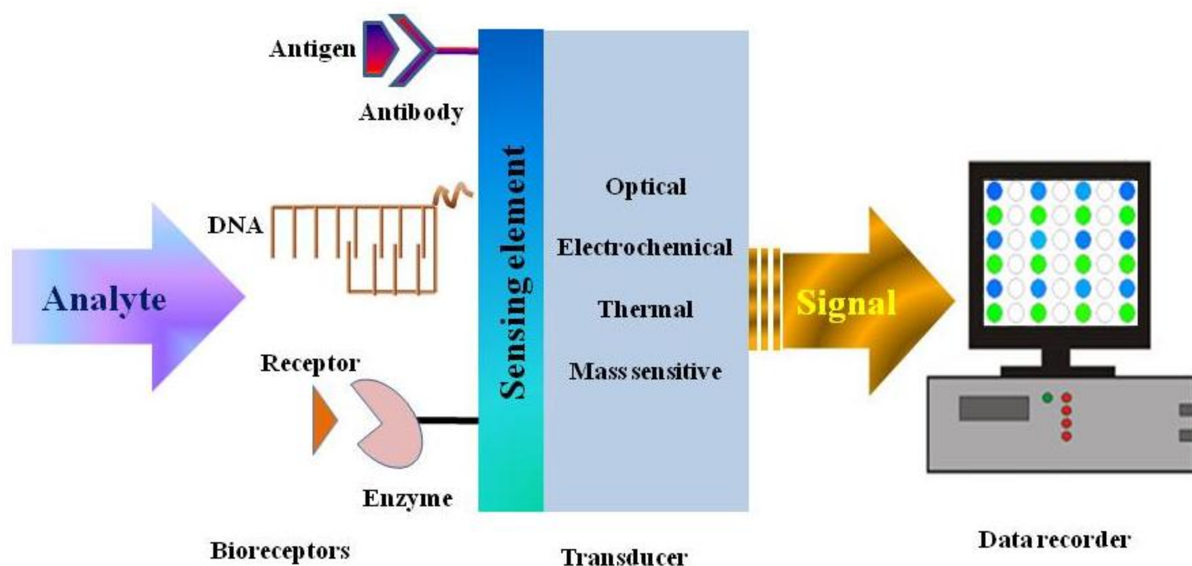



**Figure 1.1** A schematic representation of biosensor components.

### 1.1.1 Bio-recognition elements in biosensors

- **Biocatalysis-Biosensors (Enzymatic Biosensor):** These biosensors use enzymes as their biosensing element.
- **Bioaffinity-Biosensors (Antibody based Biosensor):** These biosensors employ interaction between an antibody and an antigen.
- **Cell-Biosensors (Whole cell Biosensor):** These biosensors utilize microorganisms as their biosensing element.
- **Aptamer (DNA/RNA) based Biosensor:** Aptamers are nucleic acid ligands that can be generated against amino acids, drugs, proteins and other molecules.
- **Molecularly imprinted polymer (MIP)**: MIP is a polymer with a memory of the shape and the functional groups of a template molecule.

## 1.2 Transducers in biosensor

Biosensors can also be classified according to their method of signal transduction. The transducer is an important component in a biosensor through which the measurement of the target analyte is achieved by selective interaction of a bio-molecule and analyte into a quantifiable signal. Based on the type of transducers used, biosensors may be classified as optical, electrochemical, thermal, and piezoelectric. This thesis is focused on two transducers which are described here:

**1.2.1 Optical transducer**: An optical transducer converts an optical signal into an electrical signal. It does this by generating an electrical current proportional to the intensity of incident optical radiation. Analyte undergoing a change in their optical properties, such as absorption, fluorescence, luminescence, surface plasmon resonance (SPR) or reflectance. Optical transducers represent the largest and fastest growing area in biosensor technology. Photomultiplier tubes (PMTs) and Charge-coupled devices (CCDs) are among the most extensively reported transducers for optical biosensors (Chouhan et al., 2006; Chouhan et al., 2010). PMTs are extremely sensitive detectors of light in the ultraviolet, visible and near-infrared ranges of the electromagnetic spectrum.

Numerous review articles were described about enzyme-based optical biosensors (Amine et al., 2006; Borisov et al., 2008; Ligler, 2009; Verma et al., 2005; Weetall et al., 2004). Among the various optical techniques, CL is reported to be promising technique with high sensitivity (Gracia et al., 2005). It involves production of light from a chemical reaction. In this thesis, we adopted CL technique as well as amperometric technique for OPs analysis. Luminol is used for CL measurments. Luminol ($C_8H_7N_3O_2$) oxidation leads to the formation of an aminophthalate ion in an excited state, which emits light when returning to the ground state. Horseradish peroxidase (HRP) is able to catalyze the chemiluminescent reaction of luminol in the presence of hydrogen peroxide ($H_2O_2$). This reaction proceeds at pH value 8-8.5. In the course of a series of enzymatic step in presence of luminol and $H_2O_2$, HRP is successively converted into intermediate complex. Enzymatic steps produce luminol radicals and generate luminol hydro peroxide. Application of CL based biosensor has been successfully reported for inhibitors/pesticide detection (Ayyagari et al., 1995; Chouhan et al., 2010; Guardigli et al., 2005). An inhibitor can be defined as “A number of substances may cause a reduction in the rate of an enzyme catalyzed reaction”.

**1.2.2 Electrochemical transducer**: The second and most commonly used transducers are electrochemical. Based on their operating principle, the electrochemical biosensors can employ potentiometric, amperometric and impedimetric transducers converting the chemical information into a measurable amperometric signal. Amperometric biosensors constitute the major share in electrochemical biosensors for pesticides. It functions by the production of a current when a potential is applied between two electrodes. The working electrode of the amperometric biosensor is usually either a noble metal or a screen-printed layer (surface, printed with carbon paste) covered by the biorecognition component (Lin et al., 2004). At present, there are many proposed and already commercialized devices based on the biosensor principle including those for pathogens and toxins, some even based on a multi-channel configuration (Pohanka et al., 2007; Pohanka, 2009).

## 1.3 Classification of biosensors

Biosensors can be classified into three generations according to the degree of integration of the separate components, i.e. the method of attachment of the bio recognition molecule (bio-receptor) to the base indicator (transducer) element. In the first generation of biosensors, the bio-receptor is retained in the vicinity of the base sensor behind a dialysis membrane, while in the subsequent generations; immobilization is achieved via cross-linking reagents or bi-functional reagents at a suitably modified transducer interface or by incorporation into a polymer matrix at the transduction surface. In the second generation, the individual components remain essentially distinct, while in the third generation, the bio-receptor molecule becomes an integral part of the base sensing element. It is in the second and third generations of these families that the major development effort can now be seen (Wilson et al., 2005).

## 1.4 Enzyme based biosensor

Enzymes were among the first recognition elements to be incorporated into biosensors. The first application of an enzyme in chemistry was carried out by Clark and Lyons in 1962 (Webb, 1966). Advantage of enzyme-based biosensor is to modify catalytic properties or substrate specificity by genetic engineering. The limitation is the lack of specificity in differentiating among compounds of similar classes. Two different approaches have been used for determining an analyte concentration by use of an enzymatic biosensor (i) if the enzyme metabolizes the analyte, the analyte concentration can be determined by measuring the enzymatic product, and (ii) if the analyte inhibits the enzyme, the decrease of enzymatic product can be measured and correlated to the analyte concentration. In the latter case, the device is designated as a ‘‘biosensor based on enzyme inhibition’’ or ‘‘inhibition biosensor.’’ The inhibition based biosensor comprises a major portion of the enzyme based biosensors (Wilkinson et al., 1971).

**1.4.1 Principle of enzyme inhibition:** Among the different biosensors employed in environmental analysis (Bucur et al., 2004; Sotiropoulou et al., 2005), a leading role is played by the inhibition based biosensors (Andreescu et al., 2001; Crew et al., 2004; Dzyadevych et al., 2005). The principle of operation of these biosensors is based on the interaction that occurs

between specific chemical and biological agents (inhibitors) present in the sample and the biocatalyst immobilized on the biosensor itself. The binding of an inhibitor can stop a substrate from entering the enzyme's active site or hinder enzyme catalysis. The mechanism of enzyme-substrate interaction and inhibition is shown in Figure 1.2. In enzyme inhibition based biosensors, the measurement of the target analyte is carried out measuring the enzyme activity before and after exposure of the biosensor at the target analyte (Arduini et al., 2009). The percentage of inhibition is calculated using the equation 1.1.

$$I\% = (A_0 - A_I)/ A_0 \times 100 \qquad (1.1)$$

Where

$I\%$ = percentage of inhibition, $A_0$ = the enzyme activity before exposure at the analyte;

$A_I$ = the enzyme activity after exposure at the analyte.

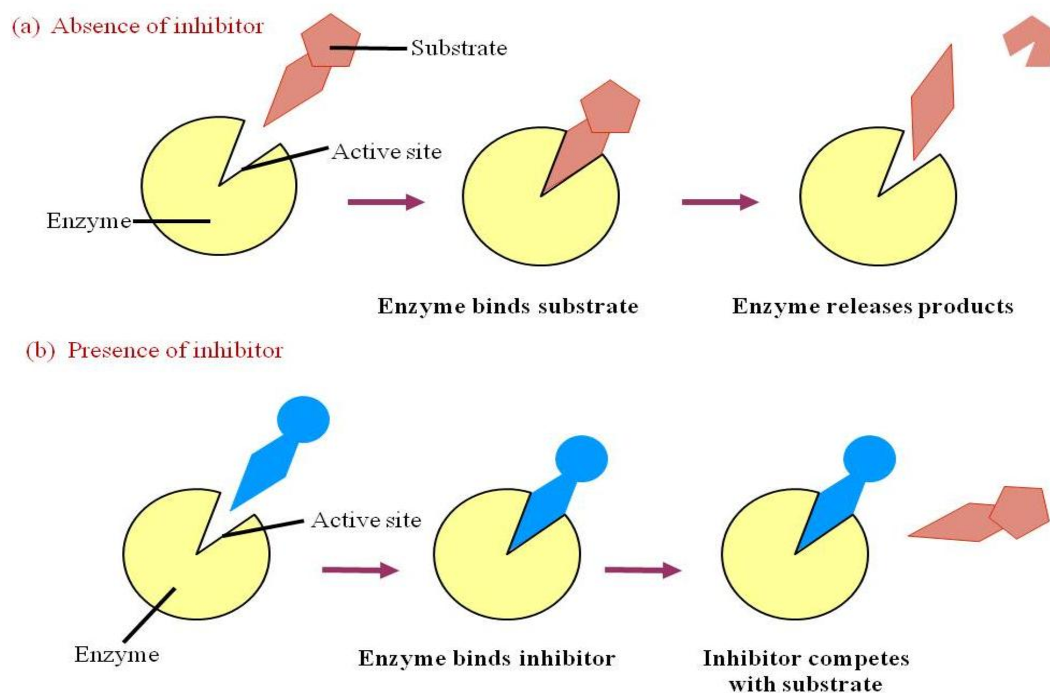



**Figure 1.2** A schematic representation of the lock and key mechanism **(a)** Enzyme-substrate reaction to produce product **(b)** enzyme inhibitor reaction, where inhibitor locks the active site and does not allow substrate to react.

The percentage of inhibition is correlated with the concentration of target analyte, hence, possible to evaluate the unknown concentration of the analyte. The choice of enzyme/analyte system is based on the fact that these toxic analytes inhibit normal enzyme function. The enzyme inhibitor reaction is often complex and has been reviewed in the current literature (Arduini et al., 2010; Luque de Castro and Herrera, 2003).

### 1.5 Factors affecting performance of enzyme inhibition

Factors influencing the enzyme activity such as enzyme concentration and immobilization, substrate selection and substrate concentration, pH, temperature, ionic strength of buffer or reaction medium, incubation time for inhibition were extensively studied by researchers (Amine et al., 2006; Bucur et al., 2006). The compiled notes about each parameter are as follows: temperature and pH, ionic strength, enzyme and substrate concentrations, incubation time, immobilization of enzyme, substrate selection and substrate concentration.

#### 1.5.1 Temperature and pH

Temperature is a measure of the kinetic energy (energy of motion) of molecules, however, when the temperature increases beyond a certain point, the increased energy begins to cause disruptions in the tertiary structure of the enzyme; denaturation is occuring. Temperature that produces maximum activity for an enzyme is known as the optimum temperature for that enzyme (Arduini et al., 2009). Enzymes are also affected by changes in pH. The pH value, where the enzyme is most active is known as the optimum pH.

#### 1.5.2 Ionic strength, enzyme and substrate concentration

The ionic strength is an important parameter affecting enzyme activity because the reaction rate depends on the movement of charged molecules relative to each other. It is important to control the ionic strength of solutions in parallel with the control of pH. Optimization of enzyme is an important parameter for enzyme-substrate as well as for inhibition studies. Active site is an area on the surface of the protein site where the reaction occurs. At low enzyme concentration, there is a great competition for the active sites and the rate of reaction is low. As the enzyme concentration increases, there are more active sites, and the reaction can proceed at a faster rate.

If amount of the enzyme is kept constant and the substrate concentration is gradually increased, the reaction velocity will increase until it reaches a maximum value or point. After this point, increase in substrate concentration will not increase the enzyme activity. For inhibition assay, optimization of substrate concentration is a critical factor. Among the reported work, Kok et al., concluded that the inhibition level increases with increase in the substrate concentration and they worked with a saturating substrate concentration for inhibition (Kok et al., 2002).

### 1.5.3 Incubation time

Incubation time for inhibition is one of the most influencing parameters in enzyme inhibition assays. The improved sensitivity and short response time represent important advantages of inhibition based biosensors.

### 1.5.4 Immobilization of biomolecules

On the basic of the numerous studies reported earlier, the enzyme immobilization is one of the most important steps involved in the biosensor design. Several different approaches for enzyme immobilization have been reported in the literature (Chauhan et al., 2011; Gouda et al., 2002). Recently, different paper based techniques were also used for coupling of AChE (Hossain et al., 2009; Pohanka et al., 2010). A novel approach was reported for immobilization of whole cells of *Sphingomonas* bacteria onto the surface of the microwell plate for pesticide detection using gluteraldehyde as the cross-linker (Kumar and D'Souza, 2010). Immobilization of AChE on SPE has been also reported extensively (Alonso et al., 2012; Cortina et al., 2008; Ivanov et al., 2003; Zhang et al., 2005).

### 1.5.5 Substrate selection and substrate concentration

The substrate concentration can affect the degree of enzyme inhibition. Kok et al. reported that the enzyme inhibition increases with increase in the substrate concentration. They have worked with a higher substrate concentration for pesticide inhibition (Kok et al., 2002). Joshi et al., have used a concentration of acetylthiocholine (ATCh) two times higher than the apparent $K_M$ for the determination of the maximum activity of AChE before and after the inhibition by the paraoxon (POx) which was selected as model pesticide (Joshi et al., 2004). Generally, acetylcholine is used

as a substrate for AChE enzymes. Other substrates were also tried in the literature such as Butyrylcholine chloride (BuChCl) and indophenyl acetate (IPA) (Hossain et al., 2009).

### 1.6 Analytical figures of merits and performance criteria of biosensor

A comparative account of analytical features of merit for enzyme biosensors as well as enzyme inhibition biosensors is summarized in Table 1.1

**Table 1.1** Analytical figures of merits for biosensor

| Parameter | Biosensor | Enzyme inhibition biosensor |
|---|---|---|
| Dynamic range | Concentration range over which signal varies in a monotonic manner with analyte concentration. | Concentration range corresponding to I% between 20 and 80% |
| Linearity | The relationship of signal to amount of analyte over a range of concentration of analyte with a constant proportionality factor. | Fall within I% of 20 to 80. |
| Limit of detection, LOD | Lowest concentration of the analyte which can be measured at a specific confidence level. | Preferably I% ≅ 10. |
| Limit of quantification, LOQ | The lowest concentration level at which a measurement is quantitatively meaningful. Normally more than LOD. | Preferably I% > 10. |
| Limit of linearity | The upper limit of quantification where calibration curve tends to become non-linear. | Normally up to I% ≅ 70. Sometimes up to 100% also. |
| Reaction time | Time for enzyme –substrate reaction. | Total time of reaction between enzyme- inhibitor and enzyme -substrate reaction. |

The performance of a biosensor is evaluated on the basis of various parameters. Parameters significant in evaluating biosensor performance are listed below in the Table 1.2 (Theavenot et al., 1999).

**Table 1.2** Performance criteria of a biosensor

| Features | Description |
| --- | --- |
| **Specificity** | Ability of a method to detect one element in the presence of another element. |
| **Selectivity**: | The extent to which other substances interfere with the determination of a substance according to a given procedure or the term selectivity is used to describe the relative rates of two or more competing reactions on a catalyst. |
| **Sensitivity** | The slope of the calibration curve. If the curve is in fact a 'curve', rather than a straight line, then of course sensitivity will be a function of analyte concentration or amount. |
| **Stability** | The operational stability of a biosensor may vary with sensor geometry, method of preparation, as well as the applied enzyme or transducer. |
| **Precision** | Measure of instrument reproducibility that is the ability to obtain the same value with repeated measurements of a process variable. |
| **Response time** | Time which elapses, when there is a stepwise change in the quantity to be measured, between the moment when this change is produced and the moment when the indication reaches a value conventionally fixed at 90% of the final change in indication. |
| **Sample throughput** | The number of results that is produced by an instrument divided by time of operation. |
| **Reusability** | The ability of biosensors to use them most number of times with same efficiency. |

## 1.7 Enzyme inhibition based biosensor for pesticide residues analysis

### 1.7.1 OPs as a potent inhibitor

OPs contain a central phosphorus atom with a double bond to sulfur or oxygen, $R_1$ and $R_2$ groups that are either ethyl or methyl in structure, and a leaving group which is specific to the individual OPs. OP compounds which contains P=O moiety are more potent inhibitors of AChE as compare to compounds which contains P=S moiety. Also, organophosphorus esters containing the P=S moiety are less reactive and more stable to hydrolytic degradation than the corresponding P=O ester (Fukuto, 1990). Figure 1.3 shows the general structure of commonly used OP pesticides such as MP, CPO, MPOx, MT, MAO etc. is also presented in the Figure 1.3.

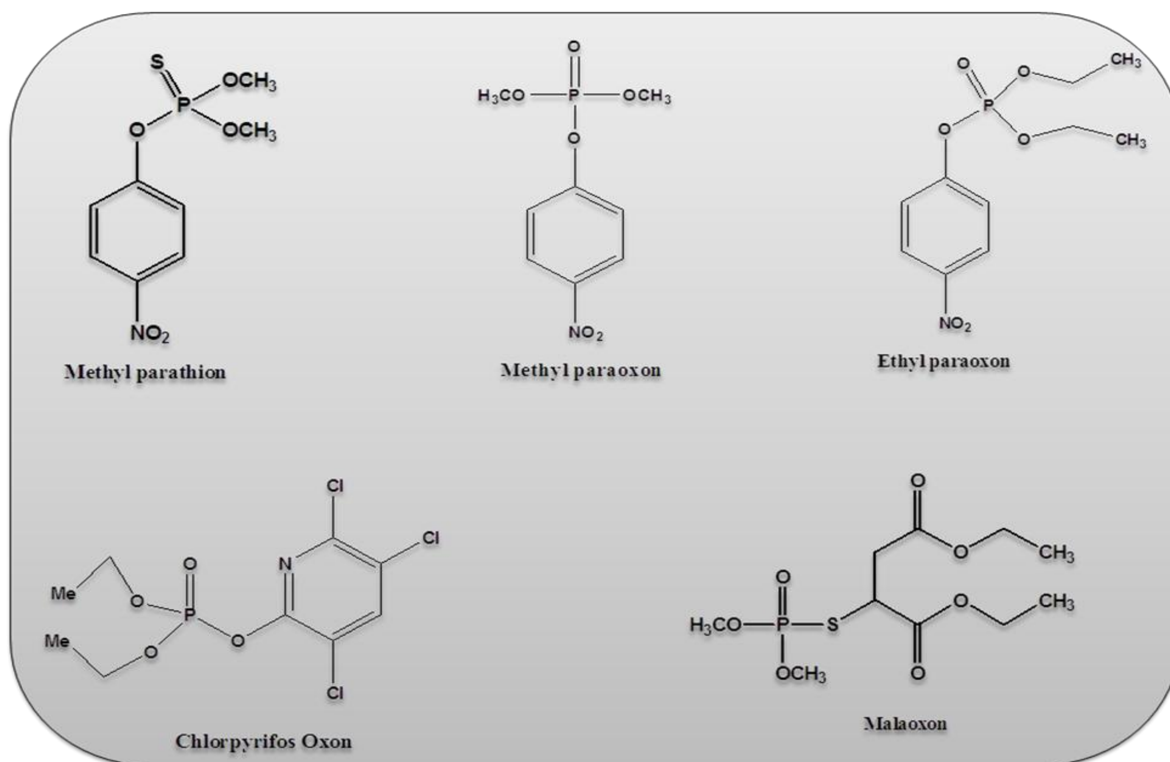



**Figure 1.3** Structures of some important OPs insecticides.

Many pesticides are designed to inhibit various enzymes such as AChE, BuChE, ALP, acid phosphatase, tyrosinase, OPH, aldehyde dehydrogenase and others were investigated for their ability to detect pesticides in water and other matrices such as soil, food and beverages. The most

commonly used enzymes for the design of biosensors are AChEs (50%) followed by BuChEs (11%). However, HRP, tyrosinase and urease represent 7% of the total enzymes used. Most of these enzymes have been incorporated into biosensors for this purpose (Amine et al., 2006).

### 1.7.2 Mechanism of cholinesterase (AChE/BuChE) inhibition

Cholinesterase comprises the major class of enzyme used for OPs quantification. When OPs come in contact with AChE/BuChE, the serine residue in the active site of the enzyme is phosphorylated. The mechanism of inhibition is as follows: the hydroxyl group on the serine residue acts as an electrophile which attacks the nucleophilic phosphorus. The phosphorylated enzyme is highly stable and the hydrolysis of acetylcholine is blocked. The serine hydroxyl group, blocked by a phosphoryl moiety, is no longer able to participate in the hydrolysis of acetylcholine. Figure 1.4 represents the phosphorylation of AChE by POx. It acts as a strong inhibitor and inhibits the activity of enzyme, due to the high reactivity of the compound (Fukuto, 1990).

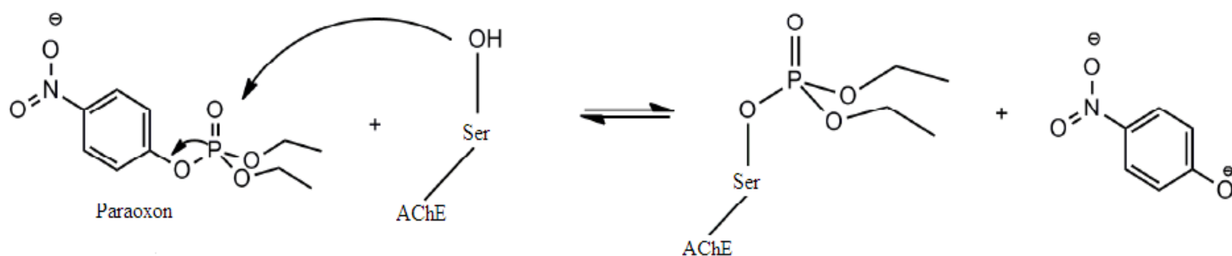



**Figure 1.4** Mechanism of cholinesterase inhibition by paraoxon (Fukuto, 1990).

AChE from different sources may vary in their reactivity to pesticide inhibition and their sensitivity (limit of detection). Recently, Dyk and Pletschke summarized the use of enzyme for the detection of organochlorine, OPs and CM in the environment (Dyk and Pletschke, 2011). Marques et al. compared various wild-type AChE as well as some genetically modified enzymes (Marques et al., 2004). ALP is another class of enzyme exploited for OP determination. ALP has not been used as extensively as AChE for the detection of pesticides in the environment. But

suitability of ALP for detection of organochlorine, OPs and CM pesticides has been studied (Ayyagari et al., 1995; Chouteau et al., 2005; Mazzei et al., 2004).

## 1.8 State of the art

### 1.8.1 Enzyme inhibition based biosensors for OPs analysis

The major enzymes exploited for enzyme inhibition based study are cholinesterase such as AChE and BuChE (Andreescu and Marty, 2006). Several enzyme inhibition assays have been developed for the analysis of the pesticides and HMs (Arduini et al., 2009; Baeumner, 2003; Bhand et al., 2005). About 71% of these enzymatic biosensors were used for the determination of pesticides including CM and OP compounds, while HMs represents only 21% of the total application of these biosensors (Amine et al., 2006). However advances have been made towards multianalyte multicomponent analysis as well as analysis of complex environmental matrices (Choi, 2004; Pirvutoiu et al., 2001). A state of the art of enzyme inhibition assay for environmental monitoring is illustrated in Figure 1.5.

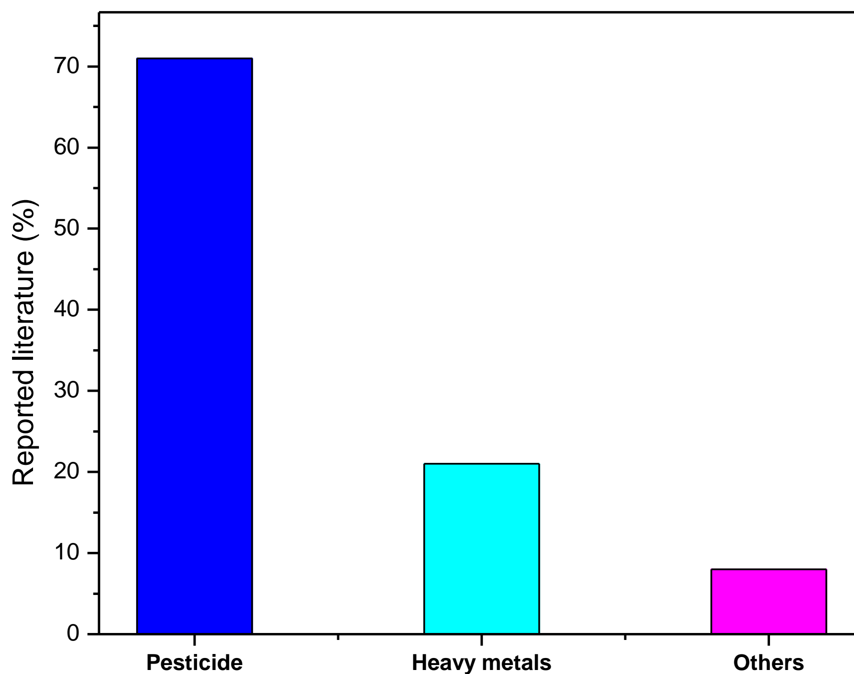



**Figure 1.5** Distribution of inhibitors in enzymatic biosensor (Adapted from Amine et al., 2006).

Several reviews based on enzyme inhibition biosensors have been published in recent years (Amine et al., 2006; Andreescu and Marty, 2006; Chauhan et al., 2010; Dzyadevych et al., 2003; Pohanka et al., 2009; Selid et al., 2009; Verma et al., 2005). Biosensor based on enzyme inhibition forms the largest category of biosensors for environmental analysis. Summary of reported biosensors for pesticides are shown in Table 1.3.

**Table 1.3**: Summary of reported biosensors for OPs using different enzymes/biomolecules and limit of detection

| Analyte | Receptor | Detection System | LOD/Range | References |
|---|---|---|---|---|
| Dichlorvos and MPOx | AChE | Amperometric | 0.1µM and 0.001µM | Valdés-Ramírez et al., 2009 |
| MP | Anti-MP IgY antibody | Optical | 0.001ng $mL^{-1}$ | Chouhan et al., 2010 |
| CPO | AChE | Electrochemical | 2µg $L^{-1}$ | Hildebrandt et al., 2008 |
| CPO and chlorfenvinphos | AChE | Electrochemical | $1.3 \times 10^{-11}$ M | Istamboulie et al., 2007 |
| POx and parathion | OPH | Electrochemical | 0.002-0.4 mM | Mulchandani et al., 1998 |
| MP | Flavobacterium sp. | Optical | 0.3 µM | Kumar and D’Souza 2006 |
| POx | AChE | Electrochemical | 0.5 nM | Joshi et al., 2004 |
| POx and CF | AChE | Electrochemical | 10 ppb & 8 ppb | Dounin et al., 2010 |
| MP | antibody | Optical | 10ppt | Chouhan et al., 2005 |
| POx and MP | OPH | Electrochemical | 5 mg/L and 3 mg/L | Wang et al., 2004 |
| Chlorpyrifos methyl oxon and POx | BuChE | Electrochemical | 1 & 4 ppb | Fabiana et al., 2006 |

Obtained LOD for OPs determinations using different biomolecules are also presented in the table. An important aspect, which is seldom addressed in research on enzymatic detection, is whether these methods are able to achieve sufficient sensitivity to detect pesticides at their maximum acceptable limits as described in regulations. However, enzymatic methods should not be seen as a means of replacing traditional methods of analysis. Enzymatic methods have a role to play as a screening tool which could allow the screening of hundreds of samples in a short period of time. Particularly, AChE activity can serve as a ‘‘toxicological index”, a measure of the toxicity of a sample (Kamanyire et al., 2004). Some obstacles still have to be overcome before enzymatic methods will be utilised on an extensive basis, but these methods could complement existing methods and allow for a more rapid assessment of problematic environments to allow appropriate steps to be taken to address contamination issues. Evaluation of combine toxic action of pesticide mixtures is one of the priority research areas due to the simultaneous occurrence of pesticides in the environment and the health risk they posed to humans and the environment as a mixture. Fabrication of surface microarrays on small chips is another important direction in miniaturization methods. Recently, disposable electrochemical printed gold chips for the analysis of POx and CF was reported (Dounin et al., 2010). Regarding the use of transducers in biosensors, a majority of reports emphasize on electrochemical transducers (44.5% amperometric, 35.8% potentiometric).

### 1.8.2 Real sample analysis using biosensors

The application of biosensors or biodevices to real environmental samples is very important in the final steps of development. Most of the reviewed systems still have some way to go before application to real samples can be realized, and the study of matrix effects, stability issues and careful comparison with established methods are crucial steps in this respect. Sample matrix can dramatically affect the application of biosensors to real samples. Matrix effect could be defined as an induced deviation from theoretically predicted parameters, caused by constituents or properties of the sample other than the analyte (Oubina et al., 1997). Since the exact composition of the matrix of environmental samples is usually unknown and can vary widely from sample to sample, the determination and quantitation of analytes constitutes a challenge in the application of the biosensors in real environmental samples.

**1.8.2.1 Milk and water, most challenging matrix:** Water is a chemical substance with the chemical formula $H_2O$. Pure water has a pH very close to 7 at 25°C. Milk is a most complex and challenging matrix and excellent source of energy, protein, minerals and vitamins (Noyhouzer et al., 2009). Cow's milk has a pH ranging from 6.4 to 6.8. The gross composition of cow's milk is 87.7% water, 4.9% lactose (carbohydrate), 3.4% fat, 3.3% protein, and 0.7% minerals. Milk composition varies depending on the species (cow, goat, and sheep). In the recent study, a reagent less paper based biosensor was reported (Hossain et al., 2009). The biosensor strip showed negligible matrix effect in detection of pesticides in spiked milk and apple juice samples when tested for pesticides POx, MT and carbaryl. Stoytcheva et al reported electrochemical biosensor for Chlorophos determination in contaminated milk. They have eliminated the matrix effect by using shaking and filtration (Stoytcheva et al., 2009). In another study, Zhang et al reported a disposable biosensor test for OPs and CMs in milk samples with good recovery rates (Zhang et al., 2005).

**1.8.2.2 Recovery of analyte from samples:** Recovery is the ratio of concentration of analyte quantified by a given method in a said sample to the actual concentration by standard method. When this ratio is multiplied by 100, it is expressed as % recovery.

### 1.9 Emerging trends in biosensors

Biosensors technology constitute a rapidly expanding field of research that has been transformed over the past two decades through new discoveries related to novel material fabrication technique, novel means of signal transduction and powerful computer software to control devices (Sheehan et al., 2003). Despite the past and current huge amount of research in biosensor development, there are still challenges to create more reliable devices. Miniaturization involves adaptation of microfabrication and nanofabrication technologies. In addition, biochip based platform provides miniaturization and point of contamination analysis with ease of operations (Suzuki, 2000). Taking advantage of miniaturization benefits, biosensors can become inexpensive and easy to handle analytical devices for fast and reliable measurments of chemical species. For environmental monitoring, the use of miniaturized technology reduces the amount of

chemicals used and also the energy needed to manipulate fluid volume, having an impact on environment and economy (Ziegler and Gopel, 1998).

The stability of enzymes remains a critical issue in biosensor. Poor stability is the inherent property of enzymes in their native form and can not be easily reused. For development of robust enzyme assay, stabilization of this native enzyme is of immense importance (Mozaz et al., 2006). Beyond stabilization, genetic modification of enzymes has also emerged as a useful alternative for improving the performance of native enzymes in terms of sensitivity and stability. The possibility of tailor-binding molecules with pre-defined properties, such as selectivity, affinity, and stability, is one of the major aims of the biosensor technology (Wong and Schwaneberg, 2003). Protein engineering is emerging as a possible strategy to improve the performance of native enzymes. The most commonly used strategy to achieve more sensitive and selective enzymes and consequently biosensors is to increase the affinity for the target analyte favouring the accessibility of the active site (Campàs et al., 2009). Genetically engineered AChEs have been widely exploited in enzyme inhibition-based biosensors for the detection of pesticides. The active site of *Drosophila melanogaster* (*Dm*) AChE, the most active wild form, is buried 20Å inside the protein and the entrance to the active site. The replacement of glutamic acid 69 (Glu69), located at the rim of the active site gorge, by amino acids with bulky side chains, such as tryptophan (Trp) or tyrosine (Tyr), has been demonstrated to increase significantly the inhibition constant (Boublik et al., 2002;  Sotiropoulou et al., 2005). Enzymes (using stabilizers as well as genetic modification) coupled with biocompatible chip has not only shown promising method for immobilization but also as an alternative to existing microwellplate assay. This thesis encloses work on the emerging trends of biosensors that are biochip technology and biochip based detection of OPs in milk.

### 1.10 Gaps in existing research

Although thousands of articles are published in the field of biosensors for pesticides every year due to its vast potential, there are some areas that still can be explored. The areas which need special and immediate attentions are:

**1.10.1 Need for ultrasensitive, highthroughput techniques for OPs analysis:** OPs are neurotoxic even when present at ultra low concentrations. Conventional analytical techniques do not meet stringent regulatory standards for monitoring their levels in milk, water, food etc and they provide low sample throughput. Therefore, we need such platforms where simultaneous analysis can be done using micro well plates. Micro well plates can be used for analysis of many samples at a time. Highthroughput techniques exploring enzyme assay has been reported in the area of clinical sciences, whereas it is yet to be explored in the area of environmental analysis (Cha et al., 2005).

**1.10.2 Need for miniaturized biochip detection systems for OPs and HMs:** OPs and HMs are major environmental pollutants. Miniaturized biosensors are getting attention owing to their portability, multifunctional capabilities, small sample volumes and low volumes of hazardous waste (Kricka, 1998). The miniaturization of bioassay comprises small size, high sample throughput of the order of a few hundred samples per assay and cost benefits. Miniaturization of the developed bioassay in 384 and 1536 well plate format and point of contamination using biochip will be a major task. The exceeding toxicity of analytes and excessive cost of biomolecules have created an urgent requirement for enzymatic assay miniaturization in plate and chip format. Thus there is a need for high sensitivity assay to determine such toxic contaminants at nano and pico level.

**1.10.3 Development of biosensor for multianalyte determination:** In environmental compartments, sample may be expected to contain multiple pesticides or HMs. Hence, it is essential to evaluate the synergistic effect of toxic contaminants co-existing with other contaminants. There is need for a novel biosensing system which can provide information on toxic effect of OPs. Real sample analysis requires development of multi-analytical biosensor. Hence, we need to develop a biosensor technique for multi analyte detection.

**1.10.4 Development of online determination and detoxification of OPs in milk:** A current survey of the literature has revealed that there is an urgent demand for biosensors in food monitoring with particular emphasis on online monitoring as well as detoxification. The dairy

industry is one among them, where continuous evaluation of milk constituents and milk contaminants is of utmost importance. Development of a robust yet sensitive online technique for food analysis will be a great step towards meeting this demand to the standard set by agencies.

## 1.11 Objectives of the present research

**The proposed research work aims at**

1. Development of novel high-throughput enzymatic optical biosensor for analysis of pesticide residues such as OPs and CM in matrices such as water and milk (Gap 1.10.1).

2. Development of novel miniaturized enzyme based biosensors/biochip for pesticide residues and HMs such as Hg, Pb, Cd, As etc (Gap 1.10.2 and 1.10.3).

3. Develop innovative biosensors for monitoring removal of contaminants such as OPs and HMs from water (Gap 1.10.4).

**1.12 Thesis structure** The thesis comprises seven chapters and each of these chapters has been described below:

**Chapter 1 Introduction** About biosensors, classification of biosensors, transducers, various aspects of biosensors, and enzyme based biosensor with respect to OPs induced inhibition, performance criteria of biosensors, various components, current state of art for inhibition based biosensors for analysis of OPs were discussed. The chapter also discusses about gaps in the existing research and what more needs to be done, objective of the proposed doctoral work and thesis structure.

**Chapter 2 Highthroughput enzyme assay for analysis of OPs residue in milk** Brief description about OPs, occurrences of OPs in milk, stabilization of BuChE, MPOx as a model inhibitor, BuChE interaction with OP, principle of enzyme inhibition exploiting CL technique for quantification, optimization of reaction parameters, assay development in PB, determination of individual analytes MPOx, EPOx, CF and MP, evaluation of toxicity of OPs mixtures, application of developed assay for milk analysis.

**Chapter 3 Development of biochip device for analysis of OPs in milk** Brief discussion about biochip, fabrication of chip and advantages of using chip based platform, application of biochip for OPs, stabilization of AChE and bi-enzyme coupling, functionalization on bio-chip, on chip assay and its detection using CL, method validation using LC-MS/MS and detection of reference pesticide mixture-2 and 174 using developed biochip devise.

**Chapter 4 A novel on chip inhibition assay for mercury analysis in water** Brief discussion about $Hg^{2+}$ contamination, limits set by regulatory agencies, device fabrication in brief, immobilization of enzyme on chip, on chip enzyme inhibition studies using $Hg^{2+}$.

**Chapter 5 A visual colorimetric bioassay for determination of pesticides in drinking water** Introduction, colorimetric technique, inhibition of ALP using OPs, reported biosensors using ALP, construction of $SiO_2$ based micro column, screening of two different sources ALP, inhibition, visual and quantitative determination of OPs and CF, analysis of pesticide mixture using ALP, interference of HMs in OPs determination, real sample analysis, cross validation.

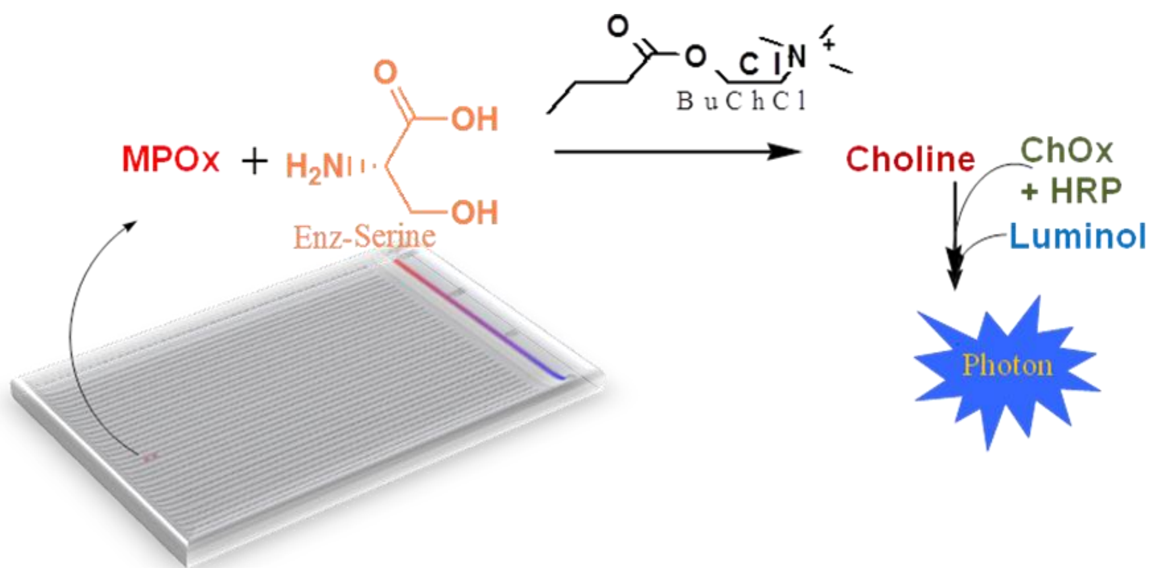
**Chapter 6 Novel strategy for automated detection and detoxification of OPs in milk** Electrochemical techniques, need for flow injection analysis, reported work for OPs, construction of genetically engineered biosensor, optimization of parameters in Flow injection analysis (FIA) system, enzyme immobilization, amperometric measurement, determination of EPOx, CPO and MAO in milk, Applicability of biosensor, recovery study. Detoxification of OPs, detoxification using biosensors, PTE based detoxification column for OPs, Influence of flow rate on OPs degradation, stability of column, optimization of Omnifit column for detoxification of OPs. Detoxification of CPO, EPOx and MAO spiked in water and milk.

**Chapter 7 Conclusions** Suggested future work, references and appendix, highlights of work Future scope.

# CHAPTER 2

## Highthroughput enzyme assay for analysis of OPs residue in milk

**Novelty Statement:** In this work, a highly sensitive, highthroughput BuChE based microwell plate assay was developed for OPs analysis in milk. A novel stabilization protocol was developed using a stabilizer composition for BuChE enzyme. BuChE showed good stability over the period of 6 weeks when pre-loaded in micro well plate. The assay is based on CL technique and based on inhibition of BuChE by MPOx. The developed assay is highly sensitive, miniaturized (8.5μL assay volume) and can determine MPOx in milk at pico level within 15 min.




Schematic representation of highthroughput analysis of OPs residues in milk

## 2.1 Introduction

The extensive use of pesticides to protect agricultural crops necessitates reliable tools for the detection of residues in food and water, thus ensuring environmental protection and consumer safety. The worldwide increasing use of OPs insecticides which are powerful neurotoxins and the resulting environmental and public concerns created a demand for the development of reliable, fast, sensitive, simple and low costing methods for their quantification, suitable for online and onsite measurments. There have been episodes of contamination of ground water, surface water and even bottled water with extensive use of pesticide residues (CSE report, 2003). OP pesticide residue levels as high as 75.7 μg/L in bottled drinking water, 26.8 μg/L in soft drinks and 227.8 μg/L in human blood have been reported (CSE report, 2002a; 2003b; 2005c). These reported levels of OPs exceed the permissible levels.

**2.1.1 Pesticide residues in milk:** Milk is an essential food for infants and the young population (Padilla et al., 2004; Zhang et al., 2005). Contamination of milk by toxic substance causes a serious problem to the agricultural and dairy industry. Among the various milk contaminants, OPs are most important due to their high toxicity even at very low residual concentration. Although, OPs have relatively low persistence in the environment, there are a number of ways in which they can reach to milk (Abou Donia et al., 2010). Presence of OP residues in milk has already been reported by many researchers (Gazzotti et al., 2009; Juhler 1997; Pagliuca et al., 2006; Salas et al., 2003; Stoytcheva et al., 2009) which is mainly due to the ability of OPs to covalently link with milk proteins (Deiana and Fatichenti, 1992). The summary of optical biosensors is presented in Table 2.1. High exposure of OPs and its concurrent toxicological effects in developing countries, like India and China is a serious concern (Bai et al., 2006; CSE report, 2005). There is a need for fast screening techniques, especially for neurotoxic insecticides in food items that are consumed to a great extent by vulnerable groups such as young children and infants. The EU has set maximum admissible concentrations of 0.1 μg $L^{-1}$ for individual pesticides and 0.5 μg $L^{-1}$ for total pesticide residues. The BIS has set permissible limit for total pesticide residues in drinking water as 1 μg $L^{-1}$ (CSE report, 2002). The EU has set a very low limit for pesticides in baby food. According to this regulation, infant formulae must not contain residues of individual pesticides at level exceeding 10 μg $kg^{-1}$ which is in practice the minimum

detectable level using the officially admitted detection methods (EU Commission Directive 1999/50/EC). Codex Alimentarius has set different-different maximum residual limit (MRL) for food items (Codex Alimentarius, Pesticide Residues in Food, 1993). Food Safety and Standards Authority of India (FSSAI) has set the limits for pesticides in different matrices. For milk and milk products the tolerance level is 0.01mg/kg.ppm for OPs.

**Table 2.1** Summary of biosensors reported for OPs using optical techniques

| Analyte | Biomolecule | Technique | MDL/Range | References |
|---|---|---|---|---|
| POx | OPH | FL | 0.05µM | Viveros et al., 2006 |
| POx | OPH | FL | 20 µM | Simonian et al., 2005 |
| MP | Antibody | CL | 10 pg/mL | Chauhan et al., 2006 |
| MP | MP Hydrolase | FL | 0.26 ng/mL | Leng et al., 2010 |
| MP | *Flavobacterium* sp. | UV-Vis | 0.3 µM | Kumar and D'Souza, 2006 |
| MP | *Sphingomonas* sp. | UV-Vis | 4-80 µM | Kumar and D'Souza, 2010 |
| Triazophos | MIP | CL | 2.5 nM | Xie et al., 2010 |
| POx | AChE | CL | 0.75 µg $1^{-1}$ | Roda et al., 1994 |
| POx | AChE | UV-Vis | 10 ppb | Weetall et al., 2004 |
| POx | AChE | CL | 25 to 250 ppb | Danet et al., 2000 |
| MP | Antibody | FL | 0.5 ng/ml | Kolosova et al., 2004 |

### 2.1.2 Need for fast screening methods for the analysis of OPs in milk

Reported high levels of residual OPs in human body and their high toxicity at trace level has forced many scientists to develop highly sensitive, selective, rapid and reliable analytical method for OPs determination. Apart from the standard analytical method used for OPs analysis, biosensor has emerged as potentially sensitive technique with added advantage of toxicity assessment. Enzyme inhibition based biosensor for OPs analysis have been reported using various enzymes (Amine et al., 2006; Arduini et al., 2009; Shulze et al., 2002; Weetall et al., 2004; Zakir Hossain et al., 2009) but its application to real samples such as milk has still remained a challenge, which is also evident from the scarcity of reported literature. Biosensors based on ChEs have emerged as sensitive and selective technique for toxicity assessment in food and agricultural applications (Andreescu and Marty, 2006). Inhibition of AChE by OP in milk reported by Beam and Hankinson (Beam and Hankinson, 1964) is considered as one of the pioneer work. Sensitive determination of MPOx has been reported using AChE (Zhang et al., 2005). OPs analysis using BuChE inhibition has been reported using electrochemical biosensors (Beattle et al., 1994; Cho et al., 1999; Renault 2001). Few reports are available for sensitive optical detection in high throughput assay formats. Measurement of OP induced inhibition using BuChE provides comparatively higher stability and sensitivity towards pesticide analysis over AChE (Imato et al., 1995).

Reliability of an enzymatic bioassay/biosensor is depends on the stability of the enzyme used, as biosensor may often be stored for weeks or months prior to its use. Enzymes such as ChEs provide limited operational and storage stability at room temperature. It has been reported that unfolding of proteins can be prevented by using stabilizers that remain in the amorphous phase with the protein and hydrogen bond to the protein in place of water during drying (Gouda et al., 2002; Vakurov et al., 2005). Development of OPs biosensor as a screening assay for milk sample is of paramount interest. For high throughput analysis, enzyme inhibition based biosensors coupled with CL technique in 384 and 1536 well plate formats, has gained immense interest over the past two decades. Co-exposure of OPs such as MPOx, MP and MT is very common in the environment. Thus, toxicity study of these OPs in combination is of imperative significance

(Laetz et al., 2009). Although the effects of individual OPs on ChEs activity have been studied for decades, the neuro toxicity of mixtures is still poorly understood.

### 2.1.3 Significant contribution

In the present chapter, we developed a novel stabilization protocol for BuChE at room temperature. This work illustrates development of a rapid miniaturized assay in 384 and 1536 well plate format for OPs residue in milk. Assay utilizes BuChE inhibition with CL technique, for the determination of highly toxic OPs such as MPOx, MP and MT in milk. The synergistic effect of OPs mixture on BuChE inhibition in milk sample was also studied. A novel stabilization protocol was utilized in the present study with pre-loaded BuChE in micro well plate. Stability provided by dextrose to BuChE may be due to the interaction of H-OH bond between protein and carbohydrate. This bond provides a higher conformational stability to BuChE and also provides long shelf life. The reproducibility of the stabilized BuChE was studied and good RSD i.e below 3.5% was obtained for the interday assay over the specified period. The stabilized BuChE exhibits significant stability over the period of six weeks.

### 2.1.4 Research gaps identified

In the current knowledge, there is a need for highly sensitive, miniaturized and highthroughput techniques for analysis of OPs in milk using CHEs.

### 2.1.5 Objectives

The aim was to develop a highly sensitive and highthroughput enzyme assay using CL technique for determination of OPs residue in milk based on BuChE inhibition.

### 2.1.6 Methodology

For highly sensitive determination of OPs, the inhibition based principle was adopted to achieve the objectives. BuChE and MPOx was used as a biocomponent and model inhibitor and CL techniques has been employed. The BuChE stabilization was tested and recoveries were obtained in good agreement. The principle is given as under:

### 2.1.6.1 Bio-assay principle

The presented assay is based on the inhibition of BuChE by OP residues. During the inhibition, serine hydroxyl moiety in the BuChE active site is phosphorylated. The serine hydroxyl group, blocked by a phosphoryl moiety is no longer able to participate in the hydrolysis of BuChCl (Fukuto, 1990; Moris et al., 1995; Valdés-Ramírez et al., 2009). Assay principle is presented as Figure 2.1. The percentage of inhibited enzyme activity (I %) that results after exposure to the inhibitor is quantitatively related to the inhibitor concentration according to following equation 2.1 (Arduini et al., 2009).

$$I\% = (A_0 - A_I)/ A_0 \times 100 \qquad (2.1)$$

Where

I% = percentage of inhibition, $A_0$ = the enzyme activity before exposure at the analyte;

$A_I$ = the enzyme activity after exposure at the analyte.

CL technique was exploited to determine the activity of stabilized BuChE.

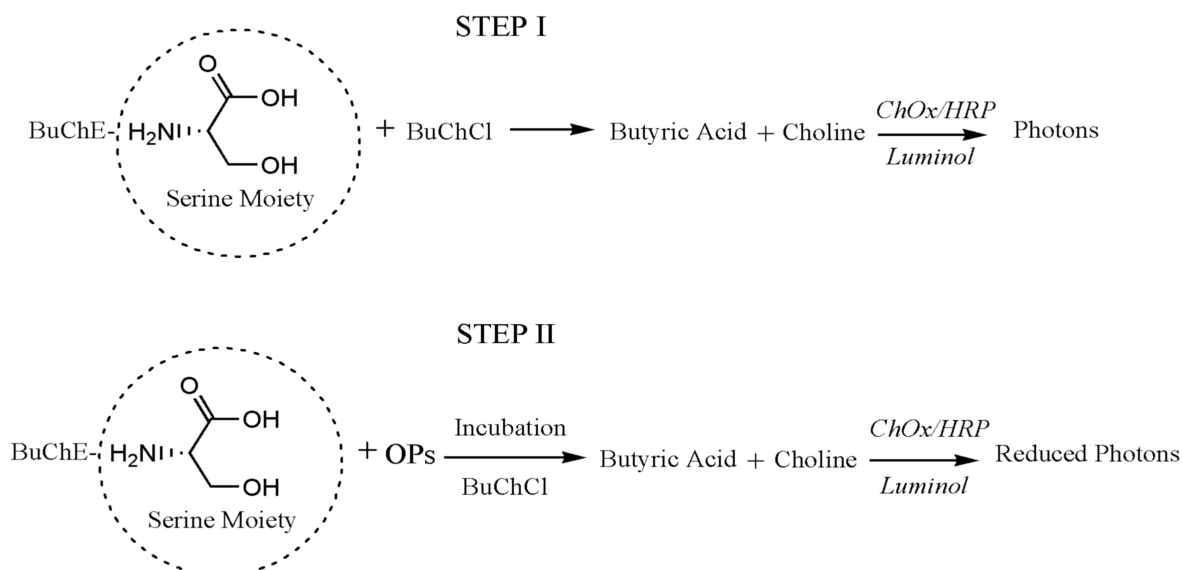



**Figure 2.1** Principle of enzyme inhibition based assay for OPs based on CL technique.

## 2.2 Experimental

### 2.2.1 Chemicals, biochemicals and instruments

Butyrylcholinesterase (E.C.3.1.1.8) from *Equine serum,* Choline oxidase (E.C 1.1.3.17) from *Alkaligenes species,* Peroxidase (E.C.1.11.1.7) from *Horseradish,* Butyrylcholine chloride, Choline chloride, Trehalose, 5-Amino-2, 3-Dihydro-1,4-Phthalazinedione (Luminol) and Protein standard, micro standard solution were purchased from Sigma Chemical Co. St. Louis (USA), Methyl Paraoxon PESTANAL™ grade purity 96.3 area%, Methyl Parathion PESTANAL™ grade purity 99.9 area% and Malathion PESTANAL™ grade purity 97.3 area% were purchased from Riedel-de Haën (Germany). Hydrogen peroxide (30%), Acetonitrile, sodium phosphate dibasic, sodium phosphate monobasic and other chemicals were of GR grade, Merck (Germany). Dextrose, anhydrous (A. R.) was obtained from High media laboratories, Mumbai (India). Multi label Reader Victor™ X4 offers a high sensitivity luminescence measurement system with capability to measure both 384 and 1536 well plate format. The detector used in the system is a red photomultiplier tube, capable of low photon counting. Micro-titer plates Optiplate 384 (Nunc, Denmark), 1536-Well Plates (Corning, USA) and micro pipettes (Eppendorf, Germany) were used for assay. Origin 6.1 (Microcal, USA) was used for processing of data.

### 2.2.2 Reagent preparation

Phosphate buffer (PB) 0.1M, pH 7.4 was prepared by mixing sodium dihydrogen phosphate monohydrate GR (0.1M, pH 4.4) and di-sodium hydrogen phosphate anhydrous GR (0.1M pH 9.2) using ultra pure water. Stock solutions (1mg $mL^{-1}$) of MPOx, MP and MT were prepared in 5% acetonitrile and kept at 4°C. Stock solutions of BuChCl (0.1M), BuChE (160U/mL), choline oxidase (ChOx) (8U/mL) and HRP (1U/mL) were prepared in PB and stored at 4ºC. Working solutions were prepared by appropriate serial dilutions in 0.1M PB. Luminol solution was prepared by dissolving 4 mg of luminol in 2 mL, 0.1 M NaOH and making up the volume to 20 mL by 0.1 M PB at pH 7.4.

### 2.2.3 Stabilization of BuChE and assay development

For enzyme stabilization, dextrose and trehalose were dissolved in protein standard solution (2% w/v). Different compositions of stabilizer and BuChE (1:1, 1:2, 1:4) were prepared. We stabilized BuChE with dextrose in protein standard solution (1ml/amp: 1mg BSA/ml in 0.15M NaCl, 0.05% $NaN_3$). The assay protocol for inhibition studies is as follows: The stabilized BuChE (0.5 µL of 0.08 IU) was dispensed in the 384 microwell plate and were dried at room temperature. Stabilized BuChE forms thin film like layer at the bottom of the well. Subsequently, 5 µL of inhibitor (in PB or Milk) was added to well and incubated for 10 min. The reaction was followed by addition of reaction mixture (14.5 µL) consisting of BuChCl (0.5 mM), ChOx (0.004 IU), HRP (0.0008 IU) and luminol (1mM). The number of photons emitted was recorded. A washing step was also performed after incubating stabilized BuChE with OPs.

### 2.2.4 Analysis in milk

Commercial milk samples containing 0.5% fat were purchased from local market of Goa, India. Milk samples were only filtered using 0.2µ filter (Whatman USA) and diluted prior to analysis. Matrix matching studies were carried out by preparing different dilutions of milk in PB (1:10, 1:100, 1:500, 1:1000, and 1:2000). Milk samples were spiked with individual pesticides (MPOx, MP & MT) and their mixture taking MPOx as a reference. The concentration of OPs in mixture is as follows. Mixture 1: MPOx, MP and MT (1 $\mu gL^{-1}$ each), Mixture 2: MPOx (1 $\mu gL^{-1}$) and MP, MT (10 $\mu gL^{-1}$ each), Mixture 3: MPOx (1 $\mu gL^{-1}$) MP and MT (100 $\mu gL^{-1}$ each). For each inhibition assay, 5 µL of milk sample spiked with OPs mixture was used and assay was carried out as described in assay protocol.

## 2.3 Results and Discussion

### 2.3.1 Optimization of enzyme substrate reaction

Optimization of kinetic parameters which may affect the activity of single enzyme as well as bi-enzyme reaction kinetics was studied. Concentration of ChOx and HRP along with their substrate was optimized. Luminol concentration was also optimized. The optimized parameters

for bienzyme reactions were used to study trienzyme reaction for kinetic control and the optimal signal for CL detection.

### 2.3.2 Parameters affecting the analytical performance of tri enzyme reaction

The analytical performance of bioassay depends on several parameters, such as the activity of enzyme, stability of enzyme, enzyme loading, pH, ionic strength, temperature of reaction and substrate concentration. Reaction parameters were optimized with stabilized enzyme in micro plate.

#### 2.3.2.1 Optimization of pH and ionic strength of buffer

Enzymes are affected by changes in pH. The most favorable pH value, the point where the enzyme is most active is known as the optimum pH. During experimental work for the optimization of pH, trienzyme reaction was initially studied in the range of pH from 6.0 to 9.0 with the difference of 0.5 pH unit. It was observed that, tri-enzyme system shows their optimum activity around pH 7.5; finally pH was varied from 7-7.8 with difference of 0.2 pH unit. The activity of enzyme reaches to maximum at pH 7.4. Thus pH 7.4 was optimized as pH for further experiment. Figure 2.2(a) shows effect of pH on stabilized BuChE activity. The ionic strength of the solution is an important parameter affecting enzyme activity. This is because the reaction rate depends on the movement of charged molecules relative to each other. Thus both the binding of charged substrates to enzymes and the movement of charged groups within the catalytic 'active' site will be influenced by the ionic composition of the medium. It is important to control the ionic strength of solutions in parallel with the control of pH. Buffer of ionic strength 0.1 M is reported for tri-enzyme reaction (Cremisini et al., 1995). Optimization of ionic strength was carried out by varying ionic strength from 0.01 to 0.2 mM of PB. From Figure 2.2 (b), we can conclude that at ionic strength of 0.1 mM of PB, tri-enzyme shows their optimum activity at 30ºC with 0.1 M PB at pH 7.4.

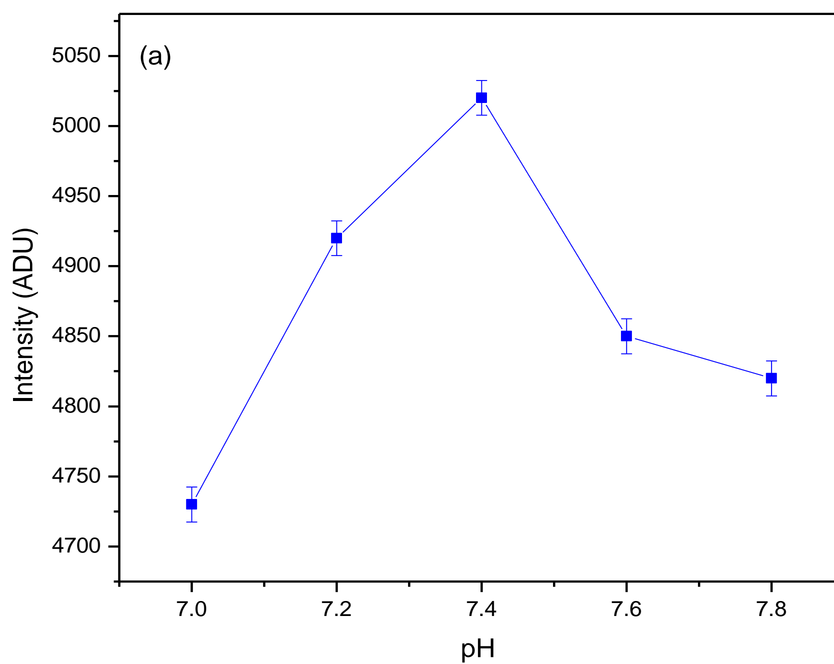



**Figure 2.2 (a)** Effect of pH on the activity of stabilized BuChE in micro well plate with 0.5 mM BuChCl, 0.1 M PB at pH 7.4.

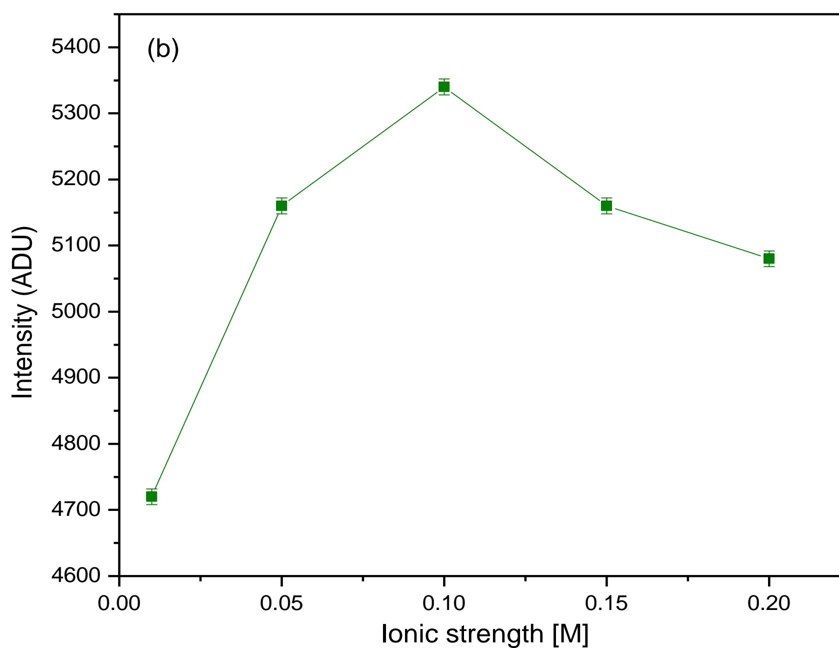



**Figure 2.2 (b)** Variation of CL intensity profiles for stabilized BuChE at different ionic strength of PB.

Experiments were performed initially with varied concentration of BuChE (0.04-0.32 IU) and subsequently with BuChCl (0.03-3mM) under optimized experimental conditions. The Michaelis constant, $K_M$ apparent was calculated 0.27 mM for BuChE using Lineweaver-Burk plot.

**2.3.2.2 Stabilization of BuChE at varying temperature:** Enzyme BuChE is sensitive and unstable at room temperature. AChE stabilization has been reported at room temperature (Weetall et al., 2004), whereas, stability of BuChE at room temperature has not been reported. In this work, we have investigated the stabilization of BuChE using dextrose and trehalose in protein standard solution. The other two enzymes ChOx and HRP are added in the presented assay after the inhibition step. Thus, their storage stability at 4ºC was also evaluated using the developed assay. Intra-day and inter-day stability of ChOx and HRP was also studies up to 6 weeks. The kinetic profile of native and stabilized BuChE at 30ºC is shown in Figure 2.3 (a).

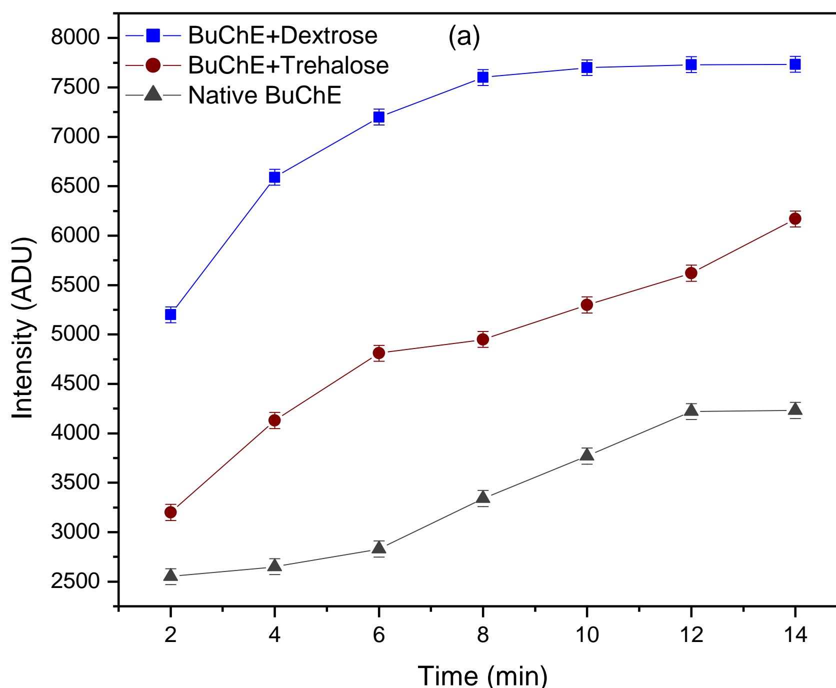



**Figure 2.3 (a)** CL intensity profile for native and stabilized BuChE with trehalose and dextrose as a stabilizer.

The intensity profile shows increasing trend where intensity saturates after six min for dextrose stabilized BuChE. This stability in the intensity also decreases the analysis time. It is clear that with stabilization, we could achieve almost double enhancement in the intensity than native enzyme. Among the two stabilizers studied, dextrose in protein standard solution provides higher stability over trehalose. With studied stabilizer composition, the data obtained is highly reproducible for longer duration. The effect of temperature on stabilized BuChE activity was studied using dextrose solution in the range 25-40ºC and shown in Figure 2.3 (b). It is evident from the graph that the activity of stabilized BuChE was found stable in the range 30-40ºC.

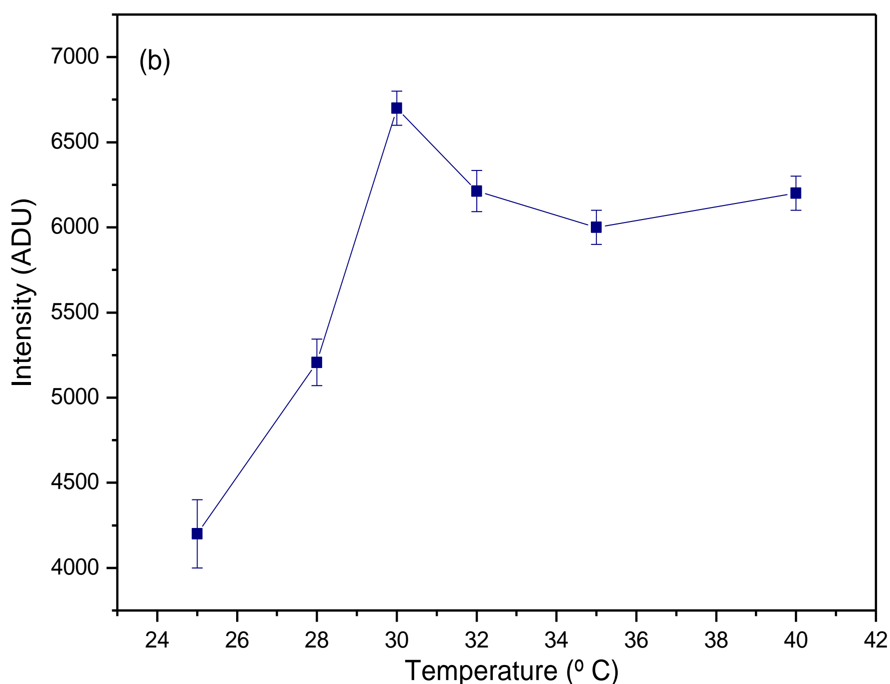



**Figure 2.3 (b)** The effect of temperature on the CL intensity of the stabilized BuChE in micro well plate with 0.5 mM BuChCl, 0.1 M PB at pH 7.4.

### 2.3.3 Matrix matching

Evaluation of the matrix effect is an important aspect of the assessment of the analytical method. The milk constituent, mainly fat can cause decrease or enhancement of signal response. In milk the fat content posed major problem. Milk was diluted with PB to optimize the compatibility of both matrices. The histogram of matrix matching is shown in Figure 2.4. Experimentally, it is

observed that inhibition from milk constituents can be reduced by diluting milk with PB and maximum activity of BuChE in diluted milk was obtained when milk was diluted with PB in the ratio 1:1000. This composition shows comparable activity with that of PB showing least inhibition and thus selected to study the performance of bioassay in milk.

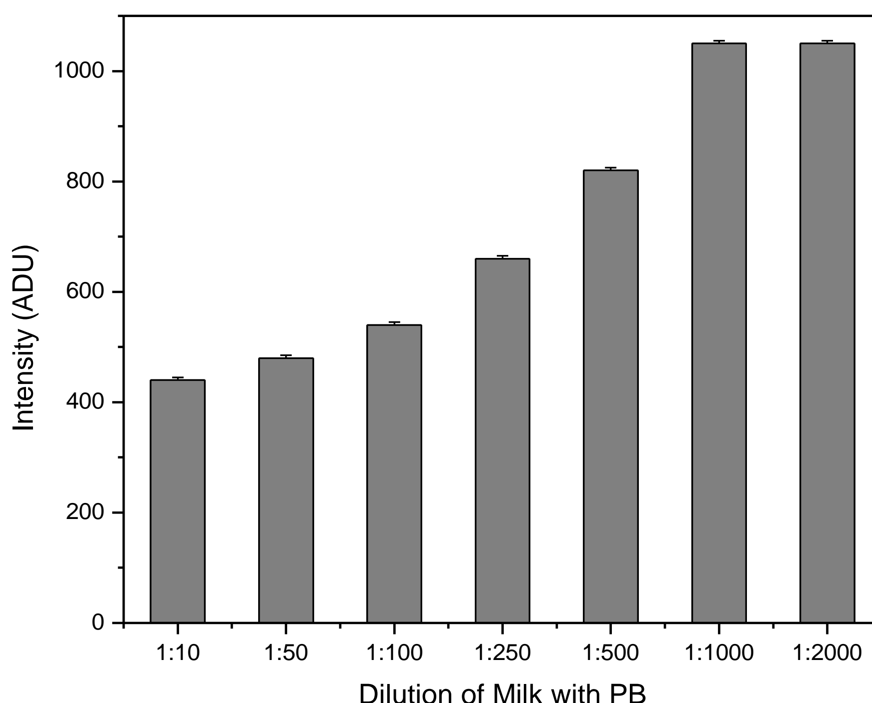



**Figure 2.4** Dilution of milk with PB for matrix matching study.

### 2.3.4 Inhibition measurment in buffer using MPOx

**2.2.4.1 Optimization of incubation time:** For irreversible inhibition**,** the degree of CHE inhibition in solution is dependent primarily on the incubation time (Arduini et al., 2009). Assays were designed to study inhibition parameters such as incubation time and inhibitor concentration. Inhibition studies were carried out in two steps. In the first step, triplicate measurements were carried out in the absence of inhibitor to obtain optimal signal for BuChE. In the second step which is actual inhibition step, triplicate measurements were performed in the presence of inhibitor and optimal signal was recorded. The difference in activity (Δ I%) was calculated.

Assay performance was tested using MPOx as a model inhibitor in PB. To avoid substrate deficiency, 0.5 mM BuChCl was used for inhibition study. In the studied range of BuChCl, substrate induced inhibition was not observed. Incubation time is a key parameter in pesticide residue analysis. Thus, effect of different incubation time (2, 5, 10, 15, 20, 25, and 30 min) on BuChE activity was studied with 50 µg $L^{-1}$ MPOx. A sigmoid curve was obtained when data for different incubation time was plotted against I% and results are presented as Figure 2.5. A good linearity was observed up to 10 min incubation, where BuChE showed 60% inhibition. It is important to note that after 10 min incubation, no significant increase in I% was observed. Therefore all the measurements were performed with 10 min incubation.

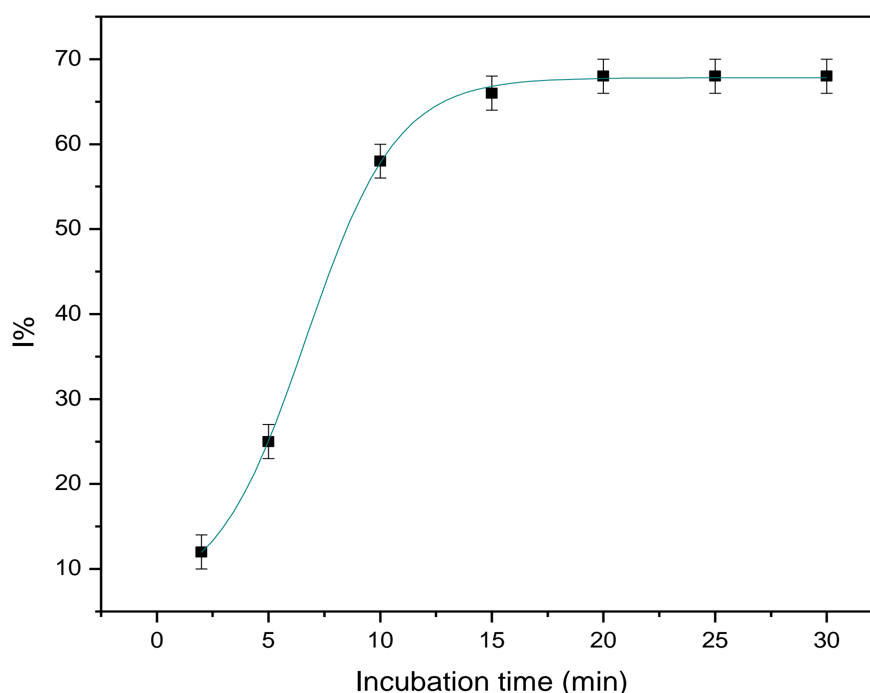



**Figure 2.5** Inhibition profile obtained for 50 µg $L^{-1}$ MPOx at different incubation time using 0.5 mM BuChCl at 30ºC.

**2.2.4.2 Calibration for MPOx:** The ability of OPs to inhibit ChE was exploited to quantitatively determine its concentration. The efficiency of proposed assay was tested by exposing BuChE to different MPOx concentration in PB. A calibration curve was constructed for MPOx in PB (as a

reference) prior to milk analysis. Figure 2.6 shows the percentage of BuChE inhibition caused by different MPOx concentration. No significant difference in the degree of inhibition was observed when an additional washing step was introduced after incubation with pesticide. BuChE as an immobilized enzyme component facilitates specific inhibition of BuChE by OPs. Each experimental data point is mean of three inhibition assays whereas in each assay, triplicate measurements were performed for each MPOx concentration. A good linearity was found in the range 0.005-50 µg $L^{-1}$ with equation of line: Y = 53.4754 + 9.3608 X, $r^2$ = 0.9931. The BuChE assay could achieve a lower LOD up to 0.001 µg $L^{-1}$ for MPOx in 384 well plate formats, which is much lower than reported values in the literature (Shulze et al., 2002; Valdés-Ramírez et al., 2009; Zakir Hossain et al., 2009; Zhang et al., 2005).

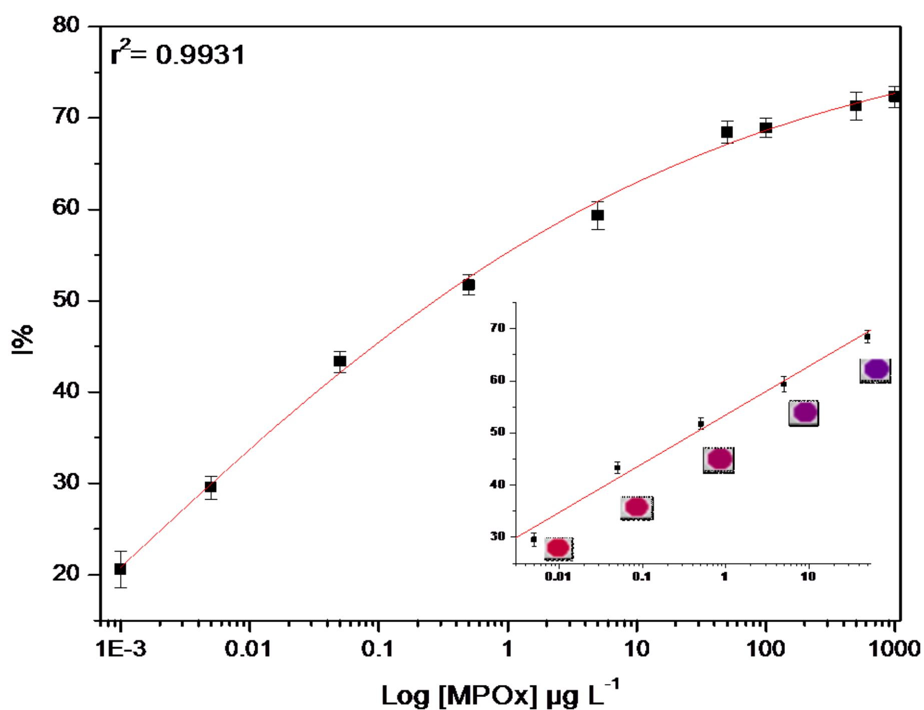



**Figure 2.6** Calibration curve obtained for MPOx in PB using 0.08 IU BuChE with 0.5 mM. BuChCl, 0.004 IU ChOx, 0.0008 IU HRP, 0.1 M PB, pH 7.4 at 30 ºC, incubation time =10 min (Inset: linear range for MPOx).

### 2.3.5 Assay performance in milk for OPs determination

Inhibition studies were extended to analyze three OPs namely MPOx, MP and MT in milk samples. Different concentrations of individual OPs (0.001-1000 µg $L^{-1}$) in milk were mixed with BuChE and allowed to incubate for 10 min in dark. The data obtained was used to construct standard inhibition curve relating to OPs concentration in milk and presented in Figure 2.7. As indicated in Figure 2.7, the MPOx curve (a) shows maximum inhibition up to 65%, whereas MP (b) and MT (c) curve shows maximum inhibition up to 60%. MPOx shows 15-20% more inhibition than other two OPs in the lower concentration range. Linear range, Limit of quantification (LOQ) and Inhibitory concentration (IC) values were calculated and presented in Table 2.2. Lowest concentration in the linear range is represented as LOQ whereas; concentration corresponding to 50% inhibition is represented as $IC_{50}$.

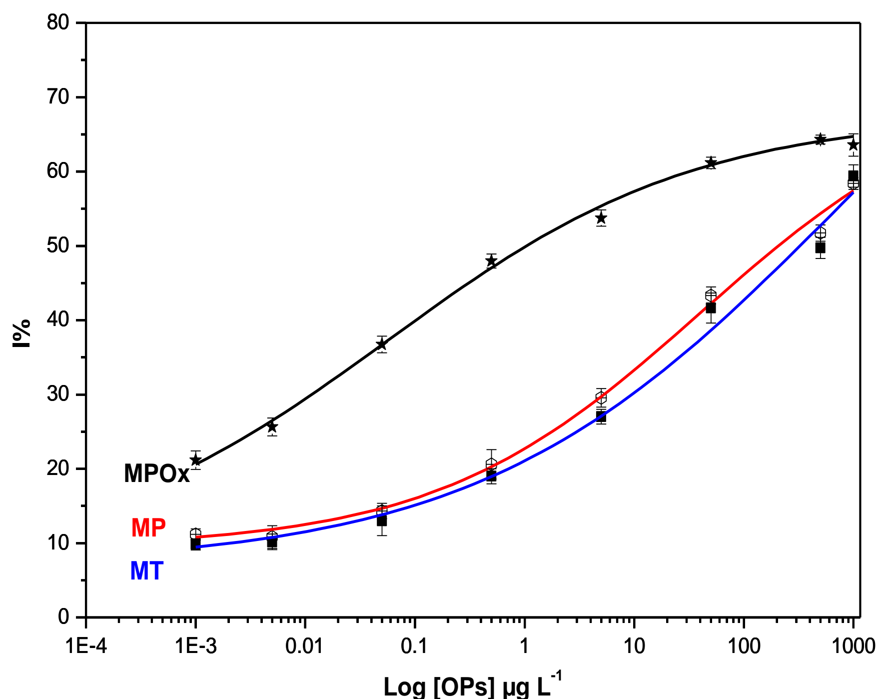



**Figure 2.7** Inhibition profile obtained for MPOx, MP and MT spiked milk samples using BuChE. The assays were carried out in 384 well plate formats with 10 min incubation.

The higher toxicity of MPOx as against MP and MT is clearly evident from the significant difference in their $IC_{50}$ values and linear range. This investigation also revealed the sensitivity of BuChE towards MPOx as against other two OPs.

**Table 2.2** Analytical figures of merit for the developed bioassay in spiked milk

| **Analytical parameters** | **MPOx** | **MP** | **MT** |
|---|---|---|---|
| Linear range (μg $L^{-1}$) | 0.005-5 | 0.5-1000 | 0.5-1000 |
| LOQ (μg $L^{-1}$) | 0.001 | 0.5 | 0.5 |
| LOD (μg $L^{-1}$) | 0.001 | 0.5 | 1 |
| Total assay volume(μL) | 8.5 | 8.5 | 8.5 |
| Analysis time ( min) | 15 | 15 | 15 |
| Apparent $IC_{50}$ (μg $L^{-1}$) | 1.02 | 202.02 | 326.70 |
| $r^2$ | 0.9953 | 0.9985 | 0.9933 |

### 2.3.6 Analysis of MPOx in real milk samples

The developed assay has been applied to the analysis of real milk samples for demonstrating the possible occurrence of the MPOx residue. Real milk samples were tested with and without spiking MPOx standard solution using bioassay. Milk samples were spiked with different MPOx concentration so that the final concentration lies within calibration range. Results obtained are presented in Table 2.3. In spiked milk samples, recoveries were found in the range 93.2-98.6% with % RSD 0.99-1.67. Measurements were performed in triplicates and each experiment was carried out three times (N=3). The obtained % RSD values support the reproducibility of developed assay in milk. Lower recoveries up to 93.2% in the studied samples denote the absence of MPOx in the samples or presence in below the LOD of the developed assay.

**Table 2.3** Analysis of MPOx in real milk samples and recovery studies

| Samples | MPOx added (µg $L^{-1}$) | MPOx found (µg $L^{-1}$) | % Recovery (N=3) | % RSD |
|---|---|---|---|---|
| Milk-1 | 0 | BDL | --- | --- |
| | 0.5 | 0.468 | 93.6 ± 0.93 | 0.99 |
| | 5 | 4.82 | 96.4 ± 1.11 | 1.15 |
| Milk-2 | 0 | BDL | --- | --- |
| | 0.5 | 0.478 | 95.6 ± 1.03 | 1.07 |
| | 5 | 4.86 | 97.2 ± 1.40 | 1.44 |
| Milk-3 | 0 | BDL | --- | --- |
| | 0.5 | 0.493 | 98.6 ± 1.0 | 1.01 |
| | 5 | 4.66 | 93.2 ±1.56 | 1.67 |

*BDL: Below detection limit of developed bioassay i.e. 0.001 µg $L^{-1}$
N: Number of analysis

### 2.3.7 Reproducibility and specificity

Reliability and reproducibility of BuChE inhibition assay was studied over an extended period of six weeks. Inhibition assays were performed for interbatch and intrabatch studies. Measurements were performed daily. The calculated % RSD was 2.5 for successive MPOx inter-batch assay performed 15 times (N=15). For intra batch assay, 40 measurments were carried out over the period of six weeks and the % RSD was calculated 3.5 (N=40).

### 2.3.8 Analysis of mixture of OPs

The interactive effects (additive, synergistic, and antagonistic) of mixture of OPs were investigated using MPOx as a reference analyte. Milk samples spiked with mixtures of MPOx,

MP and MT were studied for inhibition of BuChE. Figure 2.8 shows interactive effect of MPOx, MP and MT in three combinations of pesticide mixture. In each combination, four bars are presented, where first three bars represent the I% due to individual analyte and fourth bar represent interactive effect of mixture.

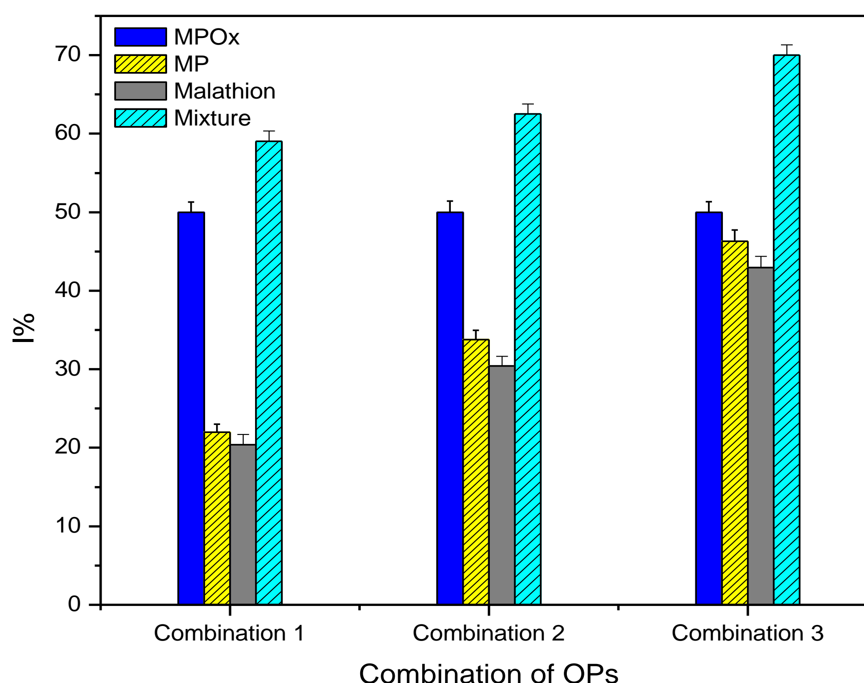



**Figure 2.8** Inhibition pattern obtained for tertiary pesticide mixtures using stabilized BuChE. **Mixture combination 1**: MPOx, MP and MT (1 μgL$^{-1}$ each), **combination 2**: MPOx (1 μg L$^{-1}$) and MP, MT (10 μg L$^{-1}$ each), **combination 3**: MPOx (1 μg L$^{-1}$) MP and MT (100 μg L$^{-1}$ each).

The objective of our study was to determine effect of MP and MT (over the varied concentration range) in presence of fixed MPOx concentration. From the obtained inhibition curve, it is observed that total inhibition activity of OPs mixture was not simply additive but synergistic (lower than the sum of individual inhibition values). From I% of the mixture, it is clear that inhibition by mixture is dominated by most potent inhibitor (i.e. MPOx). The obtained results also match with the other reports (Kok et al., 2004; Mishra, et al., 2002).

### 2.3.9 Assay miniaturization in 1536 well plate

Miniaturization of assay enables the handling of low volume samples, reduction in reagent consumption, minimizes waste generation and increased sample throughput. The developed assay was further miniaturized from 384 to 1536 well plate format with total assay volume 8.5µL without compromising assay integrity. Following remarkable features were observed as result of miniaturization of assay (i) Reduction in incubation time by half (from 10 to 5 min) (ii) Reduction in IC values by half (iii) 2 fold reduction in assay volume (iv) Broad linear range (0.0005-0.5 µg $L^{-1}$) and (v) Much higher sensitivity achieved up to 0.0005 µg $L^{-1}$. Comparative study of inhibition parameters in 96, 384 and 1536 well plate format is summarized in Table 2.4.

**Table 2.4** Comparative study of developed biosensor in 96, 384 and 1536 well plate format

| Parameters | Enzyme (Unit/Assay) | Incubation time (min) | $IC_{30}$ (µg $L^{-1}$) | Total assay volume (µL) |
|---|---|---|---|---|
| 96-well assay | 0.1 | 20 | 0.10 | 60 |
| 384- well assay | 0.08 | 10 | 0.014 | 20 |
| 1536-well assay | 0.04 | 5 | 0.0053 | 8.5 |

### 2.3.10 Stability of BuChE

The stabilized BuChE was preloaded in 1536 well plates and dried at room temperature. Preloading was done to reduce handling time and asses storage stability at room temperature. The well plate was monitored periodically for activity measurement up to six weeks. Figure 2.9 shows the activity profile of stabilized BuChE where each data point is the mean of three measurements. Stabilized BuChE retained more than 90% activity in the first two weeks whereas at the end of six weeks, BuChE retained 74% of activity. The extended stability of the enzyme is attributed to the added dextrose solution which facilitates stronger interaction of H-OH bond between protein and carbohydrate. This bond provides a higher conformational stability to

BuChE. The reproducibility of the stabilized BuChE was studied and good %RSD *i.e.*, below 3.5% was obtained for the interday assay over the six weeks.

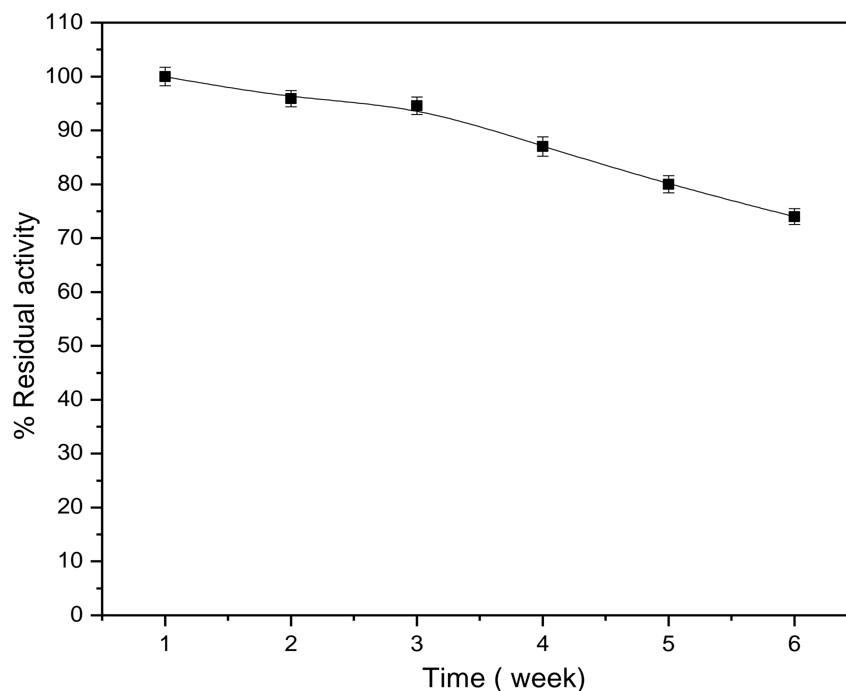



**Figure 2.9** Effect of stabilization on BuChE activity over the period of six weeks at 30ºC.

## 2.4. Conclusions

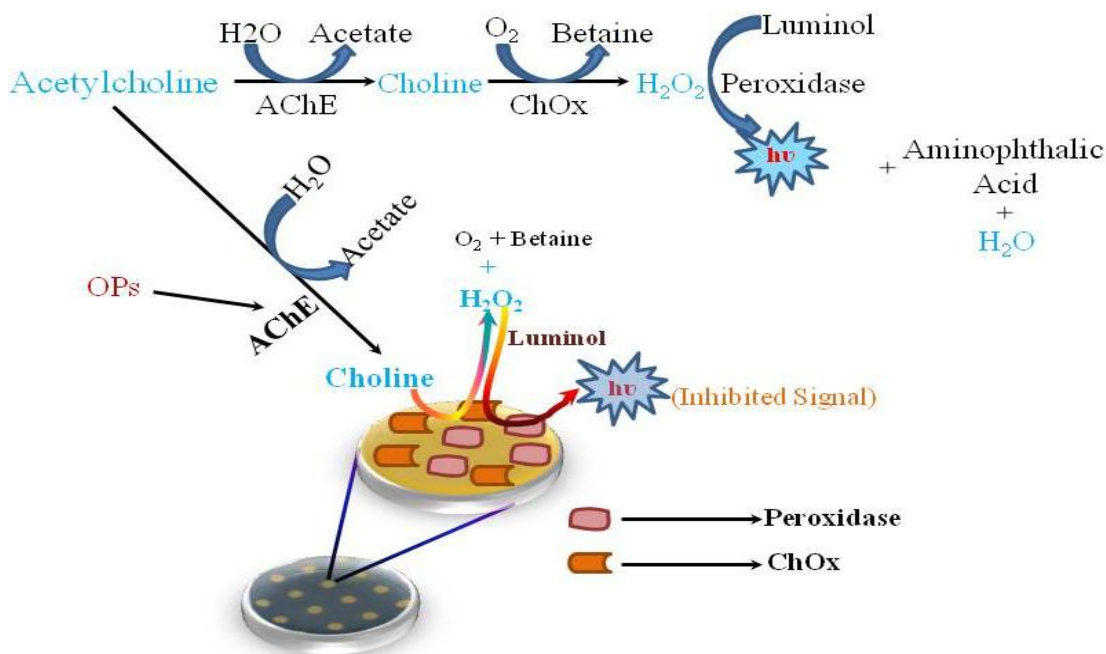
The significance of the demonstrated work lays in the facilitation of microplate based high-throughput, sensitive and economical bio-assay for determination of OPs residues. The BuChE based bioassay reported here enabled low level determination of individual OPs in milk samples with LOD 0.001 µg $L^{-1}$. A novel stabilizing protocol for long term stability of BuChE was developed. Short analysis time (12 min) and high reproducibility are the key features of the work. The bioassay was successfully tested to study interactive effect of mixture of three common OPs residue MPOx, MP and MT in milk. Recovery rates for MPOx in spiked milk samples lay between 93.2%-98.6%. The proposed method facilitates rapid analysis of milk samples in 384 well plate with further miniaturization in 1536 well plate format. The detection limit was found to be 0.0005 µg $L^{-1}$ of MPOx in milk in 1536 well plate formats.

# CHAPTER 3

## Development of biochip device for analysis of OPs in milk

**Novelty Statement:** This work presents a micro well biochip based platform using AChE as a probe for determination of OPs and pesticide mixtures in milk. A novel protocol was developed for immobilization of AChE on chip. Determination of MPOx, EPOx, MP and CF in milk was demonstrated successfully using developed biochip. The AChE showed an excellent stability when coupled on chip and stored at room temperature for more than 3 months. The developed biochip can be used as a screening tool for OPs residue in milk and simultaneously analyzes atleast 40 samples at a time.

***Note: This work is a part of Indian patent application no. 933/MUM/2012 and work involves conflict of interest due to commitment to the funding agency (NAIP) and requires in part a nondisclosure of material.***




Schematic representation of AChE inhibition using biochip

### 3.1 Introduction

During the last two decades, the exploration of biomolecules such as enzymes, antibody and cells has extensively grown based on the advancement in chip based biosensing system. This enabling technology allows sensing of ever-decreasing sample volumes and target analyte concentrations in ways that are not possible using conventional testing systems (Breslauer et al., 2006). Such technology also has the benefit of scaling the dimensions that enables a range of fundamental features to accompany system miniaturization such as reduced reagent consumption, high temporal resolution due to rapid mixing, high throughput, enhanced analytical performance, less waste, low unit cost, reduced energy consumption, and reduced dimensions when compared to macroscale techniques (Hong et al., 2003).

Chip based technologies offer a number of useful capabilities i.e. the ability to use very small quantities of samples and reagents and to carry out separations and detections with high resolution and sensitivity; low cost; short times for analysis; and small footprints for the analytical devices (Whitesides, 2006). Chip is a powerful tool holding great promise to facilitate novel experiments with unprecedented performance and has already been documented for analysis of toxic compounds (Gardeniers et al., 2004). Recently, significant development of bioanalysis has mainly been driven by the strong demand for fast and reliable results, which are essential for early screening and further analysis. These bioanalytical challenges in many cases can be solved using specifically designed and fabricated miniaturized tools called biochip. It is noteworthy that substrates having microarrays of bio-receptors are often referred to as biochips although most of these systems do not have integrated micro-sensor detection systems.

Advances in chip based biosensing methods have been met with serious challenges in the creation of devices that allow for the simultaneous detection of several types of biotargets on a single platform for environmental monitoring. Advances in technology have allowed chemical and biological processes to be integrated on a single platform (Blasco and Pico, 2009). Biochips represent the ability to miniaturize the experiments with the advantages of speed, higher order automation, volumetric reduction of sample and waste (Manz et al., 1992). Development of biochip based system facilitates field deployable techniques capable of analyzing trace analytes in contaminants on site. Recent efforts to minimize the time span between sampling and results include the use of miniaturized devices that do not depend on special infrastructure and sample

preparation procedures. Initially, the main reason for miniaturization was to enhance analytical performance, but the reduction of size also presented the advantages of reduced consumption of reagents and the ability to integrate separation and monitoring techniques within a single device (Liu et al., 2010). The use of chip-based devices will have a huge impact on environmental analysis because this will allow the creation of single platform based screening devices that are no longer bound to centralized laboratories (Jang et al., 2011). A major advantage of all chip-based biosensor is a simple and reliable method for analysis of contaminants with mere sample preparation; provide more stability to biomolecules and reusability of the chip for biosensing (Khandurina and Guttman, 2002). This is often the bottleneck for chip-based detection principles when transferring the detection principle into a real life application.

### 3.1.1 Fabrication of chip

Several techniques have been applied to generate user-defined patterned surfaces of biomolecules. As shown in Figure 3.1 some of those are microfabrication techniques such as photolithography, electron beam (EB) lithography, focused ion beam (FIB) lithography, microcontact printing (MCP) and scanning probe lithographic (SPL) techniques such as dip-pen lithography (DPL) and STM lithography.

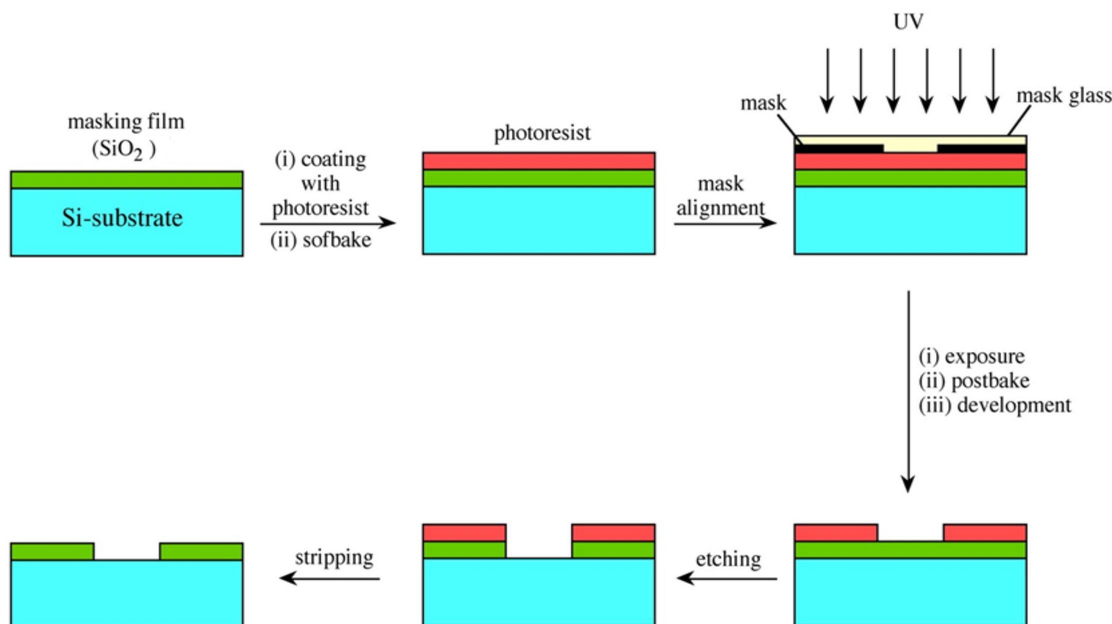



**Figure 3.1** A schematic of chip fabrication using photo-lithographical technique.

Photolithographic procedures utilize a mask that shields some areas from the UV radiation sources. EB and FIB lithography are maskless procedures that directly mill or remove the resist material by way of a highly energetic focused beam of electrons or ions.

### 3.1.2 What is a biochip?

An integrated circuit whose electrical and logical functions are performed by protein molecules appropriately manipulated (Compendium of Chemical Terminology, 2nd edition (the "Gold Book")". Broadly defined, biochips are engineered devices and systems possessing a chip component and a biologically derived component. It is noteworthy that substrates having microarrays of bioreceptors or often referred as biochips although most of these systems do not have integrated micro-sensor detection system. The chip substrate may be as simple as a glass microscope slide. With this diversity of options, biochips have emerged as a major field of study, active product development and commercial activity. There is a distinct demarcation between biochip based assay and conventional microplate based assay, which requires a relatively long assay time, and involves troublesome liquid-handling procedures and large quantities of expensive antibody reagents. Moreover, realization of point-of-care testing is difficult with conventional microplate, since rather large devices are necessary for automated practical diagnosis systems (Wang, 2005). To overcome these drawbacks, a biochip-based system is effective. Integration of analytical systems into a biochip should bring about enhanced reaction efficiency, enhance the sensitivity, simplified procedures, reduced assay time, and lowered consumption of samples, reagents, and energy (Cha et al., 2005).

### 3.1.3 Application of biochip

In addition to genetic applications, the biochip is being used in toxicological, protein, and biochemical research. Biochips can also be used to rapidly detect chemical agents used in biological warfare so that defensive measures can be taken. Biochip has wide applications ranging from clinical diagnoses, environmental analyses, and biochemical studies because of its extremely high selectivity and sensitivity. The ability to apply enzymes and antibodies on a wide variety of chip substrates is currently an area of intense research (Weller, 2005). Enzymatic assays on chip, ELISA or other immunosorbent assay systems, in which antigen and antibodies

are fixed on a solid surface are applicable to many analytes with high sensitivity using biochips. Several papers are published describing the integration of enzyme and heterogeneous immunoassay systems into microscale devices. There are already numerous examples of assays and analytical processes that have been successfully adapted to a biochip format using various biomolecules (Kricka, 1998; Wang et al., 2004).

Using biochip, a number of samples (samples from different places) can be simultaneously analyzed, or multiple assays can be run on a single platform. The power of on-chip enzymatic assays offers great promise for point-of-care testing for real environmental samples, bioprocess monitoring, or on-site monitoring of many important analytes. It foresees that it is only a matter of time before biochip devices will become a serious competitor to established enzyme strips (Wang et al., 2004).

Biochips are also continuing to evolve as a collection of assays that provide a technology platform (Cha et al., 2005). Herein, one interesting development in this regard is envisioned to couple biomolecules such as enzyme to screen pesticides at milk collection centres. At milk collection centres, milk is brought from different sources for further supply. Before sending the milk to the consumer and collecting from origin, only few hours time left for the analysis of milk. These biochips could be used at milk collection centres for quality check and also to reduce the contaminations. By adding only few micro liters of milk samples, the biochip could able to test the presence of pesticides in milk. Moreover, if a biosensor apparatus is built into these biochips, a further application might be to measure the quality of milk online. The goal is to be able to control the quality of milk and other milk products. The future will also see novel practical extensions of biochip applications that enable significant advances to occur without major new technology engineering.

### 3.1.4 Reported work using biochip

Various materials such as gold, titanium, silicon, glass, quartz, and plastic (Hadd et al., 1997; Meusel et al., 1998; Wang et al., 2004) have been tested for biochip. Lab-on-a-chip for ultrasensitive detection of carbofuran by enzymatic inhibition was reported by Llopis et al. (Llopis et al., 2009). Biochips have been reported for environmental monitoring based on the

principle of enzyme inhibition (Dounin et al., 2010; Wang et al., 2004). In practice, on chip enzyme assays helps to understand (i) how enzymatic reactions behave on a small scale (ii) can enzymatic reaction interfaced with separation biochips (iii) how the biochip can be tailored to suit the requirements of particular enzymatic assays. The summary of biochip based assay for pesticides is summarized in Table 3.1.

This chapter presents different possibilities for conducting enzymatic assays on biochip platforms, along with potential advantages and selected examples of such biochips. The developed biochip devices offer great promise for downscaling enzymatic assays to the nanoliter level and also reduce the heterogeneity of the sample. The amount of enzyme consumed in biochip protocols is thus reduced by at least ca. four orders of magnitude over conventional assays. Combining this ability of biochips to handle micro or nanoliter volumes with their versatility and speed makes them ideal vehicles for enzymatic assays.

**Table 3.1** Reported biochip based assay for pesticides

| Chip | Analytes | Biomolecule | Transducer | References |
|---|---|---|---|---|
| Plastic chip | POx, MP | OPH | Electrochemical | Wang et al., 2004 |
| Disposable printed gold chip | POx | AChE | Electrochemical | Dounin et al., 2010 |
| Carbon screen printed chip | CPO | AChE | Electrochemical | Alonso et al., 2010 |
| Lab on chip | CF | AChE | Electrochemical | Llopis et al., 2009 |
| Glass capillaries | 2-4, D | Antibody | Optical | Dzgoev et al., 1999 |
| Micro patterned electrode | Diazinon | AChE/ChOx | Electrochemical | Han et al., 2011 |

### 3.1.5 Significant contribution

This work demonstrated an enzyme based biochip where different OPs can be analyzed with small sample volume. Determination of major OPs such as MP, MPOx, EPOx and CF was demonstrated using developed biochip. We have also tested the reference pesticide mixtures using biochip to check the additive effect of pesticides on AChE. Developed assay was thoroughly investigated on chip using tri-enzyme reaction (AChE/ChOx/HRP). The most significant achievement of the developed biochip is to quantify the OP residues in milk at low level. The developed biochip is fabricated using diode sputtering and photolithography and the schematic of biochip is shown as Figure 3.2.

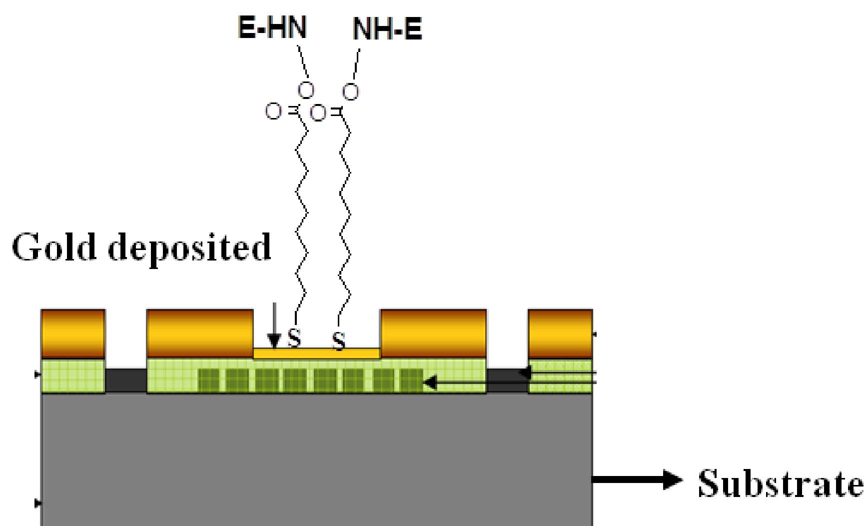



**Figure 3.2** Schematic of chip showing self assembly and enzyme coupling on gold surface.

### 3.1.6 Research gaps identified

Literature (research articles and patents) clearly indicates that there are no reports on OPs analysis in milk using miniaturized biochip which can provide highthroughput analysis and screen OPs. Thus, there is an immense need for miniaturized biochip based detection system which can provide rapid determination of OPs in milk at collection centres and requires less volume of samples.

### 3.1.7 Objectives

To develop a biochip for rapid analysis of OPs in milk at low level and also evaluate the effect of pesticide mix on developed probe (AChE).

### 3.1.8 Methodology

To determine the OPs residue in milk using biochip, enzyme AChE has been utilized. The chemistry between AChE and OPs are very well documented in the literature (Fukuto, 1990). Therefore, this chemistry has been used to determine the presence/absence of OPs in milk at low level using biochip based bioassay. The principle is described below:

**3.1.8.1 Principle:** The principle of the reaction follows the inhibition of AChE in the presence of toxic OPs. When acetylcholine reacts with AChE it produces choline. The choline produced by AChE is oxidized by choline oxidase into betaine with simultaneous production of $H_2O_2$, which is further used as substrate for the peroxidase with photon emission in the presence of luminol. The OPs or CM inhibits the AChE, hence, hydrolysis of acetylcholine does not occur and the consecutive reactions are blocked. The scheme of the principle is shown in the Figure 3.3.

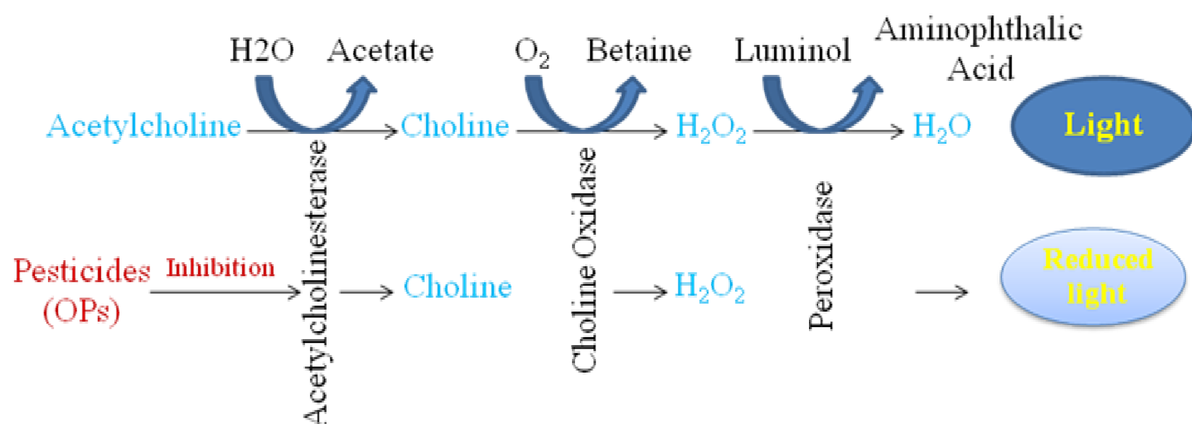



**Figure 3.3:** Principle of the AChE inhibition assay based on CL detection.

## 3.2 Experimental

### 3.2.1 Chemicals and biochemicals

All the enzyme, chemicals and bio-chemicals were prepared as per section 2.2 (chapter 2). Chips (Chip 1 and Chip 2) were fabricated in CARE, IIT Delhi by Prof Sudhir Chandra and his group using photolithography techniques. 11-Mercaptoundecanoic acid (MUA) was purchased from Aldrich (USA), Acetylcholine chloride was procured from Sigma Aldrich, USA. 1-ethyl-3(3/-dimethylaminopropyl) carbodiimide, HCl (EDC) was obtained from Calbiochem (Germany) and N-hydroxysuccinimide (NHS) was purchased from Merck (Germany).Quantification of enzyme reaction was done using method described in section 2.

### 3.2.2 Stock pesticide mixture

Pesticide mix reference solutions were procured from Dr. Ehrenstorfer. The concentration of pest mix-2 (lot no: 00709CY) was 10ng/µL (10 ppm) and Pest Mix-174 (lot no: 00622EA) was 200ng/ µL (200ppm). **Pest mix-2**: A stock solution of 1ppm was prepared by adding 10 µL of pest mix-2 in 90 µL of PB. This standard solution was further diluted with buffer to make different working solutions. Pest mix-2 contains Diazinon, Ethion, Malathion, Parathion ethyl and Parathion methyl at concentration 10 ng $\mu L^{-1}$.

**Pest mix-174**: The same procedure followed for pest mix-174. A stock solution of 2 ppm was prepared by adding 10 µL of pest mix-2 in 990 µL of 5% ethyl acetate. This standard solution was further diluted with buffer to make different working solutions. Reference pesticide mix 174 contains Azinphos-methyl, Bromophos-ethyl, Chlorpyriphos-methyl, Demeton-s-methyl and Diazinon, Ethion, Fenitrothion, Malaoxon, Malathion, Methamidophos, Methidathion, Paraoxon methyl, Phosphamidon and Trichlorphon at concentration 200 ng $\mu L^{-1}$.

### 3.2.3 Surface modification and enzyme immobilization

Surface modification was carried out in 3 steps, (i) functionalization (ii) activation, and (iii) enzyme coupling. The protocol for each step is described below:

**3.2.3.1 Functionalization of chip:** Initially, all assembly containers were rinsed with piranha solution and dried up. Two Au chips (Chip 1 and Chip 2) were soaked into piranha solution (7:1 of $H_2SO_4$ and 30% $H_2O_2$) for 5 min, then washed under the flow of distilled water and dried under a stream of high-purity nitrogen before use. For the thiolation of wells, 5 mM, 11-MUA alcoholic solution was added and kept for 24 h (assembly time) at room temperature. After assembly time, the chip was rinsed with ethanol several times so that no unbound 11-MUA left and dried under a stream of dry nitrogen.

**3.2.3.2 Activation:** Followed by thiolation, the carboxylic acid-terminated self assembled monolayer (SAM) was immersed into an aqueous solution mixture of 0.1 M EDC /0.1 M NHS (1:1) for 3 hrs. The resultant NHS ester monolayer were washed with PB (0.1 M, pH 7.4) to remove excess and unbound reactants and kept at 4°C.

**3.2.3.3 Enzyme coupling and performance evaluation:** After activation of wells on the chip, 2μL stabilized AChE (0.09 IU) was added on chip 1 and kept at 4°C. For AChE stabilization, the stabilizers (dextrose and trehalose) were dissolved in protein standard solution (2% w/v) separately. Different compositions of stabilizer and AChE were prepared to achieve best stability for inhibition studies. The effective stabilizing composition for AChE enzyme was used for stability performance in the analysis. In chip 2, 2.5 μL of ChOx (0.09 IU) and 1μL of HRP (0.04 IU) both were added and kept for 3 hrs. Further, the wells were rinsed exhaustively with PB and used immediately for enzyme activity measurement. The enzyme coupling on biochip surface was also confirmed by scanning electron micrograph images before and after the enzyme coupling. The obtained micrograph images are shown as Figure 3.4.

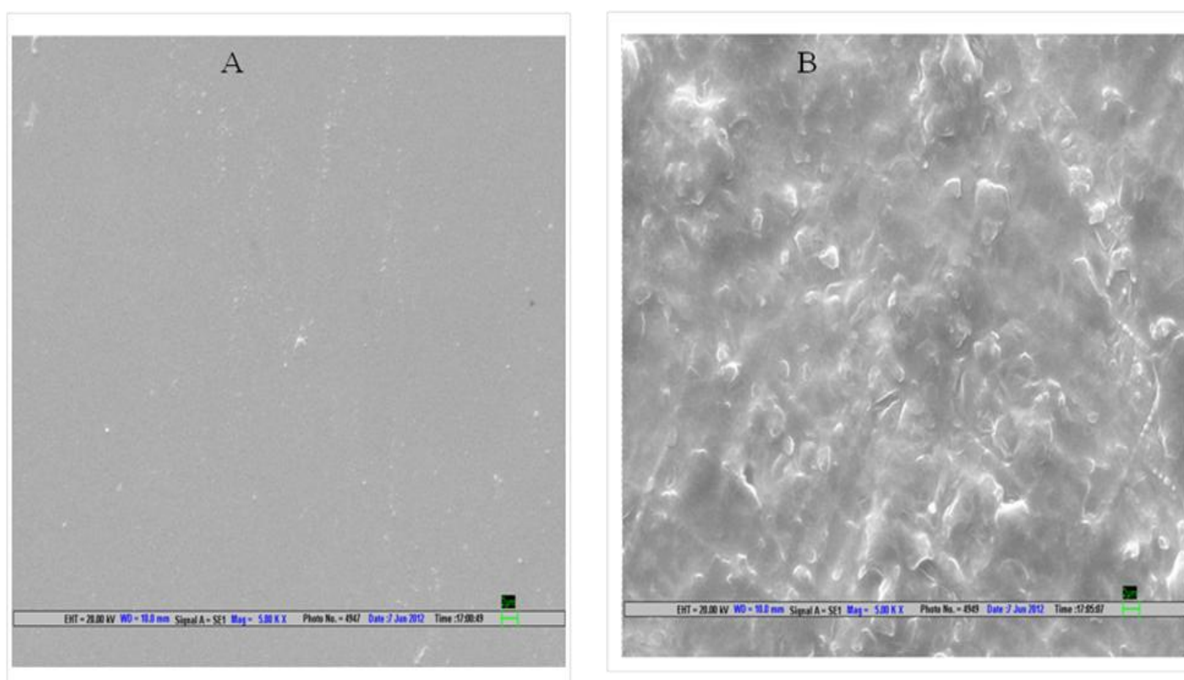



**Figure 3.4** Scanning electron micrograph of (A) bare Au surface and (B) coupled AChE on micro-biochip (Magnification= 5.00KX).

### 3.2.4 Enzymatic activity measurement on biochip (AChE/ChOx/HRP)

In the first step of analysis, enzymatic activity was evaluated separately. HRP and ChOx activity was investigated using $H_2O_2$ and ChCl. Subsequently, a fixed quantity of AChCl (2.5 µL) was dispensed on wells of chip 1. Stabilized AChE formed a thin film layer at the bottom of the well. AChCl and AChE reacted and choline produced during this reaction. Produced choline is collected from chip 1 using electronic micropipette and dispense on chip 2. Choline reacted with coupled ChOx and HRP and produced $H_2O_2$. This produced $H_2O_2$ is transferred to micro well plate with subsequent addition of luminol. Addition of luminol generates photons which confirm the enzymatic activity and also coupling on chip. The photographs of chip 1 and chip 2 are shown in Figure 3.5.

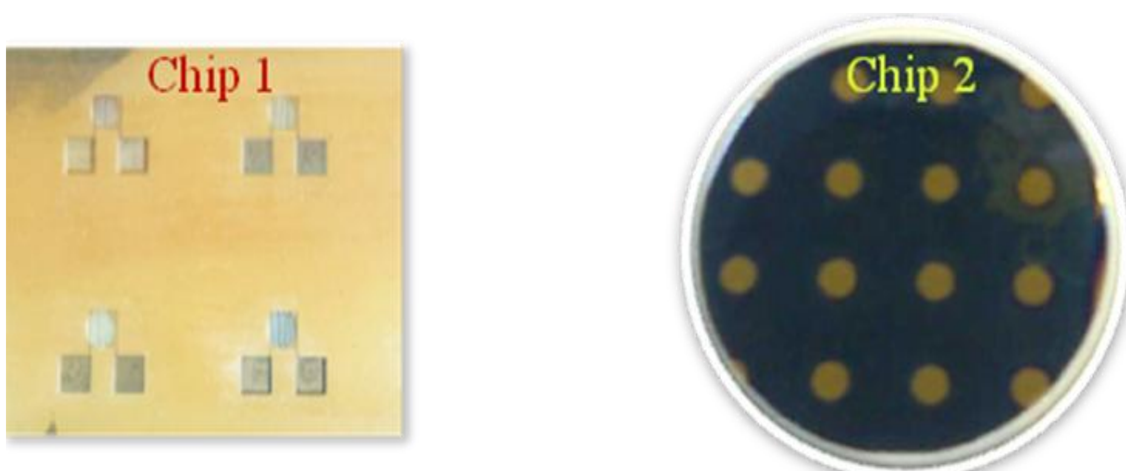



**Figure 3.5** Biochip for determination of OPs residue in milk (Chip 1 and chip 2).

### 3.2.5 Protocol for inhibition assay on chip

The OPs analysis using biochip is accomplished within 15 min. Different pesticide concentrations (0.005-200 μg $L^{-1}$) were spiked in milk for inhibition studies. The prepared inhibitor (2 μL) solution (ca MPOx) is added to the chip 1 and incubated for 10 min; 1mM acetylcholine chloride (AChCl) (2 μL) is added subsequent to the incubation step. After two-min reaction, the product (choline) is collected from chip 1 and transferred to chip 2. After two min reaction of produced choline and coupled ChOx and HRP on chip 2, the complete solution is dispensed to 1536 micro-well plate with subsequent addition of 1mM luminol (1.5 μL) to complete the reaction and measure the intensity using optical detector. The blank, reference and the sample intensities were compared to determine the inhibition of the activity of AChE and subsequent calibration curves were drawn. The schematic representing integration of on-chip enzyme assay (Chip 1 and 2) with PMT detector is depicted in Figure 3.6. The recorder shows the real time analysis display in the form of intensities in red and blue color dot. The wells are clearly visible in the picture and developed biochemistry on biochip using different cross linkers and enzymes are presented.

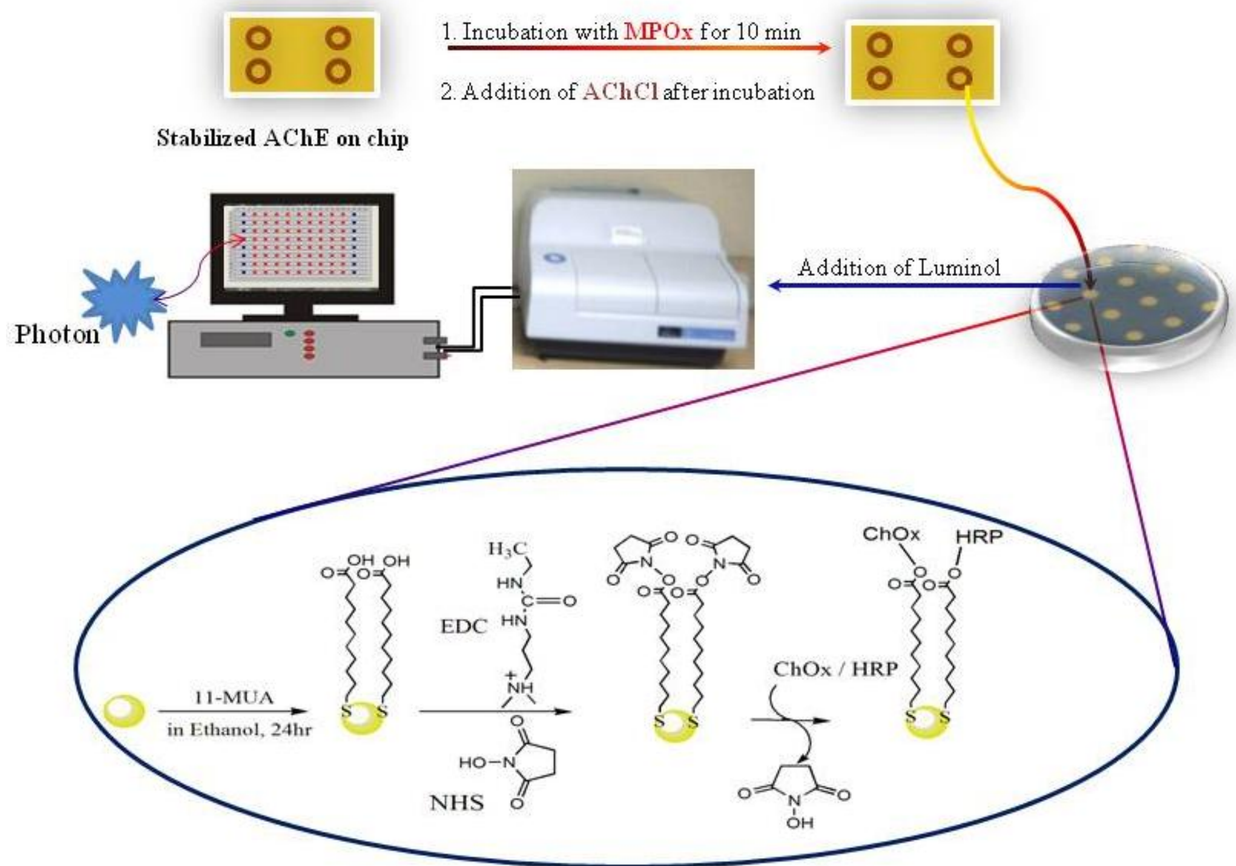



**Figure 3.6** Schematic representation of AChE inhibition using MPOx on chip.

## 3.3 Results and Discussion

### 3.3.1 Optimization of signal intensity on chip

Various substrates were tested for immobilization of AChE using developed protocol (details in section 3.2). Gold surface is reported as excellent support to carry out bio-molecular reactions as it provides a very good immobilization by surface modification chemistry (Dounin et al., 2010). The main aim of this study was to achieve a good sensitivity. Initially, signal intensity was optimized on gold chip. The signal obtained on gold chip is shown in Figure 3.7. High selectivity of Au surface toward AChE enzyme is evident from the signal intensity measured using micro-plate reader.

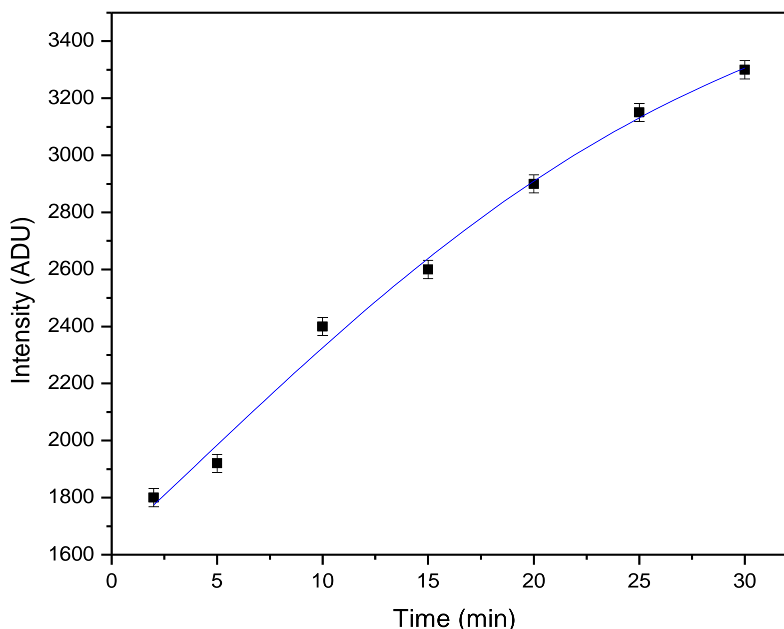



**Figure 3.7** Enzymatic activities on chip for signal optimization.

### 3.3.2 AChE activity in PB and milk

It is important to see the enzyme activity in matrix to know the actual performance in real sample. Hence, AChE activity was checked in PB and three different fat containing (0.5, 1.8 and 3.5%) milk. Experiments were performed using these matrices in the bioassay. AChE exhibits maximum activity in PB as against three different fat containing milk samples. After PB, the AChE activity is reasonably well in low fat (0.5%) milk sample. AChE shows less activity in 1.8% and 3.5% fat containing milk as shown in Figure 3.8. Further experiments were conducted using 0.5% fat containing milk samples with optimized dilution in PB (described in Chapter 2). We have also performed the experiment with spiked AChE in different fat content milk at different temperatures to see the AChE activity at high temperature with various milk samples (0.5%, 1.8% and 3.5% fat). This experiment also evaluated the stability of biochip at higher temperature using biochip 1 (inbuilt heater on chip1). The set-up for the experiment is shown as Figure 3.9 and results obtained with various fats containing milk at different temperature is shown as Figure 3.10.

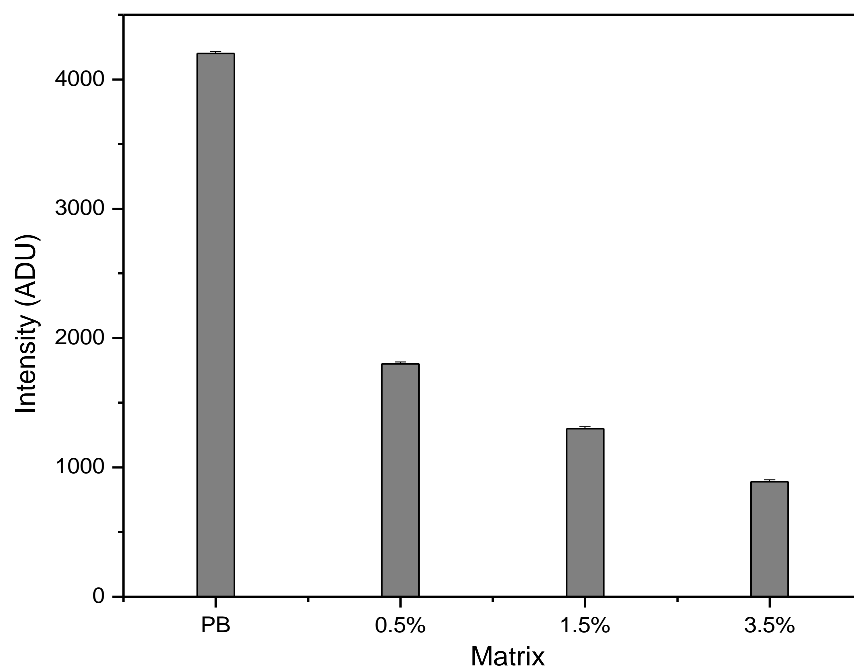



**Figure 3.8** AChE activity in PB and different fat content milk.

**Figure 3.9** Experimental set-up for OPs analysis using biochip device.

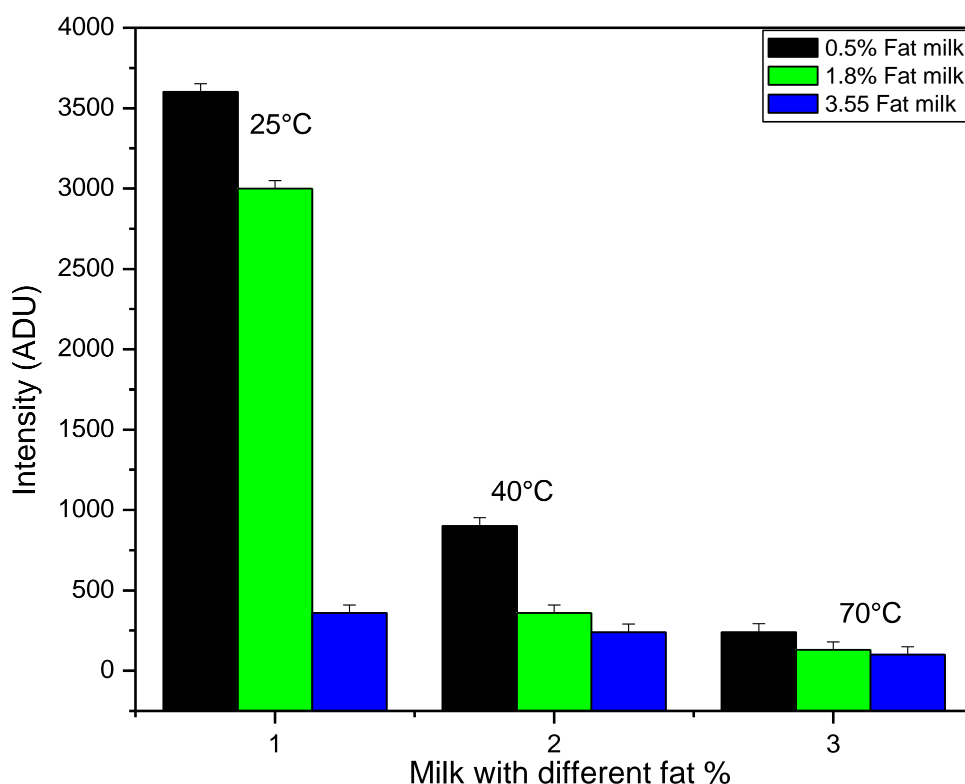



**Figure 3.10** AChE spiked in different fat containing milk at different temperatures.

### 3.3.3 Substrate calibration/optimization

Optimization of substrate is a crucial step for enzyme inhibition study and low level determination of pesticides. Therefore different AChCl concentrations (0.01-10 mM) were tested for substrate optimization. The substrate binds to the enzyme through multiple weak bonding interactions such as hydrogen bonding, Van der Waals forces, and hydrophobic interactions. AChE reacted to AChCl and product formed. Obtained AChCl curve obeys Michaelis menten profile presented in Figure 3.11. Initially, the intensity increases up to 3 mM AChCl. Then enzyme was saturated with substrate and intensity reached to a plateau. After 4 mM AChCl, enzyme shows steady performance. The kinetic data obtain was used to calculate kinetic constant, $K_M$ using Line weaver Burk plot. The $K_M$ 0.5 mM was calculated for further experiment.

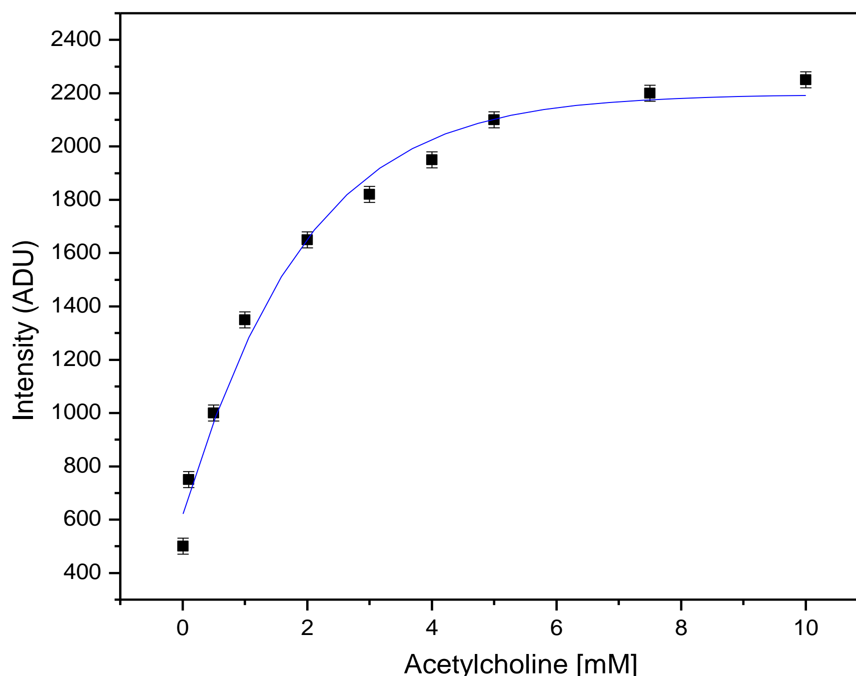



**Figure 3.11** Calibration curve of acetylcholine on biochip under optimized experimental conditions.

### 3.3.4 Calibration of MPOx, EPOx, MP and CF in low fat milk

MPOx, EPOx, MP and CF were allowed to react to stabilize AChE on chip for 10 min. Excess amount of OPs (which lies on chip surface) was withdrawn and the inhibited enzyme was allowed to react with AChCl. Produced choline was then added to the chip 2 (immobilized with ChOx/HRP) and quantified in victor using luminol. Several experiments were carried out to construct a good calibration curve. Calibration plots for MPOx, EPOx, MP and CF were constructed correlating I% with inhibitor concentrations and shown in Figure 3.12. Figure represents simultaneous inhibition studies on Au chip well. The dynamic range studied for all the pesticides were in the range 0.005-200 µg $L^{-1}$. The affinity of AChE towards these OPs can be easily distinguished by their individual values of LOD and $IC_{50}$ from calibration curve. The observed $IC_{50}$ in µg $L^{-1}$ for these four analytes are in the order MPOx (0.3039) < EPOx (0.6002) < MP (7.9174) < CF (194). For MPOx, the LOD was found to be 0.001µg $L^{-1}$ whereas, for EPOx, MP and CF the LODs were found 0.003, 0.01 and 0.0215µg $L^{-1}$ respectively.

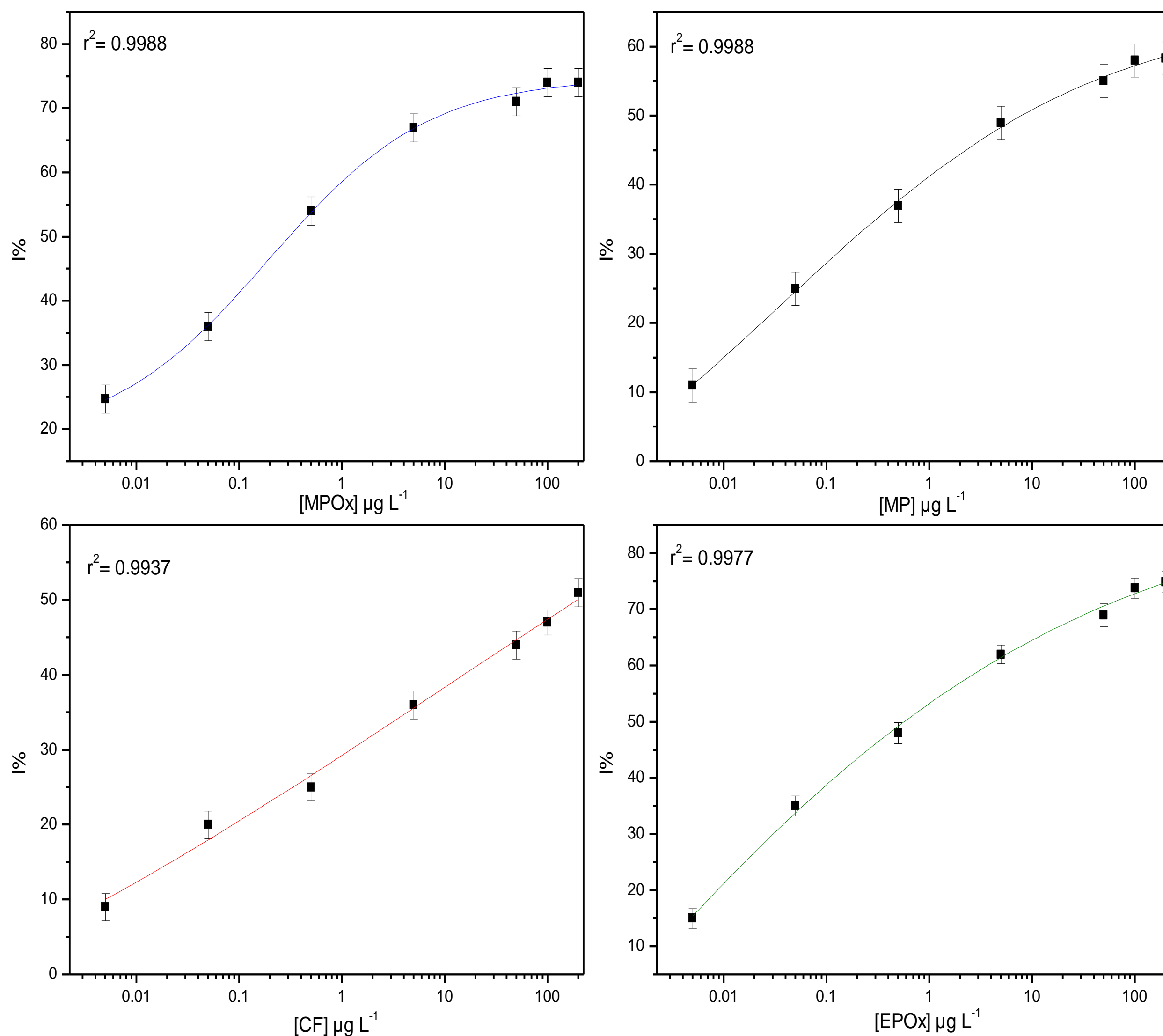



**Figure 3.12** Calibration of MPOx, EPOx, MP and CF in low fat milk under optimized experimental conditions.

The linear range obtained for MPOx was in the range 0.005-5µg $L^{-1}$ whereas EPOx showed linearity in the range 0.0029-50 µg $L^{-1}$. The obtained linear range for MP is in the range 0.01-50 µg $L^{-1}$ whereas CF showed linearity in the range 0.0215-200 µg $L^{-1}$. The $IC_{30}$ values of MP and its oxon form MPOx showed the differences of 10 times for AChE. This experimental result showed the toxicity of oxon forms as well as the affinity of AChE towards MPOx. On the basis

of obtained results we summarized the analytical figure of merits for the developed assay on chip and presented in Table 3.2. The present assay has been demonstrated with 5.5 µL assay volume. This work on biochip is among first few reports on miniaturised chip based assay (Arduini et al., 2012; Dounin et al., 2010; Wang et al., 2004).

**Table 3.2** Analytical figures of merits for proposed assay on chip for OPs determination

| Analytical parameters | MPOx | EPOx | MP | CF |
|---|---|---|---|---|
| Dynamic range (µg $L^{-1}$) | 0.001-200 | 0.001-200 | 0.005-200 | 0.005-200 |
| Linear range (µg $L^{-1}$) | 0.005-5 | 0.0029-50 | 0.01-50 | 0.0215-200 |
| LOD (µg $L^{-1}$) | Below 0.001 | 0.0029 | 0.01 | 0.0215 |
| Total assay volume (µL) | 5.5 | 5.5 | 5.5 | 5.5 |
| Analysis time ( min) | 15 | 15 | 15 | 15 |
| Apparent $IC_{30}$ (µg $L^{-1}$) | 0.0181 | 0.0346 | 0.1305 | 1.2752 |
| $r^2$ | 0.9988 | 0.9977 | 0.9988 | 0.9937 |

### 3.3.5 Comparison of assay performances between micro-plate well and biochip wells

Miniaturization of assay in different plate format leads to decrease in concentration of reagents, waste generation and analysis time due to lower well-density and decreased surface to volume ratio. Miniaturization leads to decrease in enzyme per assay requirement. In 384 well plate formats, 0.08 IU enzyme unit was utilized per assay with good signal intensity which is lower as compared to the 96 well plate based assay. In 1536 well plate assay, enzyme units were reached as low as 0.04 IU. The optimized assay with reduction in enzyme and substrate concentration and lowering the LOD by 10 times in case of 384 and 1536 was great achievement. The higher sensitivity could be possible using biochip for OPs analysis in milk. It was observed that $K_M$ is independent of miniaturization. Signal intensity variation was observed in case of different well plate format. Reasonably higher signal intensity at low reagent consumption was measured in case of 384 well plates as against 96 well plate assay. This may be due to the close proximity of

enzyme and substrate in miniaturized assay format. Close proximity also facilitates mixing of reactants. In case of 1536 well plate format, higher deviation is observed as compare to 384 well plate format due to lack of automation required for 1536 well plate format.

**Table 3.3** Comparison of assay performances using different micro well format

| Parameter | Well plate format | | | Biochip |
|---|---|---|---|---|
| | **96** | **384** | **1536** | **Au chip** |
| Incubation time (min) | 20 | 10 | 5 | 10 |
| $K_M$ /apparent $K_{M\ (mM)}$ | 0.3 | 0.27 | 0.25 | 0.25 |
| MDL (µg $L^{-1}$) | 0.2 | 0.008 | 0.003 | 0.001 |
| Analysis time (min) | 25 | 15 | 15 | 15 |
| IC 50 (µg $L^{-1}$) | 20 | 5 | 1 | 0.5 |
| Total assay volume (µL) | 100 | 20 | 8.5 | 5.5 |

### 3.3.6 Validation against standard chromatographic technique

The results obtained from the measurements on Au chips were cross validated against standard chromatographic method LC-MS/MS. As a model and most toxic inhibitors for AChE, MPOx solutions (1-12µg $L^{-1}$) were prepared, spiked in milk samples and tested using LC-MS/MS for cross validation. The LC-MS/MS was carried out in an accredited external laboratory SGS India Pvt. Ltd. Chennai. The real peak of pesticide blank and the peak obtained for MPOx spiked in milk (1µg $L^{-1}$) are shown in the Figure 3.13(a) and Figure 3.13(b).

Validation experiments against standard chromatographic methods confirmed the reliability of the biosensor test. In each data point there is a good co-relation and reliability as each point is measured 3 times. The LC-MS/MS analysis showed a detection limit of 1µg $L^{-1}$ for MPOx in milk sample. Therefore higher MPOx concentration was tested in calibration. For the pesticides to be accepted as validated the relative standard deviation of the repeatability must be less than or equal to the standard deviation proposed by the chip based method and the average relative recovery must be between 70 and 120%.

Pesticide Blank

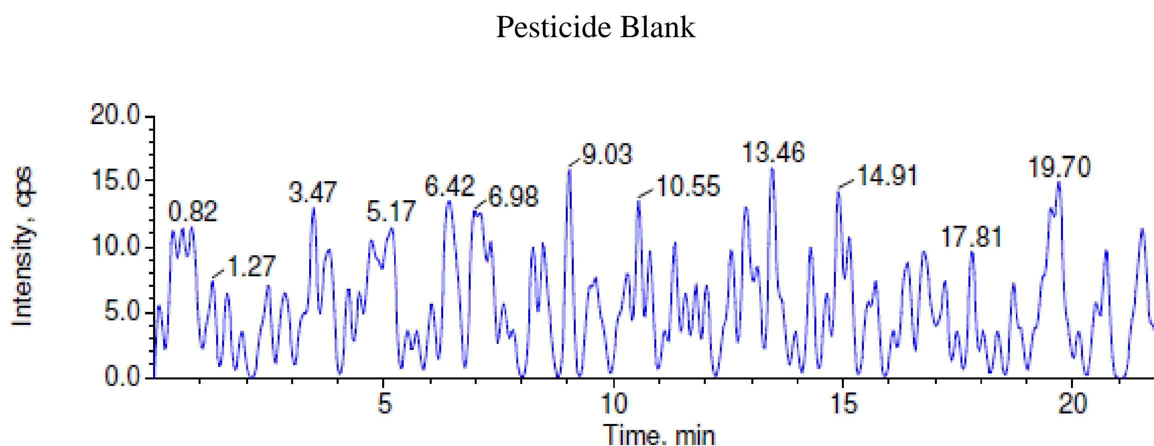



**Figure 3.13 (a)** LC-MS/MS ion chromatogram obtained for pesticide blank.

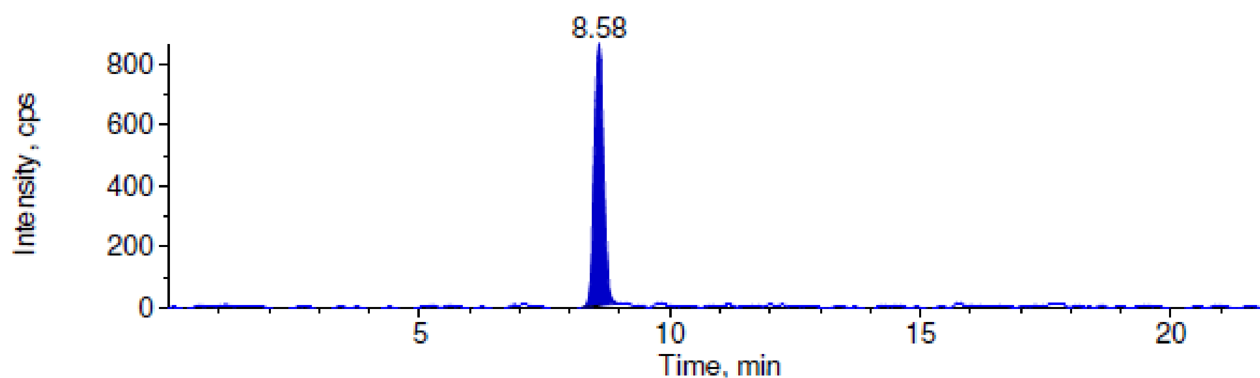



**Figure 3.13 (b)** LC-MS/MS ion chromatogram obtained for MPOx 5µg $L^{-1}$ in milk.

The co-relation observed in the data by both techniques is shown in Figure 3.14. In the graph, LC-MS/MS data is plotted as concentration vs area and biochip device data is plotted as concentration vs intensity. The recoveries obtained by both the techniques are also in good agreements.

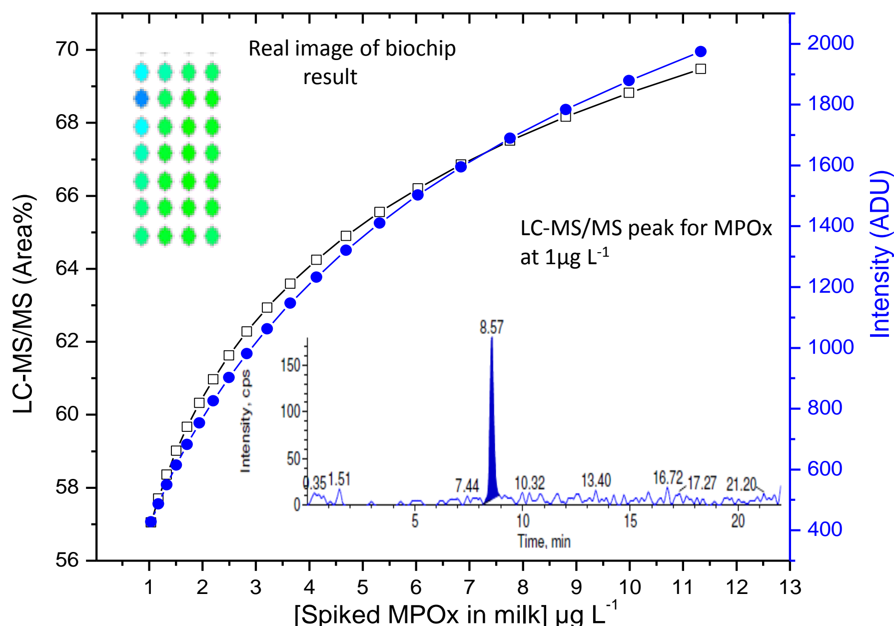



**Figure 3.14** Cross validation of results obtained through chip device and chromatographical technique.

### 3.3.7 Analysis of reference pesticide mixture in low fat milk

Analysis of pesticide mixture is done to evaluate the synergistic or cumulative inhibitions caused by different OPs toward AChE. Both pest mix-2 and 174 were spiked in low fat milk and analyzed. A calibration curve was constructed for pest mix 2 and 174. Both pesticide mixtures were tested in the similar range from 0.0001-10 µg $L^{-1}$ in low fat milk. Pest mix-2 shows linearity in the range 0.001-1µg $L^{-1}$ whereas pest mix-174 shows linearity in the range 0.001-10 µg $L^{-1}$. The calibration curve for pest mix-2 and 174 is shown in Figure 3.15. The obtained LOD for pest mix-174 and mix-2 was 0.001 µg $L^{-1}$ but with differences in I% by 10%. Importantly, pest mix 174 caused more inhibition to enzyme AChE at each concentration because of the presence of oxon forms of OPs in the mixture. The data obtained from both tested pest mix were co-related and compared with the data obtained from individual OPs. It was observed that LOD obtained for pest mix-2 was lower than LOD for MP (non oxon) whereas LOD obtained for pest mix-174 is similar to LOD of MPOx.

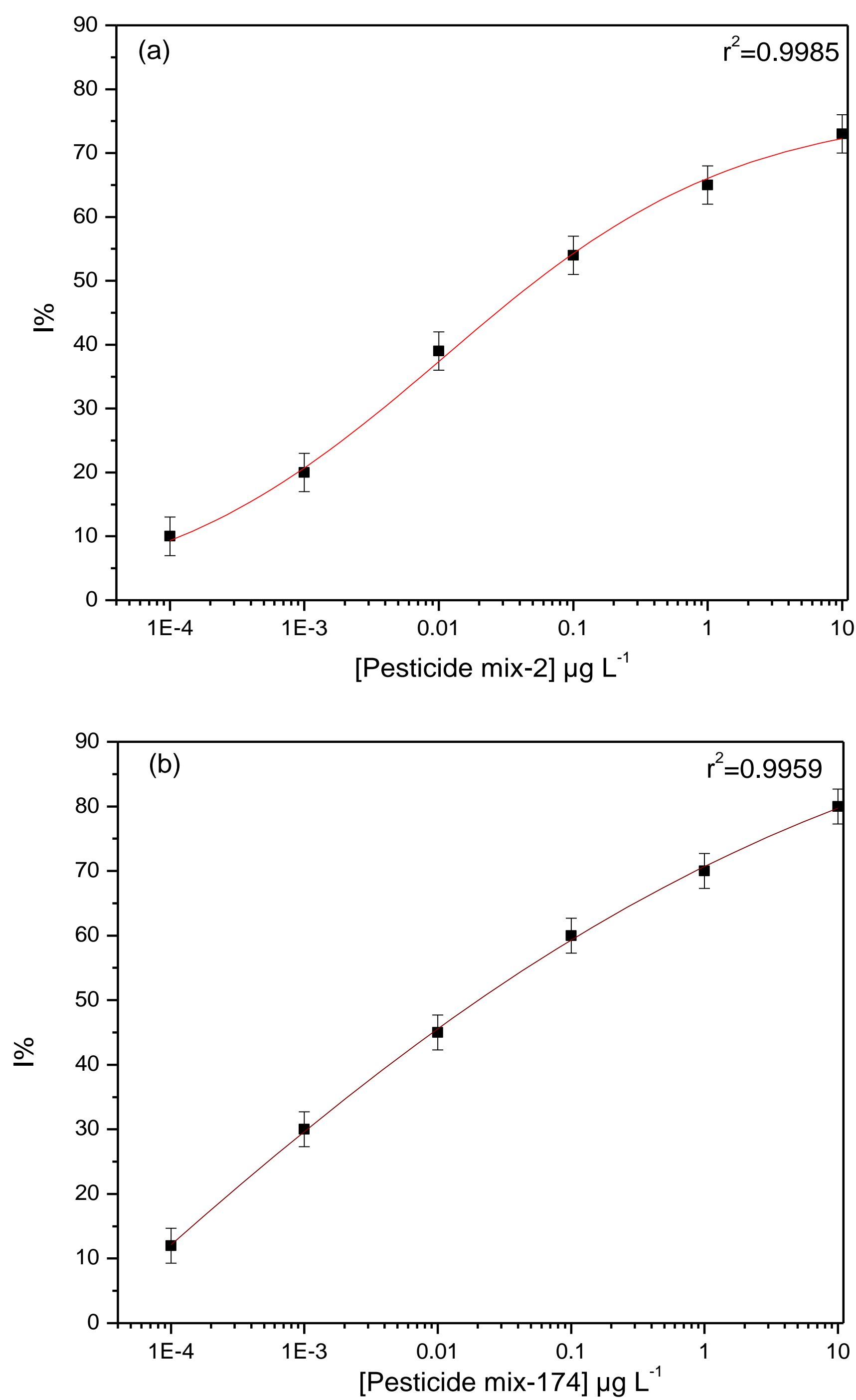



**Figure 3.15** Calibration of (a) pesticide mix-2 and (b) pesticide mix-174 in low fat milk under optimum experimental conditions.

### 3.3.8 Recovery studies

The applicability of the developed bioassay on chip for OPs determination in milk was tested for recovery studies. Low fat (0.5%) milk was spiked with different concentration of MPOx. Obtained inhibition values after incubation in milk were compared with the inhibition values obtained in calibration curve containing equivalent MPOx concentrations. Each sample was divided in two, with the first portion analyzed directly with the chip assay and the second portion was analyzed through a certified accredited lab. Three MPOx concentrations 0.1, 1 and 10 µg $L^{-1}$ were spiked in milk samples. Each concentration was spiked in duplication. One concentration was chosen at $IC_{50}$ and other two were below and above this point. At $IC_{50,}$ the % recovery lay between 90-95% whereas at 10 µg $L^{-1}$ the recovery rates are close to 100%. The recovery results are shown in the Table 3.4. From the obtained recovery rates, it is clear that LC-MS/MS could not detect concentration below 1µg $L^{-1}$ whereas for other tested concentrations LC-MS/MS showed good co-relation in % recovery. From the Table 3.4 it is evident that obtained recovery result confirms the reliability of developed method as well as the matrix. The recoveries were in the range from 92.8-99% for tested MPOx range.

**Table 3.4** Recovery studies in milk samples by spiking method

| Matrix | OPs analysis on chip | | | LC-MS/MS |
|---|---|---|---|---|
| | MPOx spiked (µg $L^{-1}$) | MPOx found (µg $L^{-1}$) | % Recovery | % Recovery |
| Milk-1 | 0.1 | 0.094± 0.3 | 94.0 | BDL |
| Milk-2 | 0.1 | 0.093 ± 0.1 | 93.0 | BDL |
| Milk-3 | 1 | 0.928± 0.4 | 92.8 | 92 |
| Milk-4 | 1 | 0.94± 0.61 | 94.0 | 89.8 |
| Milk-5 | 10 | 9.87± 0.3 | 98.7 | 90 |
| Milk-6 | 10 | 9.9± 0.1 | 99.0 | 92.8 |

### 3.3.9 Enzyme stability and reproducibility

The stability in signals response and reproducibility of the biochip performance was evaluated by the repetitive measurements ($n$=9) of acetylcholine at a fixed concentration of 1mM. Excellent storage stability was observed for immobilized enzymes in 0.1M PB at pH 7.4. Chip 1 and chip 2 both were stored at 4ºC. The corresponding % CV is 1.28 confirms that results are satisfactorily reproducible. In order to examine intraday (repeatability) and interday (reproducibility) response, intensity was recorded for a fixed concentration of substrate on Au chip. The experimental results show that the current intraday responses deviate by 2.63% suggesting thereby that biochip possesses adequate reproducibility for the determination of OPs. It is clear that the stabilized/immobilized enzymes on chip showed excellent activity for 30 days. Enzyme retained 92% activity means only 8% decreased in the activity whereas for 60 days enzyme retained 83.8% of its initial activity. When we tested the activity after 90 days, enzyme retained 78% activity. It is evident that immobilized enzyme is highly stable and retain its activity (about 80%) when stored at 4ºC. The chip stability is shown in the Figure 3.16.

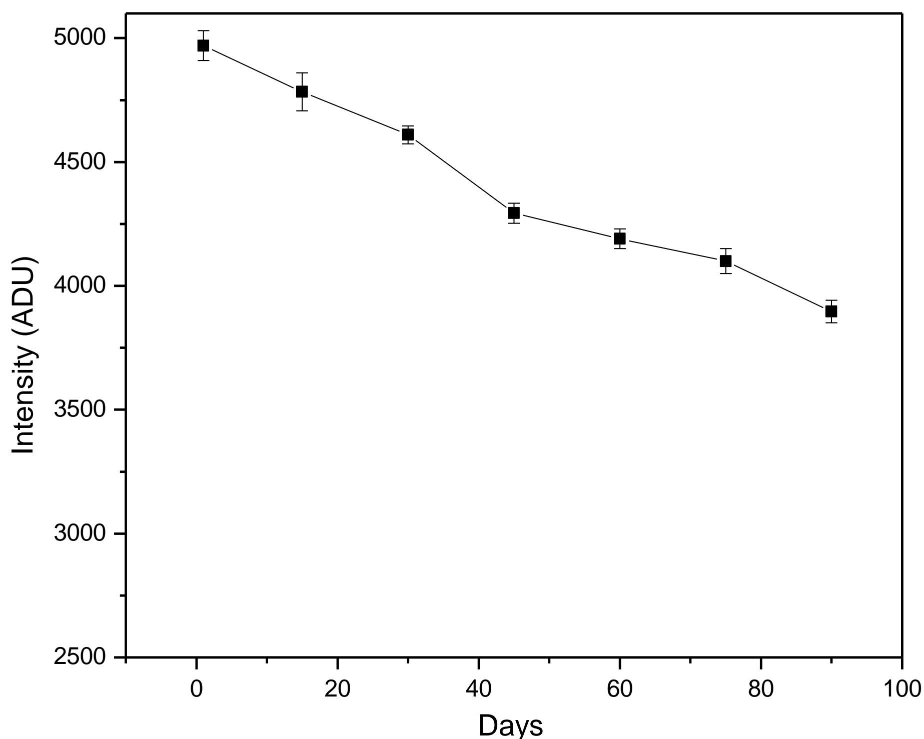



**Figure 3.16** Enzyme stability and reproducibility on chip for analysis of OPs in milk**.**

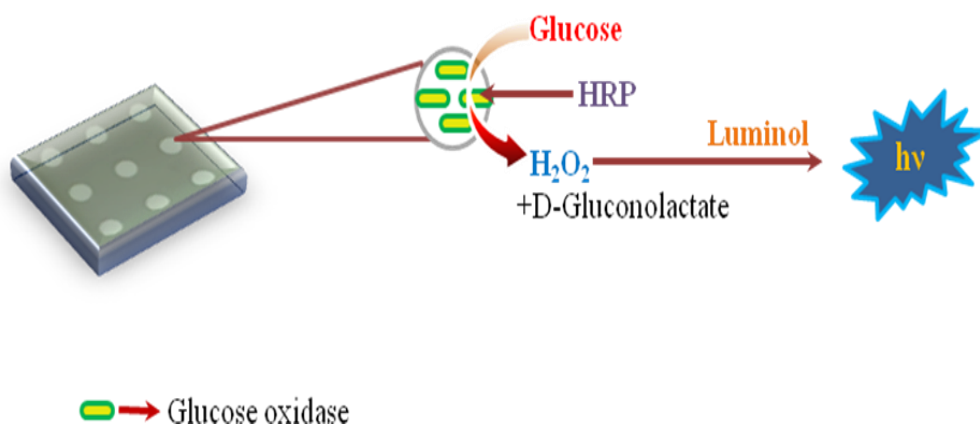
### 3.4 Conclusions

This study demonstrated the successful measurements of numerous important analytes (MPOx, EPOx, MP, CF and pesticide mix) simultaneously in a parallel operation. This chapter demonstrated OP quantification using biochip in milk as one of the significant contributions. Reduction of toxic waste, increase in sensitivity without compromising assay integrity is key features of developed biochip. The biochip could be stable at room temperature for more than 3 months without significant loss in enzymatic activity. A major achievement of the work is miniaturization of assay and point of care testing at milk collection centres with ease of operations. The developed biochip can determine OPs concentration lower down to 0.001 $\mu g\ L^{-1}$ in milk which is at par the EU standard. This work signifies application of biochip platforms as an ideal vehicle for enzymatic assays because of their versatility, efficiency, and ability to handle small volumes. The present invention has multifold applications in dairy industries and milk collection centers with ease and use of microwell chip device as a screening technology.

# CHAPTER 4

## A novel on chip inhibition assay for mercury analysis in water

**Novelty Statement:** A novel on chip inhibition assay for $Hg^{2+}$ analysis in water has been developed. GOx was immobilized on nanostructured aluminum oxide formed on oxidized silicon. Aluminium nano structures have been demonstrated as simple and cost-effective platform for $Hg^{2+}$ biosensing. The device facilitates high-throughput analysis of $Hg^{2+}$ in 20 minutes using CL technique. The device is simple, easy to operate and rugged.




GOx and glucose reaction on aluminium nanostructures

## 4.1 Introduction

Mercury contamination is a global problem because it poses severe risks for human health and the environment. Mercury originates mainly from coal-burning power plants, oceanic and volcanic emissions, gold mining, and waste combustion (Harris et al., 2003). Exposure to high concentrations of mercuric ion ($Hg^{2+}$) can damage the brain, nervous system, endocrine system, and many other organs (Tchounwou et al., 2003). Microorganisms in streams and oceans are capable of converting $Hg^{2+}$ into methylmercury ($CH3Hg^{+}$), which once entered into the food chain accumulates in the human body (Merritt and Amirbahman, 2009). Therefore, it is highly desirable to develop a sensitive $Hg^{2+}$ detection method that can provide simple, practical, and high-throughput routine determination of levels of $Hg^{2+}$ ions for both environmental and food samples. Current methods for determination of $Hg^{2+}$ include atomic absorption spectrometry, atomic fluorescence spectrometry, inductively coupled plasma mass spectrometry, and selective cold vapor atomic fluorescence spectrometry (Leopold et al., 2010). Although these instrumental techniques offer excellent sensitivity and accurate quantification for $Hg^{2+}$, they are generally rather complex, costly, non-portable, and may require complicated sample preparation procedures. Our life depends on water and its purity is of prime concern. Presence of $Hg^{2+}$ is reported in drinking water as well as coastal area in India as well as in other countries (Sahayam et al., 2010; Selid et al., 2009). According to the BIS and EU standard, the maximum allowable level of $Hg^{2+}$ ions in drinking water is 1µ $L^{-1}$. Thus, the development of a highly sensitive, facile, and practical assay for $Hg^{2+}$ ions remains a challenge.

We present a novel highly sensitive detection of $Hg^{2+}$ ions in water using GOx enzyme immobilized on aluminium surface. The developed assay of GOx using CL technique has immense potential for water quality as well as environmental analysis.

## 4.2 Experimental

### 4.2.1 Chemicals and biochemicals

$Hg^{2+}$AA/ICP Calibration /Check Standard for environmental analysis, 3-Aminopropyl-triethoxysilane, 99% from ACROS ORGANICS (USA), Dextrose from Hi Media laboratories

(India), Gluteraldehyde, 25% from Merck (Germany) and Glucose Oxidase from *Aspergillus niger.*

**4.2.2 Device fabrication**

The chip has been fabricated in collaboration with CARE, IIT Delhi. The device was fabricated by oxidation of aluminium film to produce aluminum oxide nanostructures on a 2-inch diameter silicon wafer using thermal evaporation technique. The aluminium nanostructured device is shown in Figure 4.1. The SEM of device is also shown in the callout of circular patterned aluminium.

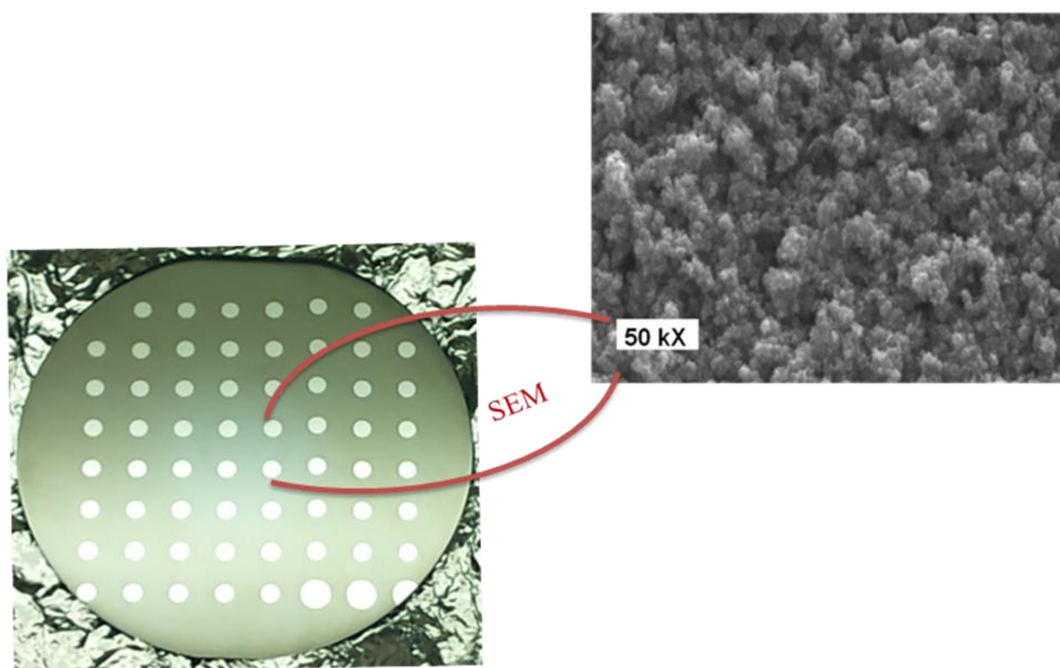



Figure 4.1: Photograph of oxidized silicon wafer having circular patterned aluminium. SEM (In callout) showing the nanostructured aluminum oxide formed after annealing of aluminium film.

**4.2.3 Immobilization of enzyme on chip**

The aluminium chip was silanized by immersing in 4% (v/v) solution of 3-aminopropyl tri-ethoxy silane in acetone at 30°C for 24 h. The silanized chip was thoroughly washed with distilled water and immerged in 2.5% (v/v) of aqueous gluteraldehyde solution for 2 h at room temperature, after being washed with distilled water and dried for 1 h, 2 µL solution of enzyme

GOx was poured on chip and kept at 4°C for 16 h. Multiple washing to eliminate the non-fixed enzymes follows the immobilization procedure.

### 4.2.4 Protocol for assay on chip

Presented chip assay exploited the properties of enzyme inhibition. Glucose (2μL) was added to aluminium chip immobilized with GOx. The reaction is followed by addition of HRP solution 1μL on the chip surface. The concentration of GOx was approximately 0.09 IU and HRP was 0.008 IU. The chip was kept at room temperature and 2 μL luminol was poured on the chip and photons generated were counted. The chip was reused for successive measurements by washing with 0.1M PB for 3-4 times. Same protocol was repeated for measurement. For inhibition studies, 2 μL of inhibitor was incubated with immobilized GOx for 10 min on chip well. Activity of inhibited GOx was compared with control assay or blank (without inhibitor).

### 4.2.5 Real sample analysis

The applicability of the developed bioassay on chip for $Hg^{2+}$ determination in water was tested for recovery studies. Water samples were spiked with 1 and 10 μg $L^{-1}$ concentrations of $Hg^{2+}$. Obtained inhibition values after incubation in water was compared with the inhibition values obtained in calibration curve containing equivalent $Hg^{2+}$ concentrations and recoveries were calculated.

## 4.3 Results and Discussion

### 4.3.1 Optimization of reaction parameters

Coupling of enzyme on aluminium surface was confirmed with enzyme-substrate reaction on chip wells. For immobilized GOx, various optimized experimental parameters were tested such as enzyme loading, pH, temperature and stability etc. The substrate specificity of immobilized enzyme was found to be similar to free enzyme, although immobilized enzyme require much more enzyme units it shows higher activities than those of free enzyme and with very less % RSD. Different glucose concentrations (0.15-10mM) were tested for inhibition measurments on chip. Obtained glucose calibration curve obeys Michaelis menten profile presented in Figure 4.2.

The signal obtained from the reaction was sufficient to carry out inhibition studies. The calculated $K_M$ was 0.75 mM for glucose. The optimized parameters are presented in Table 4.1.

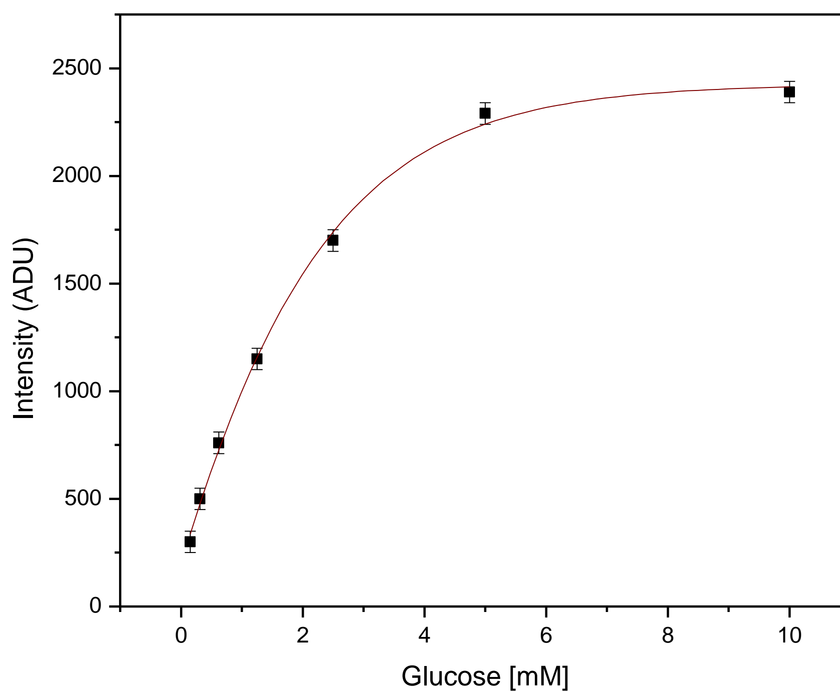



**Figure 4.2:** Calibration curve for glucose on aluminium chip

**Table 4.1:** Optimized parameters for GOx assay

| Optimized parameters | Immobilized GOx on aluminium chip |
|---|---|
| $K_M$ (mM) | 0.75 |
| pH | 7.5 |
| Temperature (°C) | 30 |
| Total assay volume ( µL) | 6.5 |
| Reusability of enzyme | Up to 5 runs |

### 4.3.2 On chip enzyme inhibition studies using $Hg^{2+}$

Experiments were conducted to determine $Hg^{2+}$ in water sample. Various $Hg^{2+}$ concentrations were tested ranging from 0.025-200 µg $L^{-1}$ under optimized experimental conditions. Glucose concentration 0.75 mM was used as substrate for sensitive determination of $Hg^{2+}$. Figure 4.3 shows the effect of $Hg^{2+}$ concentration on immobilized GOx on aluminium chip with 15 min incubation time. Good linearity was observed in the range 0.25-100 µg $L^{-1}$. The obtained LOD and LOQ for $Hg^{2+}$ determination is 0.25 µg $L^{-1}$. The graph shows that GOx is a good candidate for $Hg^{2+}$ determination at low level as exhibited the inhibition value up to 84% at concentration 200 µg $L^{-1}$. Broad linear range, low volume of sample (2 µL) and short analysis time (20 min) is the key feature of the assay. The analytical figures of merit are shown in Table 4.2.

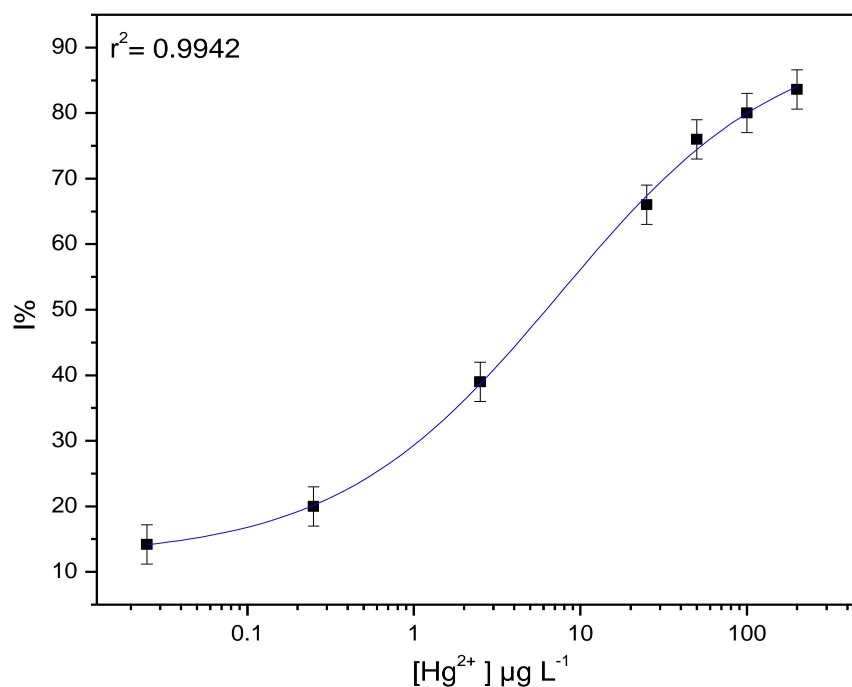



**Figure 4.3:** $Hg^{2+}$calibration curve obtained in water using GOx coupled aluminium chip

**Table 4.2:** Analytical figures of merit of $Hg^{2+}$ determination in water

| **Analytical parameters** | **Results** |
|---|---|
| Dynamic range ($\mu g\ L^{-1}$) | 0.025-200 |
| Linear range ($\mu g\ L^{-1}$) | 0.25-100 |
| LOD ($\mu g\ L^{-1}$) | 0.25 |
| Apparent $IC_{50}$ ($\mu g\ L^{-1}$) | 7.0 |

### 4.3.3 Recovery studies

To demonstrate the practicality of the proposed bioassay, the recovery test was studied by adding 1 and 10 µg $L^{-1}$ $Hg^{2+}$. For, 1µg $L^{-1}$ $Hg^{2+}$, the % recovery lay between 93.8-94% whereas at 10 µg $L^{-1}$ the recovery rates are close to 100%. The recovery results are shown in the Table 4.3. The results indicated that the chip based $Hg^{2+}$ biosensing is reliable and reproducible. It could be used for direct $Hg^{2+}$screening for water samples.

**Table 4.3:** Recovery of $Hg^{2+}$ in water sample using aluminium chip

| **Matrix** | **$Hg^{2+}$ analysis on chip** | | |
|---|---|---|---|
| | **$Hg^{2+}$ spiked ($\mu g\ L^{-1}$)** | **$Hg^{2+}$ found ($\mu g\ L^{-1}$)** | **% Recovery** |
| DW1 | 1 | 0.094± 0.3 | 94.0 |
| | 10 | 9.87± 0.3 | 98.7 |
| DW2 | 1 | 0.938± 0.4 | 93.8 |
| | 10 | 9.9± 0.1 | 99.0 |

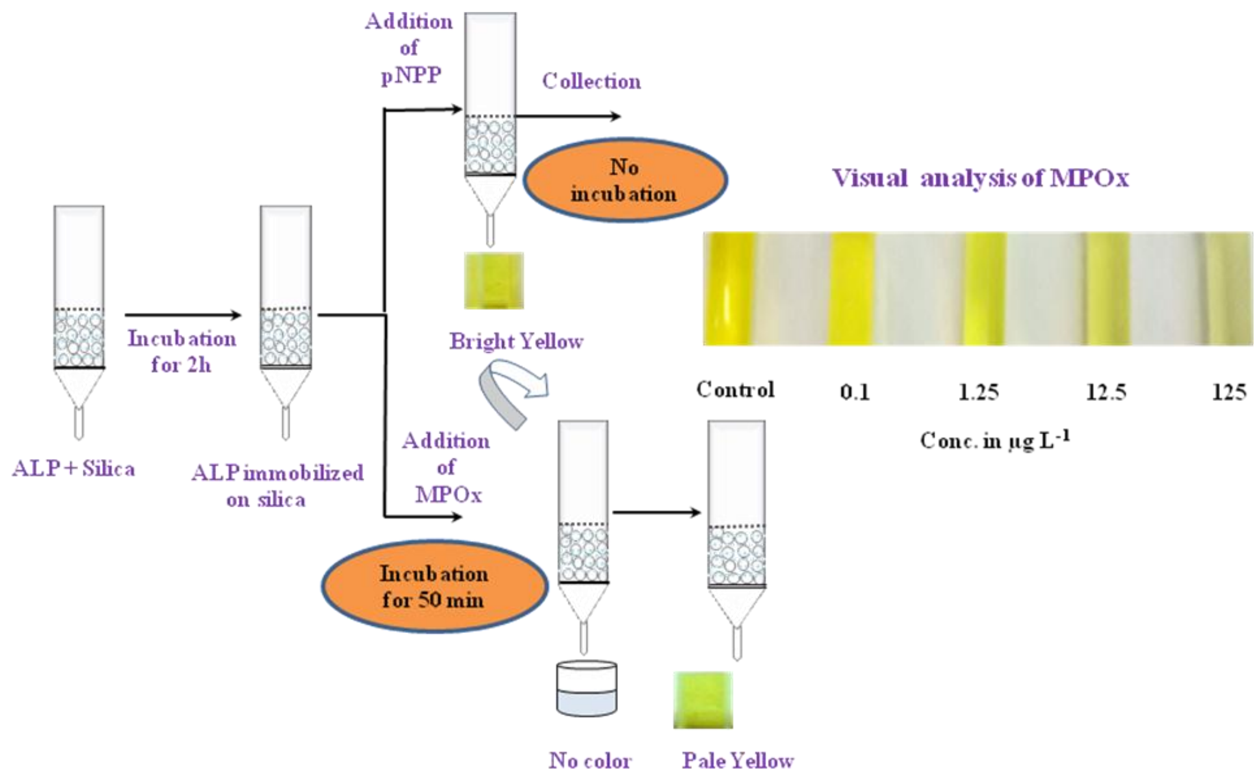
## 4.4 Conclusions

This work demonstrated a novel on chip inhibition assay for $Hg^{2+}$ analysis in water using GOx immobilized on aluminium biochip. The method is simple, economical and rapid to screen $Hg^{2+}$ in water. On chip measurement for $Hg^{2+}$ was found highly sensitive with LOD 0.25 µg $L^{-1}$. Such system will facilitate sensitive *in-situ* measurement of trace HM contaminants in water such as $Hg^{2+}$. The obtained inhibition is purely due to the enzyme immobilized on chip with total assay volume of 5µL. Besides the drastic reduction in the size of the analytical system, such miniaturization should lead to increase speed, minimal reagent consumption and disposal. A good repeatability is observed without significant changes in the response signal over 5 continuous measurements for glucose. Assay on chip allows testing for trace metals to be performed more reliably and easily. Further improvements can be done in the assay using more selective enzymes with other heavy metals.

# CHAPTER 5

## A visual colorimetric bioassay for determination of pesticides in drinking water

**Novelty Statement:** This work demonstrates a simple and visual technique for pesticides detection in water. Novel approach of micro-silica column for analysis of four different pesticides was used. The technique is free from interferences of HMs at low level, affordable to common population, simple to perform and interpret the results visually. The assay is optimized to meet the drinking water quality criteria for safe drinking water.




Schematic representation of visual colorimetric bioassay for OPs analysis

## 5.1 Introduction

A large human population in India is concentrated in rural settings and relies on open wells for drinking water. Lack of constant monitoring of water quality puts them at a higher risk of exposure to pollutants. The human health in developing countries such as India, suffers greater damage either due to lack of affordable techniques or due to the expensive technology involved for the analysis of water samples. This certainly affects the quality of life.

### 5.1.1 Affordability of assays

The high operative costs and the requirement of specialized personnel for pesticide detection, incentive to develop alternative solutions such as the simple, cheap, rapid, and affordable set up to use of biosensors. The quality of available drinking water is a powerful environmental determinant of health. Consequently, fast yet inexpensive visual testing assays suitable for field applications are the need of the hour in developing world (Pohanka et al., 2010). Affordability of a given method is a key parameter in the adaptation of bioassay/biosensors for the routine screening. This characteristic, combined with the miniaturization, low waste generation, ease of handling and low volume of sample will fulfill the requirement at field level.

### 5.1.2 Colorimetry as a simple technique

In recent years, a series of researches were performed on the development of inhibition based biosensors for pesticide determination using enzymes such as AChE, BuChE, OPH, ALP etc (Ayyagari et al., 1995; Deo et al., 2005; Imato and Ishibashi, 1995; Zhang et al., 2012). These studies include application of various transduction techniques such as amperometry, fluorometry' colorimetry and luminometry. Among them, colorimetric biosensors (Pohanka et al., 2010) have been reported extensively as simple, rapid and economical technique which can be easily extended for field studies as it is perceivable to the human eye. Colorimetric biosensors can exploited for indirect monitoring of organic (e.g. pesticides) or inorganic substances (heavy metals) that inhibit biocatalytic properties of the biosensor. The sensitivity and range of detection of a colorimetric biosensor depend on the ease of approach of the analyte to the active sites of the immobilized enzyme. The development of portable colorimetric assays requires a method for immobilization of bio-component (enzyme) on to a suitable substrate as well as an appropriate

method to generate a detectable color change (Luckham and Brennan, 2010). Direct monitoring of analytes (enzymes or substrates) has been the major application of colorimetric biosensors (Wang et al., 2009). Disposable biosensors for analysis of OPs and CB have also been reported in the literature (Joshi et al., 2005; Montesinos et al., 2001). Application of developed assay for real sample analysis is the most important step towards biosensor development. A wide number of analytical methods, based on the most commonly employed physicochemical techniques for the identification of organic compounds (HPLC, GC-MS etc.) are available to detect and determine pesticides quantitatively in different matrices in real samples; nevertheless, none of the above mentioned techniques is affordable for common population. Many research groups have utilized ALP for determination of different class of pesticides and HMs (Mazzei et al., 2004; Pande et al., 1996).

### 5.1.3 Detection techniques for OPs

Several studies have been published regarding the use of ALP in biosensors for determination of pesticides that inhibit biocatalytic properties and presented in Table 5.1.

**Table 5.1** Summary of reported bioassay and biosensors for pesticide analysis using ALP

| Technique | Analytes | LOD/Range | References |
|---|---|---|---|
| CL | POx and MP | 500-700 ppb | Pande et al., 1996 |
| CL | POx and MP | 50 ppb | Ayyagari et al., 1995 |
| CL | POx | 50 ppb | Ayyagari et al., 1995 |
| FL | Fenitrothion | 45.5 µM | Sanchez et al., 2003 |
| Electrochemical | MT | 0.1 $\mu g\ L^{-1}$ | Mazzei et al., 2004 |
| Chemometrics | Dichlorvos, POx | 1 $mg\ L^{-1}$ | Danzer and Schwedt, 1996 |
| Electrochemical | CF | ppb | Shyuan et al., 2008 |

ALP a non-specific phosphomonoesterase, is a dimeric metallo enzyme containing zinc ($Zn^{2+}$) and magnesium ($Mg^{2+}$) ion co-ordinated to its active site. This enzyme has been used for monitoring pesticides that inhibit biocatalytic properties. CL technique has been used extensively for OPs analysis using ALP enzyme.

### 5.1.4 Significant contribution

This chapter demonstrates the visual detection of both OPs and CB using visual as well as colorimetric bioassay. Researchers have reported use of cost affordable and stable enzyme for OP determination. The combination of ALP and functionalized $SiO_2$ as a biosensing platform for OPs determination using colorimetric technique so far has not been exploited. The presented technique will ensure supply of safe drinking water to the common population by screening OPs and CB residues present in drinking water. To best of our knowledge; this is among the few most simple yet sensitive field screening method.

### 5.1.5 Research gaps identified

Although, sensitive techniques are reported for OPs analysis but they need sophisticated instrumentation and time. The available techniques are lacking in ease of operation and focused on single analyte detection. Thus there is immense need for robust, simple visual technique which can be easily deployable and affordable to common people for safe drinking water. This work also demonstrated the cumulative toxicity of OPs in water.

### 5.1.6 Objectives

To develop a visual colorimetric affordable bioassay for OPs screening in drinking water using ALP and $SiO_2$ based column.

### 5.1.7 Methodology

Inhibition based biosensor for OPs analysis has been reported using ALP enzymes. But its application to screen the OPs in real water samples has remained a challenge. Development of OPs bioassay as a screening assay for water was undertaken and demonstrated the bioassay in disposable $SiO_2$ based column. The same bioassay was demonstrated successfully in 60 µL

assay volume in microwell plate using ALP inhibition through OPs and quantitatively determined the I %. The following principle was adopted to execute the experiments.

### 5.1.7.1 Assay principle

ALP catalyses the hydrolysis of phosphate esters in alkaline buffer and produces a yellow color compound *para*-nitro phenol and inorganic phosphate which can be measured at 405 nm. Enzyme (ALP) on incubation with inhibitor such as MPOx forms a complex due to which the approach of the substrate to catalytic site is affected and hence the reduced activity on reaction with pNPP. Thus the colour of the product and also the absorbance is reduced. The principle of ALP inhibition is presented in Figure 5.1.

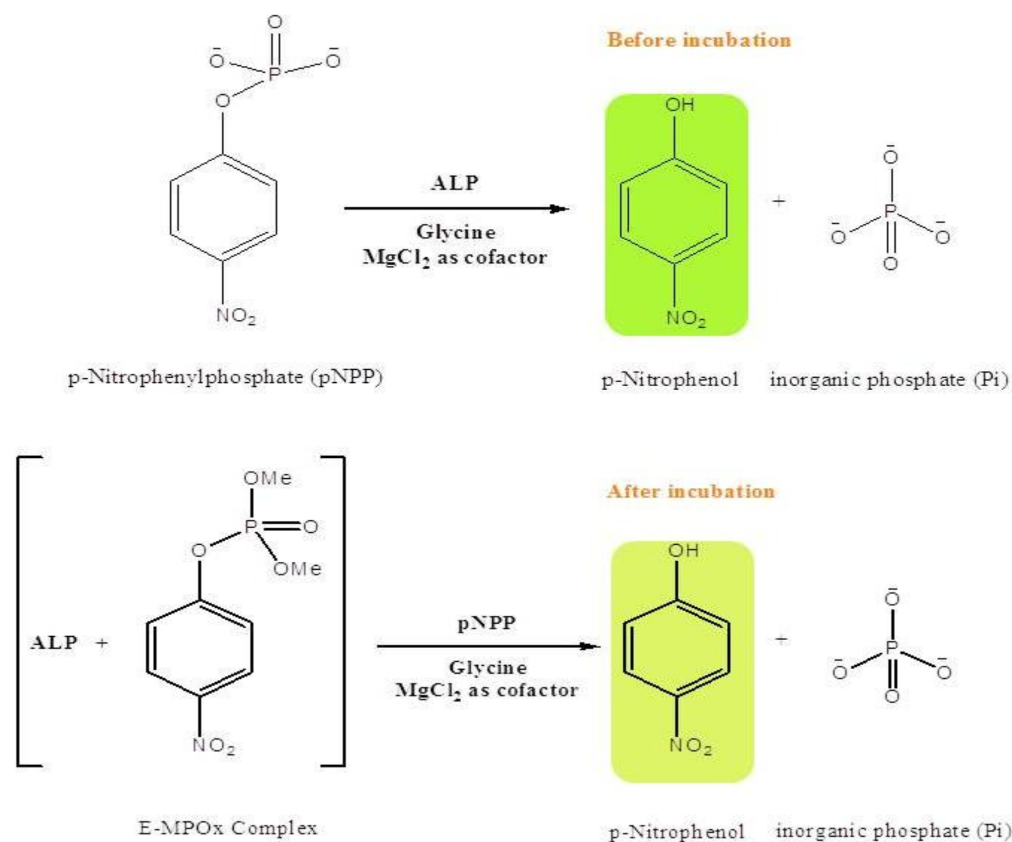



**Figure 5.1** Structural representation of principle of ALP-MPOx colorimetric assay.

## 5.2 Experimental

### 5.2.1 Chemicals and biochemicals

Alkaline phosphatase, Type IV from *porcine intestinal mucosa* 1.5 units/mg/solid purchased from Sigma Chemical Co. St. Louis (USA), Phosphatase, alkaline from calf intestine, grade 1, from Roche Diagnostics (USA), 4-Nitrophenyl phosphate disodium salt hexa hydrate (pNPP) from Fluka (UK). Silica gel, functionalized, Amino-3 was procured from Acros organics New Jersey (USA). $MgCl_2$ and actetonitrile was procured from Merck (Germany). Mercury AA/ICP calibration/check standard for environmental analysis, from Sigma Chemical CO. (MO, USA), Cadmium standard solution from Merck Germany and Lead powder from sigma Aldrich Germany were procured. Other chemicals were of AR and GR grade from Merck (Germany). Micro pipettes (Eppendorf, Germany) were used for assay. Corning, Costar 96 strip-well plates were obtained from Sigma (USA). Colorimetric measurements were recorded using 2030 Multilabel Reader Victor™ X4, Perkin Elmer (USA). UV-visible spectra was measured on the model V-570 Spectrophotometer, Jasco (Japan), multichannel pump was used for liquid dispensing in the column and micro-well plates Gilson (France). Conductivity and pH meter, model seven multi, six digit balance from Mettler Toledo (Switzerland) was used.

### 5.2.2 Preparation of stock solution and standards

7.5 g of Glycine purified was dissolved in 1L of double distilled water ($ddH_2O$) to prepare 0.1M glycine buffer (GB) and pH was set to 10.6. Stock solution of ALP from *Porcine intestine mucosa* was prepared by dissolving 3 mg of ALP in 1mL GB. Supplied ALP from *Calf intestine* solution contains 1500 units in 82μL solution. 2μL solution was taken and added in 498μL GB. ALP was further diluted prior to the experiments. 0.01M pNPP was prepared by dissolving 30 mg powder in GB. Working dilutions of the enzyme was prepared same day when assay was carried out. 0.01M $MgCl_2$ was prepared using 20 mg solids in 100mL $ddH_2O$.

### 5.2.3 UV- Vis analysis for the screening of ALP activity

ALP from two different sources (porcine intestinal mucosa and calf intestine) was tested to evaluate the sensitivity and affinity towards the OPs residue for visual detection using bioassay.

UV-Vis spectra (320-500 nm) were recorded for both ALP's (porcine intestinal mucosa and calf intestine) in the presence and absence of MPOx at the concentration 5µg $L^{-1}$. Initially, 10µL ALP (0.5U) and 100µL MPOx was incubated for 40 mins, after incubation 20µL pNPP and 10µL $MgCl_2$ was added in the disposable cuvette and then volume was made up to 1500µL using GB.

### 5.2.4 Experimental set-up

The experimental set-up consist of reagent reservoir for keeping the substrate, connected through multichannel pump for dispensing the liquids to $SiO_2$-ALP column as a biosensing platform, 96-well plate for analysis and Multilabel reader to provide the final signal. The photograph of experimental set-up is shown in the Figure 5.2

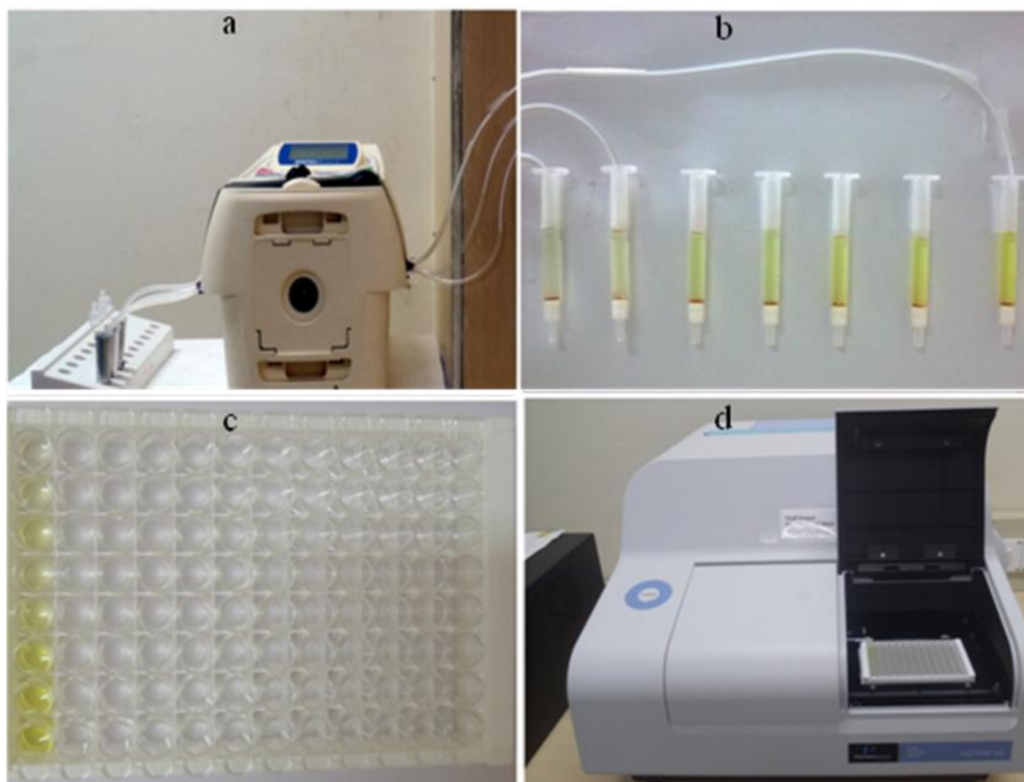



**Figure 5.2** Experimental set up for analysis of MPOx, EPOx, MP, CF using $SiO_2$-ALP micro column. Experimental setup; **a**: reservoir and multichannel liquid dispensing system, **b:** disposable column with $SiO_2$, **c**: assay performed in 96-micro well plate, **d:** micro-well plate reader.

### 5.2.5 Construction of $SiO_2$ based micro column

30 mg aminated $SiO_2$ was taken in a 2 mL capacity centrifuge tube. 1 mL 2.5% gluteraldehyde was added in centrifuge tube containing $SiO_2$ and desiccated for 1hr. Shortly, the gluteraldehyde-$SiO_2$ mixture was filtered using 0.22 micron filter paper followed by a thorough washing step using dd$H_2O$. Subsequently, different ALP units (0.011-0.22 IU) were dispensed in the $SiO_2$ column using multichannel pump. The coupling was allowed to take place overnight with gentle shaking. After shaking, $SiO_2$ column was washed by running the dd$H_2O$ on $SiO_2$- ALP micro-column. Tri-ethanolamine was added to terminate all the unreacted groups on the matrix and then washed with 0.1M GB, pH 10.6. Scanning electron micrographic analysis was carried out to see the morphological changes on $SiO_2$ micro particles by ALP.

### 5.2.6 Substrate optimization

$SiO_2$-ALP packed columns were used to optimize enzyme-substrate reaction, various concentrations (0.05-1mM) of pNPP was tested with fixed ALP coupled in $SiO_2$ column. 20 µL of each p-NPP concentration was dispensed in $SiO_2$-ALP column and incubated for 20 min. The resulting yellow colored solution was analyzed in 96-well plate at 405 nm.

### 5.2.7 Inhibition studies using disposable $SiO_2$-ALP column

Inhibition studies were carried out using four different inhibitors (MPOx, EPOx, MP and CF) as well as with binary and tertiary combination of pesticides. 30 µL of each inhibitor was added in $SiO_2$-ALP column and incubated. Initially, experiments were started with 10 min incubation followed by addition of 20 µL pNPP and 10 µL $MgCl_2$ and absorbance was taken at 405 nm. Absorbance of the reaction confirmed that 10 min incubation time did not contribute significant inhibition on the ALP activity. Later on ALP was incubated with the inhibitors for different incubation time ranging from 10-50 min. Incubation time of 30 min was found optimal. All the experiments were performed at room temperature (25ºC). We also investigated the inhibition phenomenon of potent inhibitors (MPOx and EPOx) with 50 min incubation time to see the extended I% of ALP.

### 5.2.8 Real sample analysis using bioassay and LC-MS/MS

The presented bioassay was tested for MPOx determination in drinking water, tap water, and ground water. To preserve the chemical nature of sample and minimize interferences due to suspended solids, a simple one step filtration strategy was adopted for recoveries of MPOx in spiked water samples. Water samples were collected from the local source on the same day. The collected samples were filtered through 0.22µ filter assembly and then diluted with freshly prepared 0.1M GB, pH 10.6 in series of dilutions. The diluted samples were spiked with 0.1 and 10 µg $L^{-1}$ MPOx and assay was carried out to study matrix effect and recoveries. The same samples were also analyzed using standard chromatographic method LC-MS/MS for recovery study from accredited external lab SGS India Pvt. Ltd. Chennai.

## 5.3 Results and Discussion

### 5.3.1 Screening of sensitive ALP

The UV-Vis spectra of ALP inhibition in cuvettes showed that ALP from porcine intestinal-mucosa was found more sensitive as against ALP from calf intestine. Therefore, further experiments were carried out using ALP from porcine mucosa for the all pesticides. The difference in the absorbance can be seen from Figure 5.3. The I% was calculated from the recorded absorbance. It was found that at 12.5 µg $L^{-1}$ MPOx concentration, ALP (porcine intestinal mucosa) was inhibited 58.22% whereas ALP (calf intestine) was inhibited only 35.27%. Obtained results confirm that ALP from porcine intestinal mucosa is more sensitive and suitable as a bio-component for this study towards pesticide determination. Although the absorbance is close to 1 but it is importance to have more absorbance in the inhibition studies. Therefore, higher absorbance values were recorded for inhibition studies.

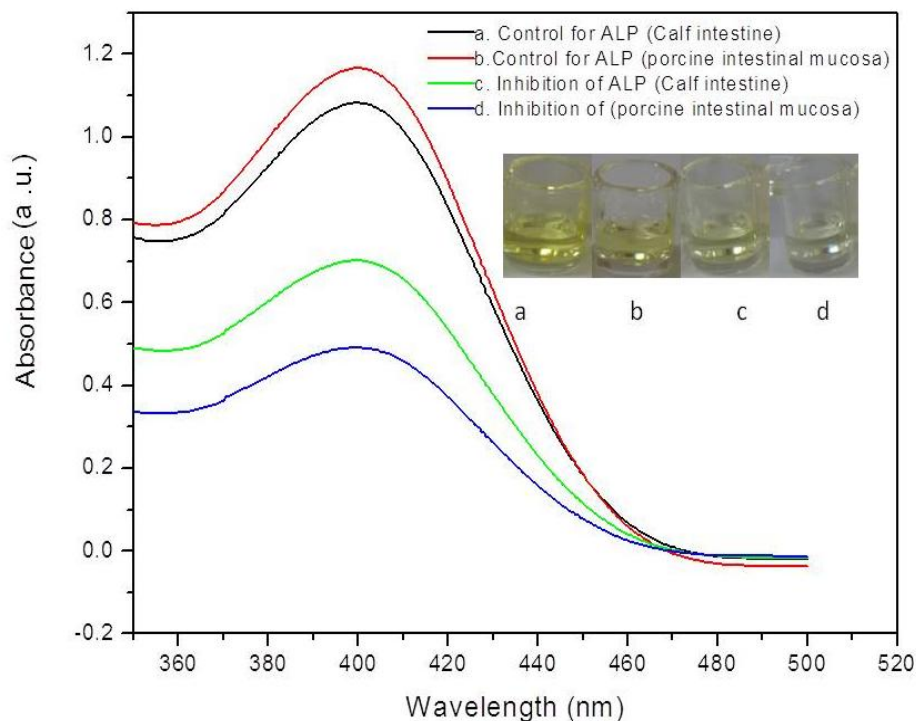



**Figure 5.3** Visual and quantitative UV-Vis analysis of ALP before and after inhibition with MPOx for evaluation of ALP sensitivity at 405 nm.

### 5.3.2 Optimization of pH and ionic strength

The colorimetric assay using $SiO_2$-ALP was performed using the consecutive reactions involving pNPP and $MgCl_2$ as a co-factor for ALP. The intensity of the color produced was directly proportional to ALP activity where the other reaction parameters such as substrate concentration, pH, temperature and ionic conductivity were at optimized condition. ALP activity in a pH range 8.7-12 was tested. It is evident from Figure 5.4 that the maximum enzyme activity was observed at pH 10.6 and as the pH increased further, there was no significant decrease in the activity. For inhibition studies, 0.005-0.2M GB was prepared and tested as reaction medium. The results for optimization of ionic strength are presented as Figure 5.5.

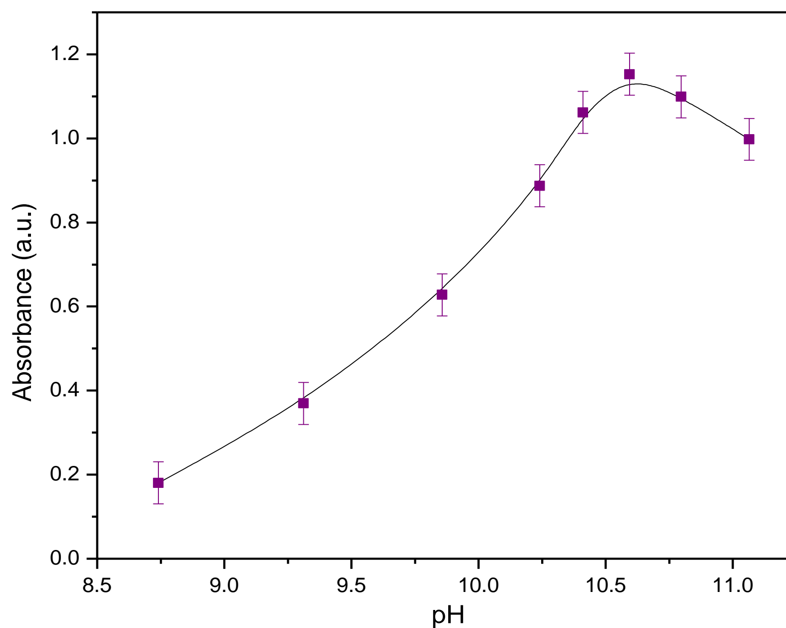



**Figure 5.4** Optimization of pH of GB for ALP and p-NPP reaction at 405 nm.

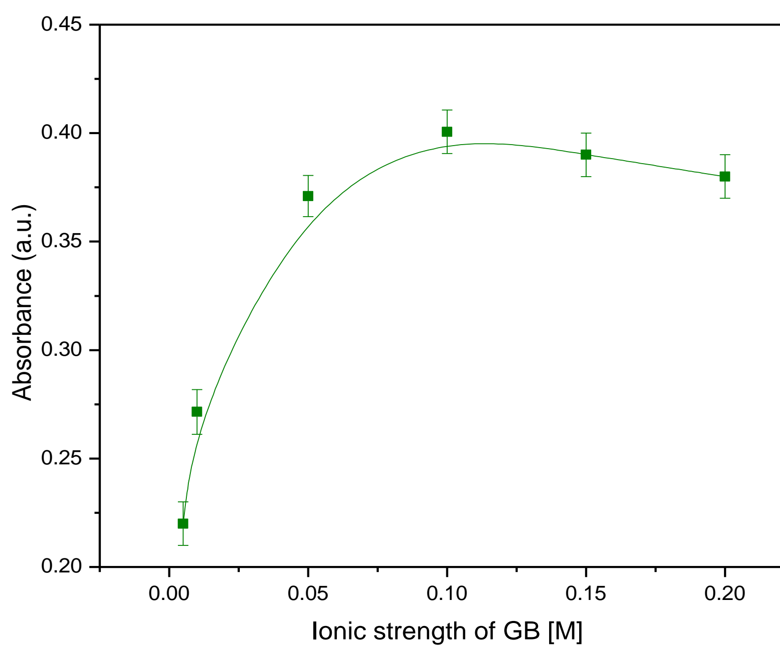



**Figure 5.5** Effect of ionic strength of GB on ALP activity in $SiO_2$ column at 405 nm.

### 5.3.3 Substrate optimization

The optimal substrate (pNPP) concentration for OPs determination was obtained from the linear zone in the kinetic curve. Figure 5.6 shows the response of the $SiO_2$-ALP to the addition of 0.05-1mM pNPP. The apparent $K_M$ was estimated to be 0.143 mM according to Lineweaver-Burk plot. The high affinity of enzyme may be attributed to the confined space in the $SiO_2$ pores that would prevent the immobilized enzymes from unfolding. Further assays were carried out with 0.1M GB, pH 10.6 and 0.143 mM pNPP.

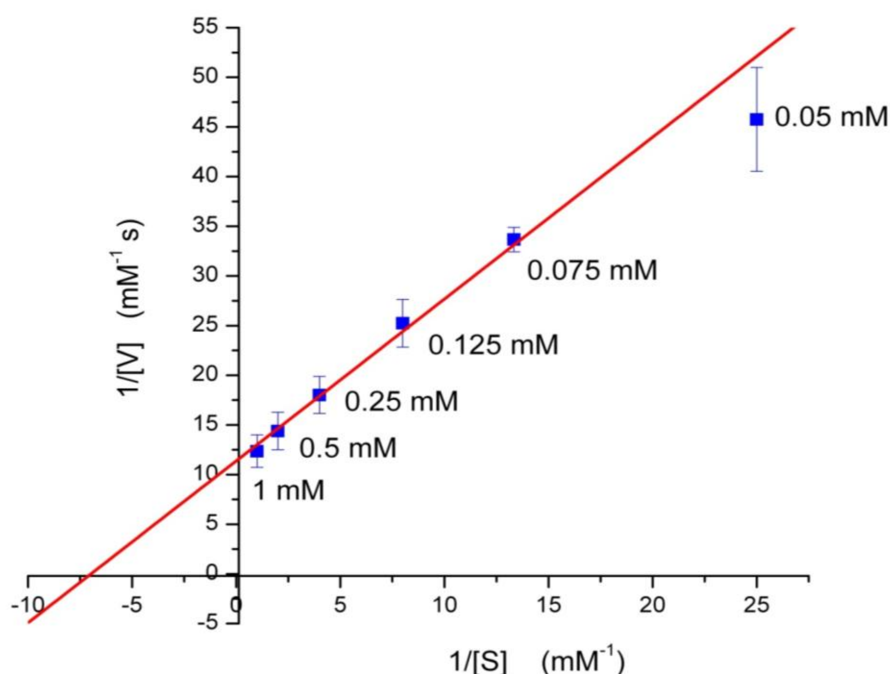



**Figure 5.6** The apparent Michaelis-Menten constant for pNPP obtained through ALP inhibition in $SiO_2$-ALP column.

### 5.3.4 Optimization of ALP loading on $SiO_2$ column

Different ALP units were allowed to react with 0.14 nm pNPP. Enzyme concentrations from 0.011-0.22 IU per assay volumes were tested for the optimization of enzyme concentration. Considering the total assay volume versus colorimetric signal, it was possible to achieve good optical signal with 0.09 IU of immobilized ALP. The bioassay response on ALP inhibition may

be affected by the ALP loading on $SiO_2$ column. It is evident from Figure 5.7 that as the ALP units were increased, absorbance of the reaction also increased. In order to establish a relationship between ALP loading and inhibition caused by pesticides, I% was measured against the ALP loading keeping the pesticides and substrate concentrations constant. It has also been reported in the literature that for sensitive determination of analytes, low enzyme concentration is preferable (Pirvutoiu et al., 2002). In this work, we could successfully achieve LOD as low as 0.1 µg $L^{-1}$ for MPOx using low concentration of ALP (0.09 IU), which is also visually distinguishable.

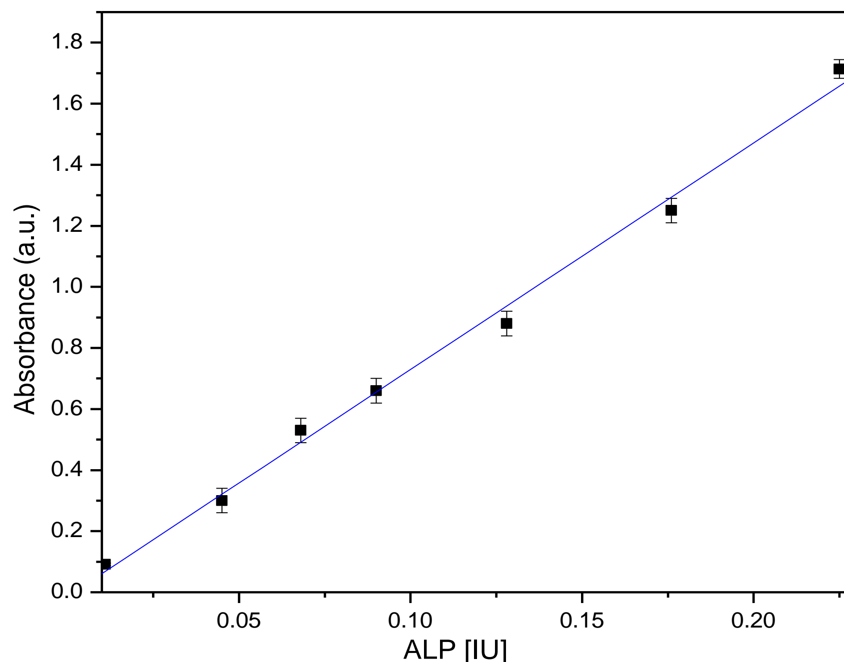



**Figure 5.7** Optimization of ALP loading for construction of silica based disposable column at 405 nm.

### 5.3.5 Incubation time

It is experienced from the experiments that ALP is less sensitive enzyme than AChE or BuChE for OPs. The incubation time required for ALP inhibition was investigated at different time from 10-60 min at the MPOx concentration of 50 µg $L^{-1}$. It was observed that I% increased with increasing incubation time and reached to maximum value. However, a longer incubation time

means a longer analysis time. Therefore, incubation time of 30 min was chosen as optimum for inhibition studies. Importantly, increase in incubation could also increase the sensitivity of the assay. Therefore, simultaneously, measurements were also done with incubation time 50 min resulting in improved limit of detections for potent inhibitors, MPOx and EPOx.

### 5.3.6 Visual and quantitative determination of OPs and CF

The developed assay was evaluated by measuring the activity of ALP in the presence of commonly found inhibitors such as MPOx, EPOx, MP and CF. Calibration plots were obtained correlating I% with inhibitor concentrations. Calibration curves for MPOx, EPOx, MP and CF were obtained under optimized conditions by plotting the graph of concentration versus I%. The dynamic range studied for the four pesticides was in the range 0.01-500 µg $L^{-1}$. LOD was obtained for each analyte separately. For MPOx and EPOx, the LOD was found to be 0.01 and 0.1µg $L^{-1}$ respectively whereas, for MP and CF, LOD was obtained 1µg $L^{-1.}$ Among the MPOx and EPOx, MPOx contributed higher inhibition over EPOx in each concentration level. Thus MPOx can easily be discriminated from ethyl analogue. The linear range obtained for MPOx and EPOx ranging 0.1- 250 µg $L^{-1}$. The real picture of color changes in the linear concentration range of MPOx is shown in Figure 5.8.

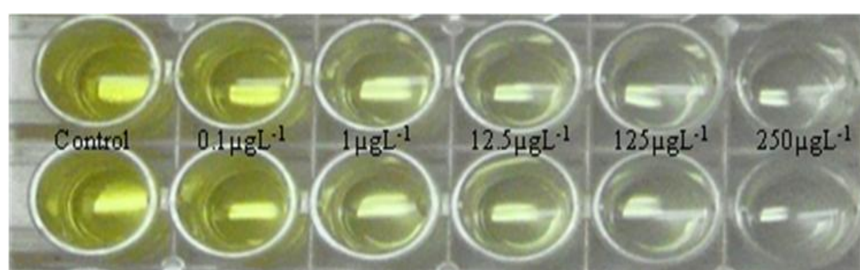



**Figure 5.8** Color changes observe during the analysis of MPOx.

The $IC_{50}$ values of MPOx and EPOx showed the differences of almost 10 times for ALP. This experimental finding showed the affinity of ALP towards MPOx is stronger than other tested pesticides. The inhibition mechanism of ALP by the OPs investigated was found to be reversible. The measured I% values and the corresponding pesticides concentration for MPOx and EPOx

with incubation time 30 mins and 50 mins are presented in Figure 5.9 (a) and 5.9 (b). More specifically, the $IC_{30}$ for MPOx was found 100 fold higher over its parent compound MP. This approach to the quantitative evaluation of the activity of ALP demonstrated is particularly suitable for the development of miniaturized high throughput assay. The present colorimetric assay has been demonstrated with 60 µL assay volume. This work is among first few reports on miniaturised visual colorimetric bioassay. The analytical figures of merit such as linear range, detection limit, % RSD and regression coefficient for inhibitors determination are indicated in Table 5.2. The relative standard deviation was found between 1.38-3.86%.

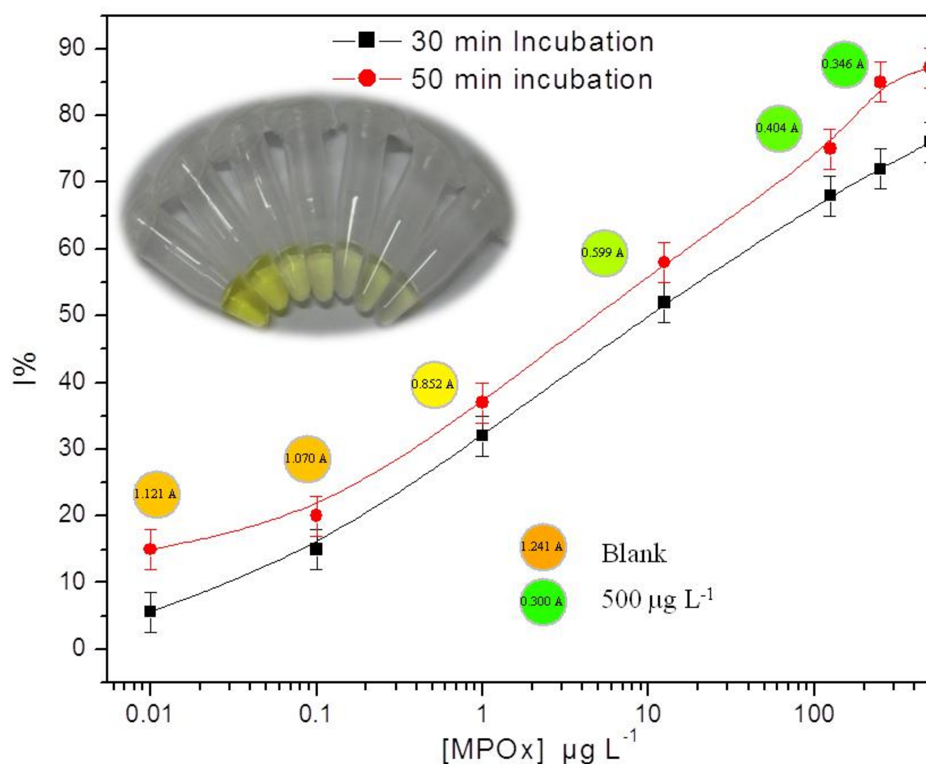



**Figure 5.9 (a)** Visual and quantitative determination of ALP inhibition caused by MPOx with two different incubation times.

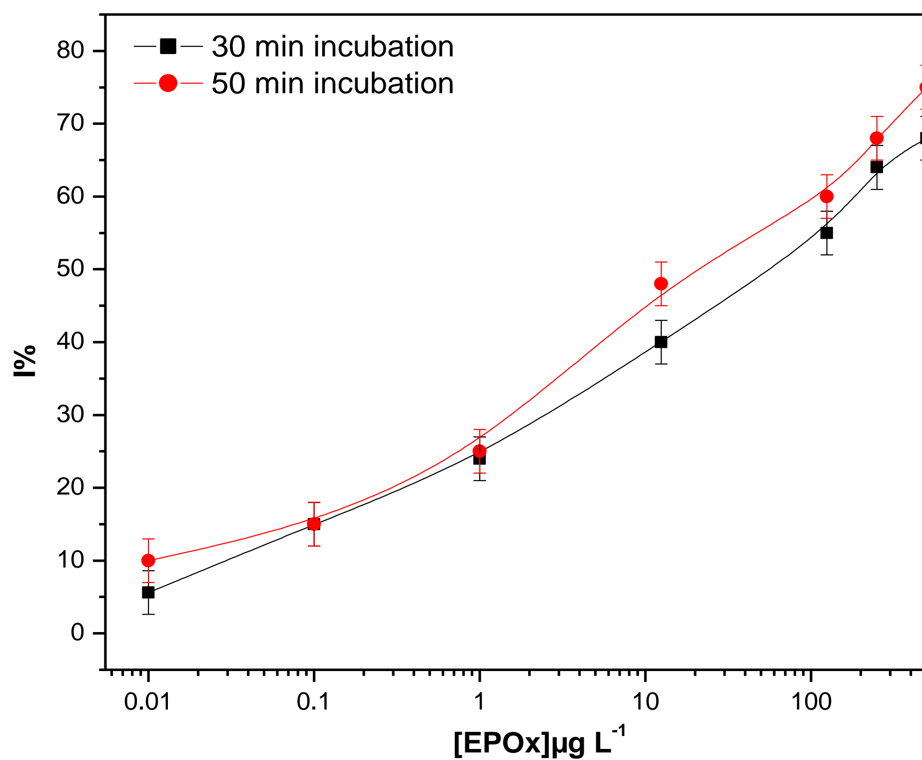



**Figure 5.9 (b)** Visual and quantitative determination of ALP inhibition caused by EPOx with two different incubation times.

**Table 5.2** Analytical figures of merit for MPOx, MP, MT and CF using colorimetric bioassay

| **Analyte** | **Linearity ($\mu$g $L^{-1}$)** | **LOD ($\mu$g $L^{-1}$)** | **$IC_{30}$ ($\mu$g $L^{-1}$)** | **$r^2$** | **% RSD** |
|---|---|---|---|---|---|
| MPOx | 0.1-250 | 0.01 | 0.4 | 0.9970 | 0.75 |
| EPOx | 0.1-250 | 0.1 | 1.6 | 0.9902 | 1.28 |
| MP | 1-250 | 1.0 | 40 | 0.9914 | 3.86 |
| CF | 1-250 | 1.0 | 75 | 0.9927 | 3.12 |

### 5.3.7 Analysis of pesticide mixture

Co-existence of OPs is commonly encountered in water and other parts of the environment as a result of human and natural activity. During pesticide analysis, the presence of other pesticides in the same matrix may elicit antagonistic, additive or synergistic effects. In order to understand the toxic effect of these chemicals in totality, it is essential to study the inhibition at various concentration levels. Experiments were carried out to determine relative inhibition of ALP by binary combination of MP+EPOx and MP+MPOx and compared the inhibition values with individual analytes. In this study, concentrations were taken at low level. Four different concentrations 0.01, 0.1, 1 and 10 μg $L^{-1}$ were tested individually as well as in binary form. In this study MP is a parent compound for MPOx. Hence, evaluation of toxicity for both of the analytes was also justified. In the tested analytes, MPOx showed higher inhibition in each concentration as against EPOx and MP. Inhibition data obtained for binary mixtures show synergistic effect in the tested range for MPOx+MP but the I% is less for EPOx+MP than the individual EPOx at 1, 0.1 and 0.001μg $L^{-1}$. The obtained results are summarised in Table 5.3 and the real time absorbance from the experiments are shown in Figure 5.10.

**Table 5.3** Profile for ALP inhibitions by EPOx, MPOx, MP and mixture of EPOx + MP and MPOx + MP

| **Conc (μg $L^{-1}$)** | **I% (EPOx)** | **I% (MPOx)** | **I% (MP)** | **I% (EPOx+MP)** | **I% (MPOx+MP)** |
|---|---|---|---|---|---|
| 10 | 39.80 | 57.23 | 30 | 47.82 | 59.9 |
| 1 | 22.96 | 37.5 | 14.56 | 20.87 | 39.80 |
| 0.1 | 18.61 | 20.97 | 9.74 | 14.27 | 22.80 |
| 0.01 | 13.69 | 16.94 | 4.37 | 4.54 | 13.8 |

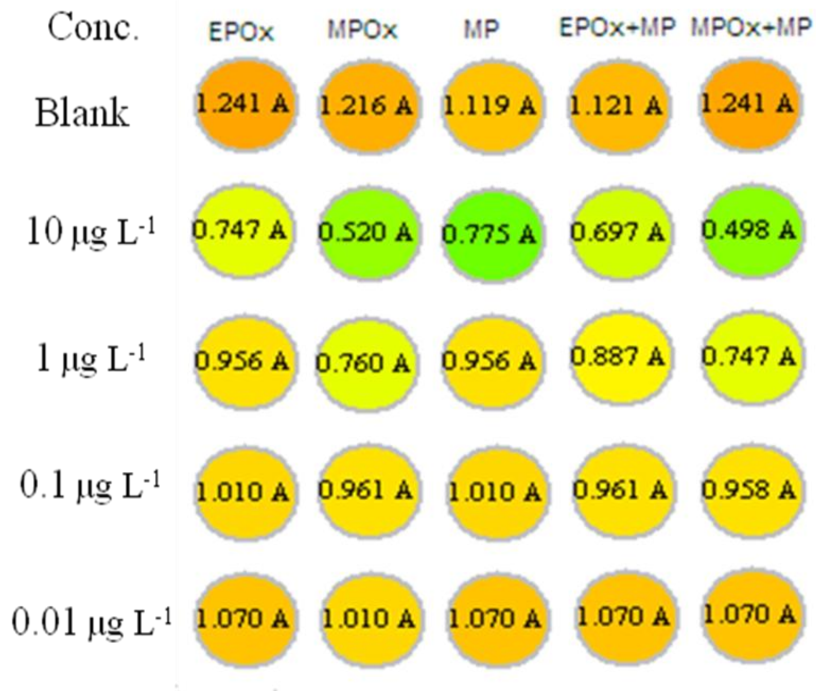



**Figure 5.10** Real time absorbance of individual and mixture of OPs at 405nm.

In another test, MPOx, EPOx, MP and CF were incubated with ALP for inhibition at higher concentration level (1, 125 and 250 µg $L^{-1}$). Mixture of MPOx + EPOx + MP + CF was also tested simultaneously. Inhibition obtained by tertiary pesticide mixture was always more than individual pesticide (synergistic but not additive). These experimental findings are shown in Figure 5.11. Results revealed that the tested OPs were more toxic when they were present in combination than when administered individually.

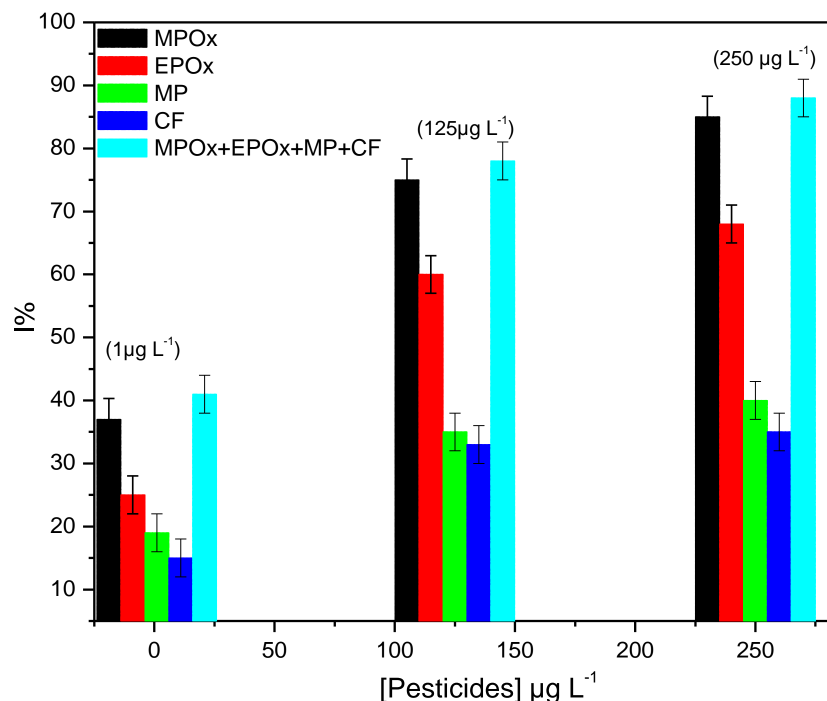



**Figure 5.11** Analysis of individual and mixture of tertiary pesticides to check the effect of cumulative toxicity on ALP activity.

### 5.3.8 Interference study

Interference studies were done in order to explore the specific determination of OPs in different water samples using the proposed bioassay. These experiments included investigation of most commonly found metal ions in real samples of water such as $Hg^{2+}$, $Cd^{2+}$ and $Pb^{2+}$. No obvious interferences were noticed with the presence of these selected metal ions for determination of OPs. The metal ions were tested at the Maximum residual limits (MRL) and 10 times below the MRL fixed by EU standards. $Hg^{2+}$ was tested with 0.001 μg $L^{-1}$ and 0.0001 μg $L^{-1}$, $Cd^{2+}$ tested with 0.05 μg $L^{-1}$ and 0,005 μg $L^{-1}$ whereas $Pb^{2+}$ tested with 0.01 μg $L^{-1}$ and 0.001 μg $L^{-1}$. In all the tested concentration of three different metal ions, only $Hg^{2+}$ at 0.001 μg $L^{-1}$ inhibited ALP activity by 3% if present in the presence of pesticides. The results confirm that ALP is less sensitive to inhibition by low level metal ions. The results are shown in the Figure 5.12.

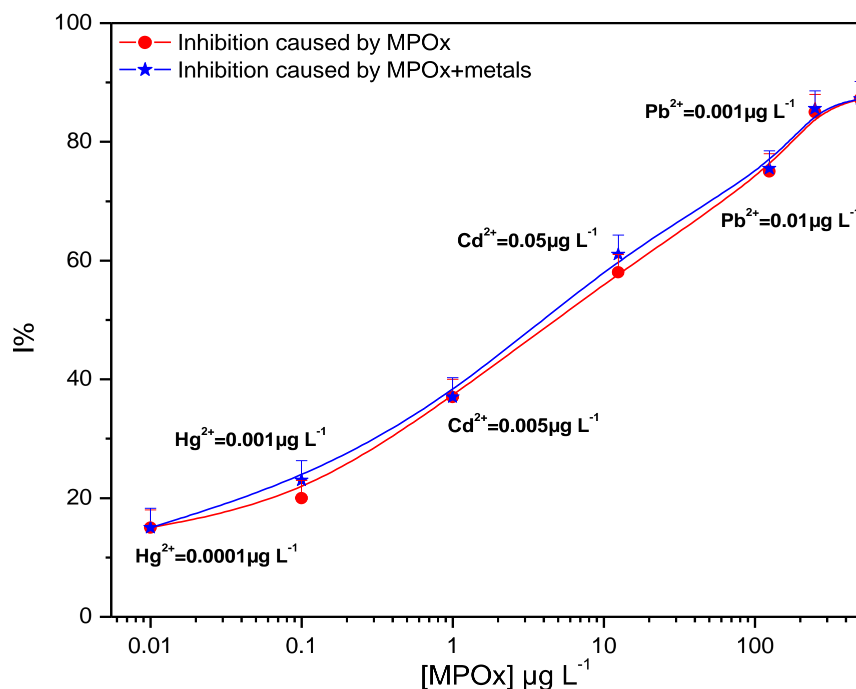



**Figure 5.12** Interference of metal ions ($Cd^{2+}$, $Hg^{2+}$ and $Pb^{2+}$) in analysis of OPs using developed bioassay.

### 5.3.9 Response time and stability of column

The stability of the immobilized enzymes during operation and storage was also examined. It was found that continuous experiments on 8 days, immobilized ALP retained more than 93% activity. After subsequent storage at 4ºC for 15 days the activity of the immobilized ALP retained more than 70%. However, after one month storage at 4ºC, the immobilized ALP retained only 50% of the initial activity. This evaluation of enzyme activity proved that ALP was successfully coupled on $SiO_2$ particles in the intact micro-column. To investigate the long-term storage stability and multiple uses capability, the biosensor was used once every 24 hrs to measure the response towards MPOx in the triplicates with pNPP and then stored at 4ºC in 0.1M GB, pH 10.6. This stability is superior to the other developed ALP based biosensors (Mazzei et al., 2004). ALP has not been reported as a disposable biosensor element in the literature.

### 5.3.10 Validation against standard chromatographic method

Measurements with the colorimetric bioassay in water were validated against standard analytical method. MPOx solutions were tested for validation purpose. Six different concentrations (1, 2, 5, 8, 10 and 15μg $L^{-1}$) of MPOx were analyzed using LC-MS/MS. The calibration of MPOx is plotted as concentration vs area and shown in Figure 5.13.

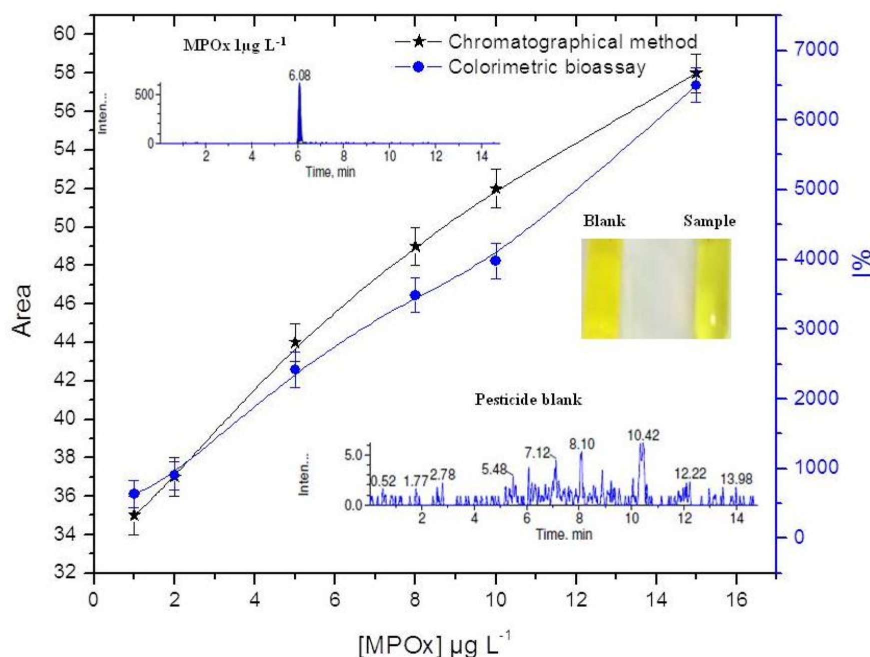



**Figure 5.13** Validation of bioassay against standard chromatographic method (LC-MS/MS).

The linear range was obtained in the range 1-15 μg $L^{-1}$ using LC-MS/MS. The real peak of pesticide blank and the peak obtained for MPOx 1μg $L^{-1}$ are also shown in the same Figure. The LC-MS/MS technique could not discriminate the concentration above 15 μg $L^{-1}$ and below 1μg $L^{-1}$ in water. Validation experiments against standard chromatographic methods confirmed the reliability of the biosensor test. The LC-MS/MS analysis showed a detection limit of 1μg $L^{-1}$ for MPOx in water sample. Therefore higher MPOx concentration was tested in calibration.

### 5.3.11 Real sample analysis

To demonstrate the practicality of the proposed bioassay, the recovery test was studied by adding 0.1 and 10 μg $L^{-1}$ of MPOx into ground water, tap water and drinking water. No significant

interference was observed in the water samples. Analysis of real samples using developed biosensor is summarized in Table 5.4.

**Table 5.4:** Recovery study of MPOx in real untreated water samples and spiking studies using developed bioassay and LC-MS/MS

| Sample matrix | No. of samples (N) | Spiked concentration (µg $L^{-1}$) | Found concentration (Bioassay) (µg $L^{-1}$) | Found concentration (LC-MS/MS) (µg $L^{-1}$) | SD (N=5) |
|---|---|---|---|---|---|
| GW | 3 | 0.1 | 0.097 (97%) | ND | - |
| GW | 3 | 10 | 9.80 (98%) | 9.28 (92.8%) | 3.4 |
| TW | 3 | 0.1 | 0.095 (95%) | ND | - |
| TW | 3 | 10 | 9.82 (98.2%) | 9.9 (99%) | 3.9 |
| DW | 3 | 0.1 | 0.097 (97%) | ND | - |
| DW | 3 | 10 | 9.62 (96.2) | 9.5 (95%) | 3.82 |

GW= Ground water, TW= Tap water, DW= Drinking water, ND= Not detected

The results indicated that the proposed method is reliable and reproducible. It could be used for direct screening of drinking water samples. Different values of I% were observed for ALP activity depending on the matrix analyzed. ALP activity was measured in untreated real samples to see the effect of matrix. An initial 6% inhibition was observed in case of ground water, whereas in case of other matrices, the activity of ALP increased and thus no inhibition was observed. These results were confirmed by spiking studies, where, 0.1 and 10 µg $L^{-1}$ MPOx concentration was spiked in all samples. The study reveals that these matrices containing MPOx can be successfully analyzed using proposed ALP assay. The developed assay is advantageous in

terms of simple to use, economical and enables to meet drinking water quality monitoring standards. However, as usually associated with visual techniques, such assay before deployment may need specific analysis to ascertain the nature of analyte in the matrix. The calculated MPOx concentrations fit very well to the added insecticide concentrations in the water sample. The concentration 0.1μg $L^{-1}$ was not detected by LC-MS/MS whereas bioassay showed expected recoveries ranging from 95-97% recoveries for the same concentration for three different water samples. The recovery rates of MPOx in tested water samples were close to 100% both for biosensor test and LC-MS/MS analysis. Furthermore, 0.1 μg $L^{-1}$ MPOx could be found with the biosensor, whereas LC-MS/MS investigations could not detect MPOx in such small concentration.

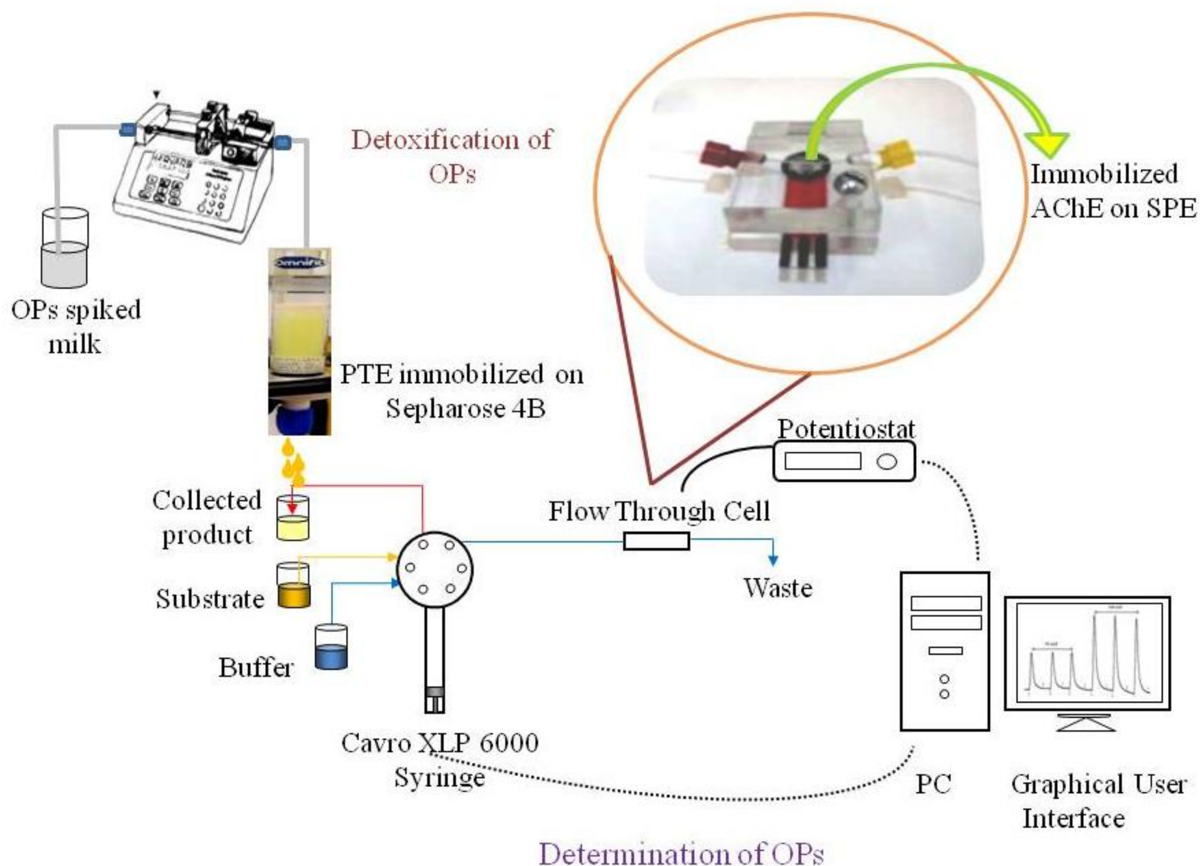
## 5.4 Conclusions

The aim of the work was to develop and demonstrate the analysis of pesticide residues visually as well as colorimetrically. The presented work utilizes immobilized ALP on a disposable micro column with the following merits; (a) The technique is affordable and simple to perform and interpret the results visually (b) The assay is optimized to meet the drinking water quality criteria for safe drinking water in fields and (c) ALP coupled micro-silica column as a disposable bio-component for analysis of pesticides in water. In this study, micro sized $SiO_2$ particles have been successfully employed as sensitive matrix to immobilize ALP enzyme to fabricate a novel visual, disposable column assay. The colorimetric results showed that the sensor could rapidly and sensitively determine OPs and CM under optimized condition with very small assay volume. The assay is free from interferences of tested metal ions at low level. The presented bioassay exhibited a color change, good linear detection range and high sensitivity for OPs. Furthermore, the developed column based assay is economical and column matrix $SiO_2$ is non-hazardous, an easier enzyme immobilization mediator which simplifies the experimental design and reduce the consumption of expensive reagents. Thus, the proposed bioassay can be useful to provide safe drinking water to the common population.

# CHAPTER 6

## Novel strategy for automated detection and detoxification of OPs in milk

**Novelty Statement:** This work demonstrates the novel strategy for detection and detoxification of three most potent OPs (CPO, MAO, and EPOx) in milk. We developed an automated electrochemical flow based biosensor using genetically modified AChEs. The automated flow based biosensor provides sensitive detection of OPs and evaluate the degradation product during the detoxification of OPs using PTE.




Automated flow based biosensor for detection and detoxification of OPs

## 6.1 Introduction

Contamination of water and milk by OPs is a worldwide problem. Reported literature includes electrochemical detection for OPs in milk using batch analysis (Zhang et al., 2005). The study also highlights use of genetically modified enzyme for early detection of OP neurotoxins is thus important for protecting water resources and food supplies for monitoring detoxification processes. Simultaneous monitoring as well as detoxification of pollutants has always remained a key interest to researchers. Application of flow system for simultaneous determination and detoxification has not been reported till date. This work presents a novel strategy for simultaneous determination as well as detoxification of OPs in milk.

### 6.1.1 Need for online analysis of OPs

Among the different types of biosensors, the electrochemical ones are especially interesting due to the high sensitivity inherent to the electrochemical detection and the possibility to miniaturise the required instrumentation providing compact and portable analysis devices. In order to improve experimental parameters, current research efforts focus on the use of genetically modified enzymes with tailor-designed properties (Campàs et al., 2009). The structural changes caused by the genetic mutations may imply variations in the enzyme functionality, thus affecting the operational and storage stability of the corresponding biosensors (Bucur et al., 2006). Genetically engineered AChEs have been widely exploited in enzyme inhibition-based biosensors for the detection of pesticides, e.g. OP and CM insecticides. The combination of biosensors with FIA techniques offers the possibility to control the whole procedure, simplifying the sequence of steps, high sensitivity and allowing an easier optimization of the reaction conditions (Schmid et al., 1990). In the past 25 years, FIA has been the most widely proposed method of automation, due to its efficiency and versatility (Gorton et al., 1991).

### 6.1.2 State of the art for OPs determination using electrochemical biosensors

The reported flow systems are coupled with a wide variety of detectors (Del Valle M, 2010), however, electrochemical methods such as potentiometry and amperometry are particularly

interesting because of their low cost, small size and the possibility of performing in situ measurements. Several AChE-based biosensors (Hildebrandt et al., 2008; Istamboulie et al., 2007; Valdés-Ramírez et al., 2008; Valdés-Ramírez et al., 2009) and flow-based biosensors have been reported for the detection of OPs (Bucur et al., 2005; Crew et al., 2011; Jeanty et al., 2002; Prieto-Simón et al., 2006; Shi et al., 2006;). Table 6.1 shows the reported enzymes for OPs determination.

**Table 6.1** Reported electrochemical biosensors using SPE for OPs determination

| Analyte | Biocomponent | MDL/Range | Reference |
| --- | --- | --- | --- |
| MPOx | B394 | $2.7\times10^{-9}$ M | Valdés-Ramírez et al., 2008 |
| Dichlorvos & CF | B1 and B394 | 0.79 nM & 4.1 nM | Cortina et al., 2008 |
| Methamidophos | dmAChE | 1ppb | Oliveira et al., 2004 |
| Dichlorvos & MPOx | B394 &B1 | $1\times10^{-4}$ to 0.1μM<br>0.001 to 2.5 μM | Valdés-Ramírez et al., 2008 |
| CPO | B394 | $1.113\times10^{-10}$ M | Alonso et al., 2012 |
| CPO & CFV | B394 | $1.3\times10^{-11}$ | Istamboulie et al., 2007 |
| Paraoxon | NbAChE | 1 μg/L | Zhang et al., 2005 |
| CPO, MAO | B421, B394 & B65 | $10^{-5}$ to $10^{-7}$ M | Crew et al., 2011 |

### 6.1.3 Need for OPs detoxification in milk

The hazardous nature of OPs and their wide usage have led to concerted efforts for efficient destruction methods for these compounds as well as their quantification (Gill and Ballesteros, 2000). Degradation of OPs is very mandatory for the detoxification of waters and milk devoted to human consumption. Conventional methods generally involve incineration and chemical hydrolysis of these products (Holm, 1996). However these techniques are hindered by their high environmental impact as well as logistics and operational safety issues (Gill and Ballesteros, 2000). Efforts have thus been made to find safer technologies, mainly based on biotechnological degradation techniques. New methods of environmentally friendly and safe OPs detoxification are urgently needed. The current methods for the detoxification of OP compounds are harmful and possess serious environmental consequences. They are classically based on extraction, cleanup and analysis using gas chromatography or liquid chromatography coupled to sensitive and specific detectors (Ballesteros and Parrado, 2004; Kuster et al., 2006). These methods are highly sensitive and allow the determination of a large number of compounds but expensive and time consuming with complex procedure. Alternative methods are mainly based on the biosensor technology.

### 6.1.4 Current status of OPs detoxification in milk

Enzymes that are capable of hydrolyzing and detoxifying such agents are of significant utility. The present work demonstrated that the potential detoxification of OPs in milk is indispensable. Bacterial enzymes capable of hydrolyzing the lethal OP nerve agents are of special interest. The use of OPs, though very important to the success of the agricultural industry, affects the environment. PTE displays a significant rate enhancement and substrate promiscuity for the hydrolysis of OPs, including chemical warfare agents like sarin or soman (Ghanem and Raushel, 2005; Raushel, 2002). Various researchers have studied detoxification of toxic analytes such as paraoxon, parathion, chlorpyriphos etc. exploiting various biomolecules such as enzymes and whole cells. The insecticides paraoxon and parathion were successfully hydrolyzed by PTE that was immobilized on a trityl agarose matrix (Caldwell and Raushel, 1991a; 1991b). The application of PTE based detoxification column for chlorpyriphos and CFV was demonstrated very recently using flow set-up (Istamboulie et al., 2010). OPH enzyme was also used for

detoxification of OPs such as coumaphos with 99% degradation using prolong incubation time (2-3 hrs) (Mansee et al., 2005). PTE has been also described as a potential catalytic scavenger for the treatment of OP poisoning (Masson et al., 1999). The matrix reported in the literature is mostly water. Reported OPs and biomolecules which hydrolyze OPs are summarized in Table 6.2.

**Table 6.2** Reported biomolecules for OPs detoxification/degradation

| Analytes | Enzymes | Detoxification | Support | Matrix | References |
|---|---|---|---|---|---|
| Coumaphos | OPH | 99% | Cellulose | Buffer | Mansee et al., 2006 |
| POx | PTE | 155mL/min | Nylon | Buffer | Caldwell and Raushel, 1991 |
| CFV & CPO | PTE | 99% | Sepharose gel 4B | Water | Istamboulie et al., 2010 |
| Malathion | Fungal cutinase and east esterase | 60 and 35% | --- | --- | Kim et al., 2005 |
| POx | OPH | 93.9% | Cellulose | Water | Wang et al., 2005 |
| MP | Parathion hydrolase | --- | --- | Soil | Pakala et al., 2012. |

### 6.1.5 Research gaps identified

Existing literature revealed that there is an immense need for automated online monitoring of OPs detection and detoxification in milk using genetically modified enzymes. Therefore, an automated flow based biosensor using a genetically modified AChE B394 based probe was

developed for online determination of OPs in milk and extended to evaluate the detoxification of OPs using enzyme PTE. Detoxifications of mixture of OPs were also investigated.

#### 6.1.6 Objective

To develop an automated flow based biosensor for detection and detoxification of OPs in milk.

#### 6.1.7 Methodology

After a careful literature survey, it is clear that, there is no report available for OPs detection and detoxification in milk using automated flow based biosensor. The SPEs were used to couple genetically modified AChE on working electrodes based on the principle given below:

##### 6.1.7.1 Principle

The activity of the AChE is measured by anodic oxidation of the TCh produced by hydrolysis of ATCh. The decrease in biosensor response is correlated with the amount of pesticide present in the sample and the time of incubation. The principle is shown in the Figure 6.1.

**Figure 6.1** The principle of hydrolysis of ATCh on SPE surface.

### 6.2. Experimental

#### 6.2.1 Chemicals, biochemicals and stock solutions

Wild type enzyme AChE (B131) and genetically modified enzymes AChE B394 and B4 from *Drosophila melanogaster* were produced by IMAGES, Perpignan, France. PTE recombinant enzyme was kindly provided by Prof. D. Fournier (Toulouse, France). This enzyme was first isolated from *Flavobacterium sp.* and cloned in *Escherichia coli*. Acetylthiocholine iodide (ATChI), acetylthiocholine chloride (ATChCl), 5,5′-dithio bis (2-nitrobenzoic acid) (DTNB), and phosphate buffer (PBS, 0.1 mol $L^{-1}$ $K_2HPO_4/KH_2PO_4$ pH 7 containing 0.1 mol $L^{-1}$ KCl) were obtained from Sigma Aldrich (Steinheim, Germany). A 0.1 M ATChCl stock solution was

prepared daily and stored at 4°C. HPLC-grade acetonitrile was supplied by Carlo Erba (Italy) and photocrosslinkable poly (vinyl alcohol) (azide unit pendant water-soluble photopolymer, PVA-AWP) was purchased from Toyo Gosei (Japan). The pesticides ethyl paraoxon and malaoxon were obtained from Fluka, Sigma Aldrich (Germany). Chlorpyriphos-oxon was procured from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of pesticides ($10^{-3}$ M) were prepared in acetonitrile and stored at 4°C. Working solutions of pesticides were prepared daily in distilled water by dilution from the stock. Slide-A-Lyzer Dialysis Cassettes were purchased from Thermo Scientific (Rockford, USA) for purification of enzyme powder in the liquid phase. The paste used for screen-printing, i.e. Electrodag PE-410, 423SS and 6037SS, were obtained from Acheson (Plymouth, UK) and Timrex T15 graphite was supplied by Timcal (Lonza, Switzerland). TCNQ-modified carbon-paste was prepared as reported previously (Andreescu et al., 2002). A glycerophthalic paint (Astral, France) was used as an insulating layer. Hydroxyethyl cellulose (HEC) medium-viscosity was purchased from Fluka (France). Transparent PVC sheets (200 mm × 100 mm × 0.5 mm) were used as printing supports. Milk samples were bought from local market of Perpignan (France) for analysis. Sepharose 4B activated by cyanogens bromide was purchased from Amersham (Buckinghamshire, UK). Peristaltic pump (Gilson, France) was used to dispense liquid in PTE based Omnifit column.

### 6.2.2 Spectrophotometric measurements

To determine the enzymatic activity and inhibition constants, spectrophotometric measurements were performed. The Ellman method was used to determine AChE activity. This method is based on the enzymatic reaction product (TCh) that reacts quantitatively and irreversibly with DTNB, producing a yellow compound (5-thio-2-nitrobenzoate) that can be spectrophotometrically detected at 412 nm (Ellman et al., 1961). The assay was performed using PBS (600 µL), 300 µL 1mg/mL DTNB (in PBS), 100 µL 10 mmol $L^{-1}$ ATChI (in 0.9% NaCl) and 10 µL enzyme was added to a spectrophotometric cell. The kinetic spectrometric measurements were performed within 1 min and the enzymatic activity in mU $mL^{-1}$ was calculated. PTE activity was measured in 10 mM PB pH 8 by monitoring the formation of p-nitrophenol at 405 nm using 1mM of the reference substrate paraoxon (Istamboulie et al., 2009).

### 6.2.3 Apparatus

Spectrophotometric measurements were performed using a Hewlett–Packard model 8451A diode-array spectrophotometer. Amperometric measurements were carried out with a 641 VA Potentiostat (Metrohm, Switzerland) connected to a BD40 (Kipp and Zonen, The Netherlands) flatbed recorder. SPEs were fabricated using a semi-automatic DEK248 printing machine (Andreescu et al., 2002) in a three-electrode configuration. The working electrode was a 4 mm-diameter disk, the auxiliary electrode was a 16mm × 1.5mm curved line and the Ag/AgCl pseudo-reference electrode was a 5mm × 1.5mm straight line.

### 6.2.4 Automated Flow based biosensor (Instrumentation)

The Electrochemical measurements were carried out with an automated flow-based system. The schematic of automated flow-based biosensor is presented in Figure 6.2.

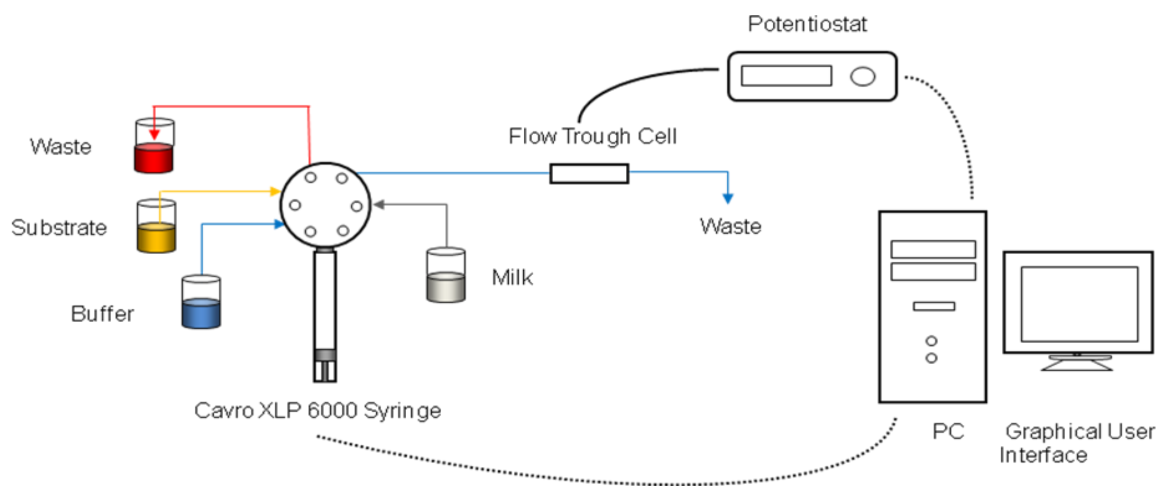



**Figure 6.2** Schematic of the automated flow-based biosensor for the analysis of OPs in milk samples.

The flow system is comprised by a modular syringe pump coupled to a multiport valve (Cavro XLP 6000), a custom flow-through cell, a 12 bits data acquisition card (National Instruments) and a 641VA potentiostat (Metrohm, Switzerland). A graphical user interface was developed in Lab-View 8.5 to control the whole system. The reagents were arranged in different reservoirs.

### 6.2.5 Construction of the biosensors

**6.2.5.1 Fabrication of SPEs:** The biosensors were manufactured in groups of 24 (reference, working, and auxiliary electrodes) on PVC sheets. The following layers were consecutively printed: silver conducting film, a carbon pad, an Ag/AgCl layer on the reference electrode, a CoPC layer on the working electrode, and, finally, an insulating layer. After each deposition the electrodes were dried at 60°C for 40 min. The working electrode was a 4 mm-diameter disk, the auxiliary electrode was a 16 mm×1.5 mm curved line, and the Ag/AgCl pseudo-reference electrode was a 5 mm×1.5 mm straight line.

**6.2.5.2 AChE immobilization in PVA:** Three different kinds of biosensors were prepared using genetically modified AChEs, B394 and B4, and wild type B131. Each enzyme was immobilised on the working electrode surface by entrapment in a polymeric matrix of PVA-AWP. On the working electrode surface, 3 µL of enzymatic solution containing 30% enzyme and 70% PVA-AWP was manually spread in order to immobilize 1 mU enzyme per electrode. Afterwards, the electrodes were exposed to neon light (15 W) for 4 h at 4°C to promote photo-polymerization between azide groups. After drying for 72 h at 4°C, the biosensors were ready to use. Figure 6.3 represents the immobilization of AChE on SPE.

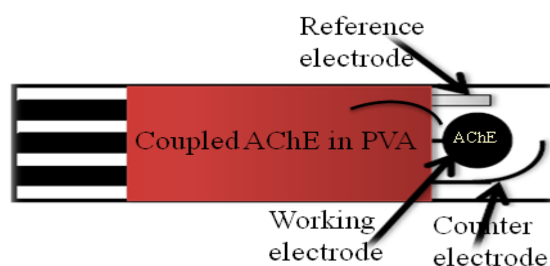



**Figure 6.3** Immobilization of AChE on SPE surface

### 6.2.6 Amperometric measurement in PBS and milk

In order to perform the amperometric measurements, the biosensor strip was vertically inserted in to the flow cell of the automated flow system and integrated into the potentiostat. A working potential of 100 mV was applied to the reference electrode and 500 µL of the substrate was dispensed three times using an automated pump. The current produced (Amplitude) was

recorded using the data acquisition card. The pesticide analysis was completed in a three-step procedure as follows: first, the initial response of the biosensor to the substrate ATChCl (1mM) was recorded three times; then, 500 µL $min^{-1}$ of each pesticide was dispensed through the flow cell for 10 min to inhibit the biosensors. The stock pesticide solution ($1\times10^{-3}$ M) was diluted 1000 times to $1\times10^{-6}$ M, and then further diluted. Each pesticide was tested against each biosensor. Finally, the residual response of the biosensor was recorded again. The electrodes were cleaned between each measurement.

**6.2.7 Recovery studies in water and milk**

To demonstrate the viability of the proposed method, we applied the developed method to real samples with water from the lake of Villeneuve de la Raho (France). The accuracy of the biosensor test was also checked by spiking milk samples with EPOx, CPO and MAO using biosensor B394. Two different fat-containing milk samples (15.5% and 30%) were examined as a complex matrix. Initially, milk samples were analyzed directly. There was a reduction in peak height by 20-30% between milk and PBS. Therefore, we filtered the milk samples and passed the filtrate through the biosensor. This was followed by a washing step to clean the tubing with PBS using the automated flow system. The matrix effect was eliminated and the peak heights were quite similar. After elimination of matrix effect, the recovery studies were carried out using CPO, EPOx and MAO. Two different concentrations were chosen from the linear range of calibration curve. The first concentration was near the lower concentration of the calibration curve and the other one is the maximum. Milk and water samples were spiked with each pesticide (CPO: $5\times10^{-11}$, $5\times10^{-7}$ M, EPOx: $5\times10^{-9}$, $5\times10^{-7}$ M and MAO: $5\times10^{-10}$, $5\times10^{-7}$ M) and recoveries were calculated.

**6.2.8 Application of developed biosensors to OPs detoxification**

**6.2.8.1 OPs detoxification using PTE:** The activity of PTE for different OP substrates was already investigated and described in a previous work (Istamboulie et al., 2009). PTE was shown to exhibit a high affinity for all the tested OPs namely: EPOx, CPO and MAO but with differences in % detoxifications. Paraoxon is the main substrate for PTE activity and the product

of hydrolysis is *p*-nitro phenol. The original photograph of the experimental set-up is shown in Figure 6.4. It is important to mention that these detoxification products are not inhibitors of AChE and are not pointed out as harmful chemicals by European regulation and WHO recommendations on water quality (Sogorb and Vilanova, 2002).

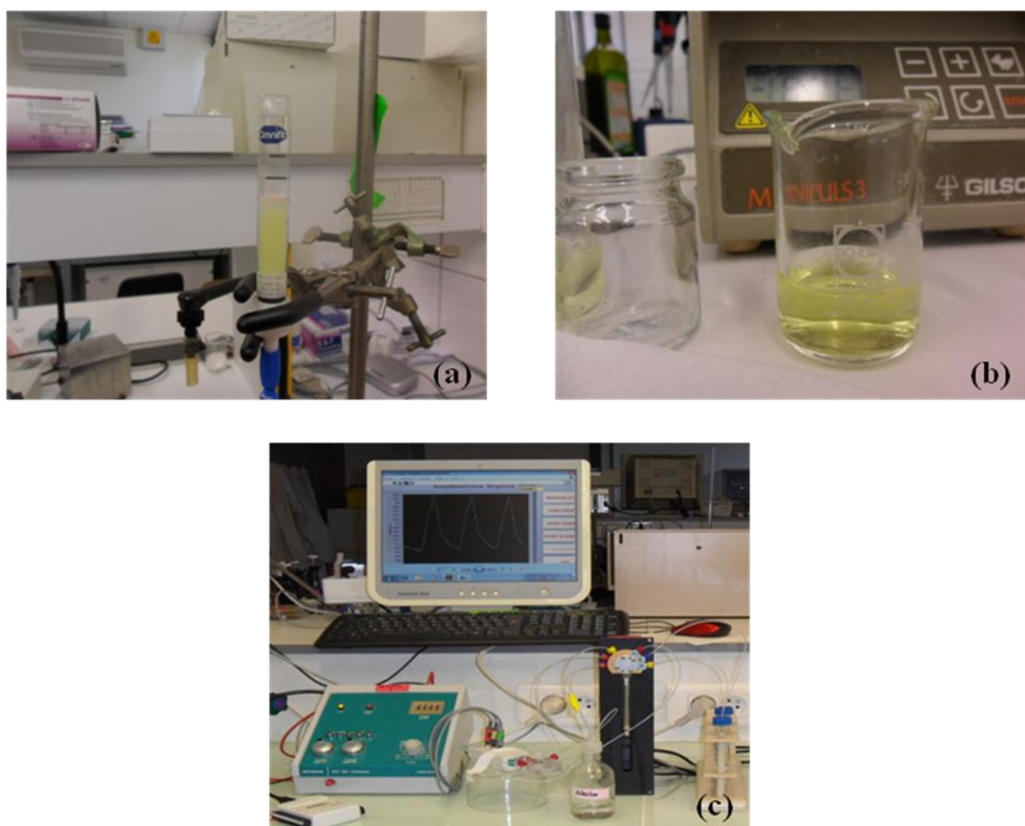



**Figure 6.4** The real picture of experimental set up used for OPs detoxification in water and in milk: (a) Omnifit column immobilized with PTE (b) collected end product after detoxification (c) electrochemical set-up to evaluate degradation product.

### 6.2.8.2 Immobilization of PTE on Sepharose Gel 4B

The gel was prepared by mixing 1 g of Sepharose 4B in 10 mL of 1 mM HCl, this suspension was then gently casted in an Omnifit column (internal volume 10 mL) (Cambridge, England). The column was cleaned with 200 mL of 1 mM HCl and then equilibrated using 50 mL of 0.1 M

PB, pH 8. After the determination of enzyme activity, PTE solutions of different concentrations were gently mixed with the Sepharose gel for 2 h at room temperature (25°C). The saturation step was then performed by passing 10 mL of 1 M ethanolamine through the gel for another 2 h. Finally, the gel was cleaned using 20 mL of 0.1 M acetate buffer pH 4 and equilibrated with 20 mL of 0.1 M PB, pH 8. The final bed volume was 4 mL, before use, the column was stored at 4°C.

#### 6.2.8.3 Detoxification measurments

Detoxification of EPOx, CPO and MAO were performed using PTE immobilized Omnifit column connected with peristaltic pump. Different concentrations of EPOx, CPO and MAO ranging from $1\times10^{-6}$ M to $1\times10^{-9}$ M were spiked in water as well as in milk and passed through the Omnifit column. The pesticide solutions were passed with an optimum flow rate of 0.5 mL $min^{-1}$ corresponding to a contact time of 6 min. Total volume of pesticide solution passed through the column was 5 mL. PTE immobilized in Omnifit column, hydrolyzes pesticide solution and a yellow color solution was collected from tubing's of peristaltic pumps. The obtained product was evaluated using a highly sensitive flow based biosensor to confirm the % detoxification.

## 6.3 Results and Discussion

### 6.3.1 Optimization of reaction parameters

To characterise the biosensors, the calibration curve, stability, and reproducibility were determined for each enzyme. The biosensor response to successive additions of ATChCl substrate and the operational stability were tested. The electrodes showed good amperometric response to the ATChCl substrate. To evaluate the operational stability of the electrode, the response of the biosensor to additions of 1 mM substrate, while rinsing the cell between measurements, was repeatedly measured. Figure 6.5 (a) represents the stability of the biosensor as a peak response in 8 consecutive injections of 1mM ATChCl.

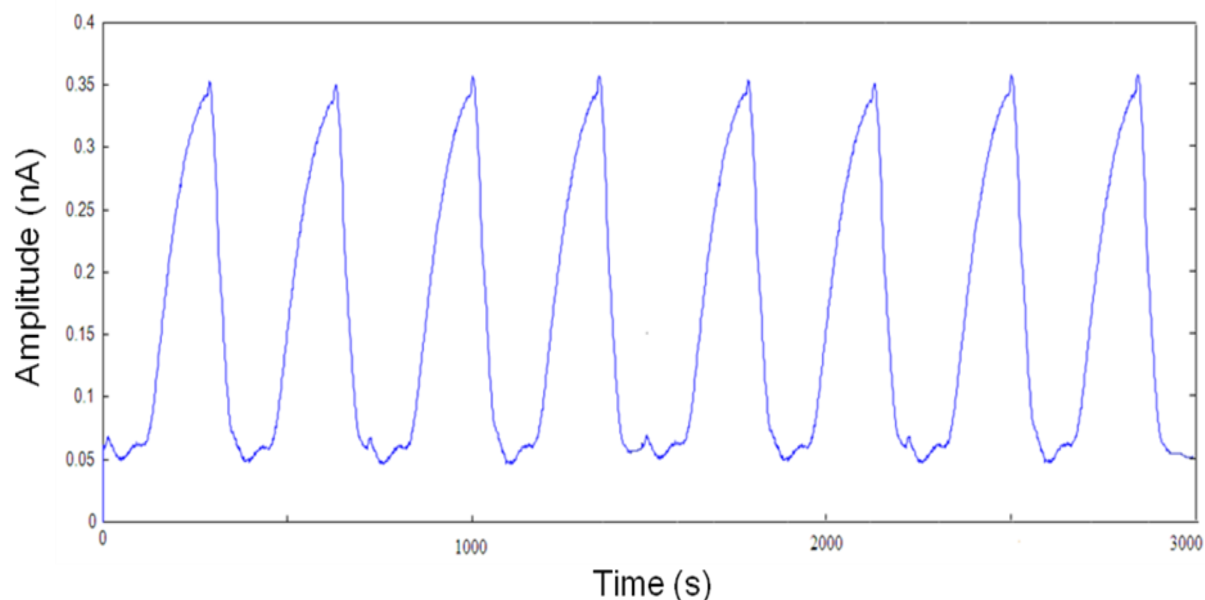



**Figure 6.5 (a)** Stability of the biosensor in peak responsiveness following 8 consecutive injections of 1mM ATChCl.

All electrodes showed good operational stability and had no obvious enzyme leaching. This can be due to the strong electrostatic binding between the enzyme and the electrodes. All biosensors were stable for at least up to N=10 assays/tests, with a response variation of less than 5%. The response time of the biosensors was approximately 3 min.

The electrodes retained full enzymatic activity after storage for up to 1 month. For the storage experiments, the electrodes were dipped in PBS and stored at 4°C in a covered container to prevent PBS evaporation and contamination. These storage conditions are mild and easy to achieve a good response. The electrodes can be re-used if the milk does not contain OPs. Flow rates of 0.5 mL $min^{-1}$ and 1 mL $min^{-1}$ were tested to achieve the optimum current. The flow rate of 500 µL $min^{-1}$ was optimized as the best compromise between the peak amplitude and speed of the pump used in the automated flow system. Pesticide detection studies were carried out at saturation substrate concentrations (1 mM). Three OPs, CPO, EPOx and MAO, were tested at a concentration of $2.5\times10^{-7}$ M to determine their effect on enzyme activity at different incubation times (5, 10, 15 and 20 min). It can be seen in Figure 6.5 (b) that the level of inhibition of the enzyme increased with increasing incubation period.

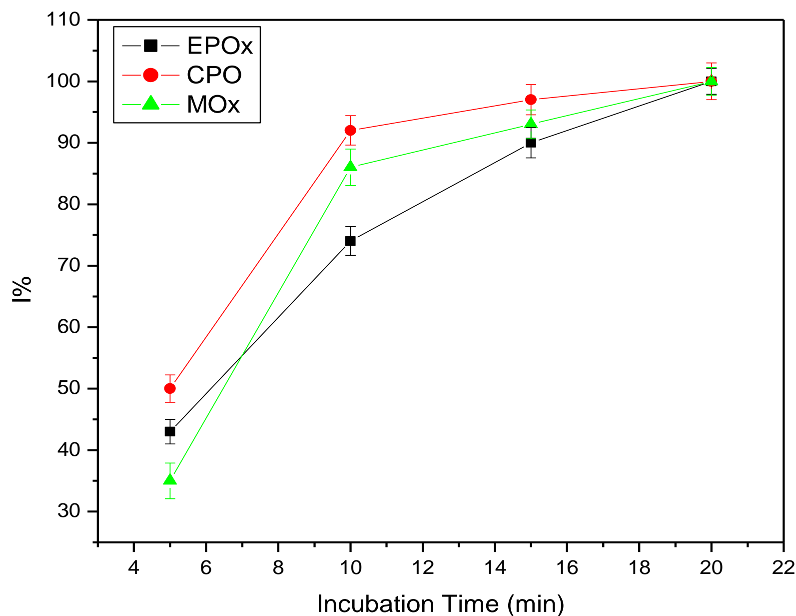



**Figure 6.5 (b)** Effect of incubation time on the performance of biosensor B394 using EPOx, CPO and MAO (ATCh =1mM).

There is always a tradeoff between incubation time and sensitivity. However, many practical analytical applications require rapid analysis. Given that the slope of the sensitivity is less than that of the incubation time, it is useful to choose the lowest incubation time that leads to a reasonable sensitivity. An increase in the inhibition of biosensors was less pronounced beyond 10 min for each pesticide. Therefore, an incubation time of 10 min was used. This value is comparable with most of the data reported in the literature (Istamboulie et al., 2007; Sinha et al., 2010).

### 6.3.2 Biosensor performance in PBS

Numerous relationships between the I % and the inhibitory concentration and/or inhibition time are described in the literature (Zhang et al., 2001). The inhibitory effect of the three OPs in PBS was evaluated with three constructed biosensors, namely B394, B131 and B4. For each insecticide, three calibration curves corresponding to the biosensors were plotted at different concentration ranges. The inhibition responses of all three biosensors were evaluated in PBS

using CPO, EPOx and MAO prior to milk analysis. Each experimental point was the mean of three measurements. The inhibition experiments showed a high level of intra-laboratory reproducibility with a coefficient of variation of 3.43%. The dynamic range for all the pesticides was kept similar to observe the affinity of the biosensors to the pesticides. The dynamic range for CPO was determined to be $5\times10^{-6}$ to $5\times10^{-13}$ M. The LODs obtained for B394, B4 and B131 biosensors were $5\times10^{-12}$, $5\times10^{-10}$ and $5\times10^{-11}$ M, respectively, with an average % RSD of 2.82. The calibration curve of CPO is shown in Figure 6.6 (a).

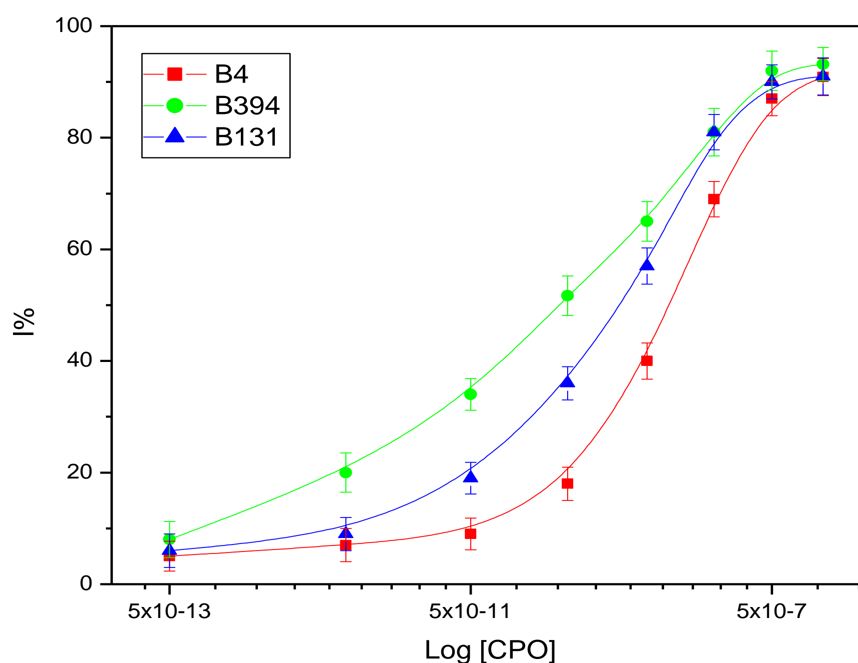



**Figure 6.6 (a)** Calibration curves of CPO in PBS obtained using biosensors B4, B394 and B131 with optimized experimental condition.

For EPOx, the dynamic range was determined to be $5\times10^{-6}$ to $7.5\times10^{-9}$ M for each biosensor. Figure 6.6(b) shows that the LODs obtained for B394, B4 and B131 were $7.5\times10^{-9}$, $1.5\times10^{-8}$, $3\times10^{-8}$ M, respectively (% RSD = 2.934). In the case of the inhibition caused by MAO, the dynamic range was determined to be $7.5\times10^{-11}$ to $1\times10^{-8}$ M. The LOD obtained for B394 was $1.5\times10^{-10}$ M. For B4 and B131, the LODs were similar ($3\times10^{-10}$ M) though there were differences in I% (B4 = 17% and B131=19%). The inhibition caused by MAO is shown in Figure 6.6 (c).

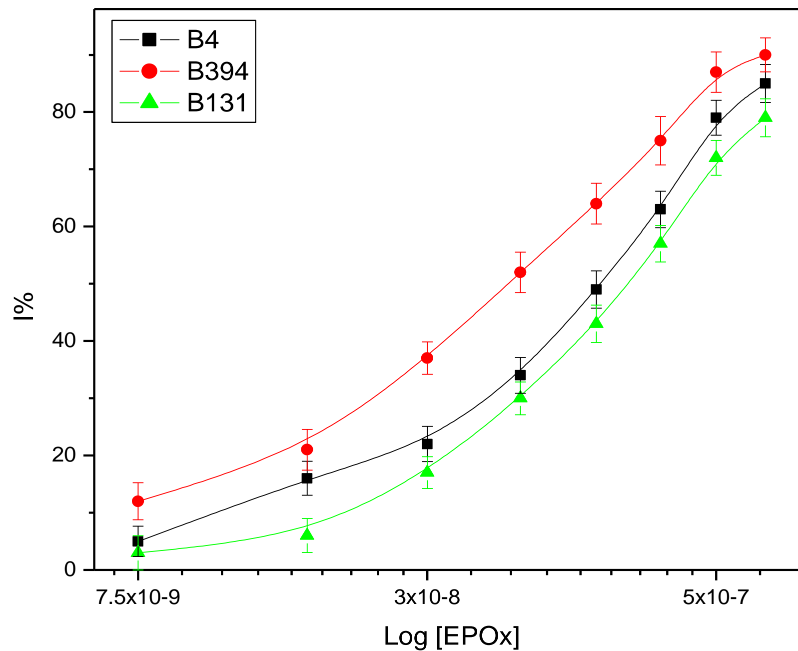



**Figure 6.6 (b)** Calibration curves of EPOx in PBS obtained using biosensors B4, B394 and B131 with optimized experimental condition.

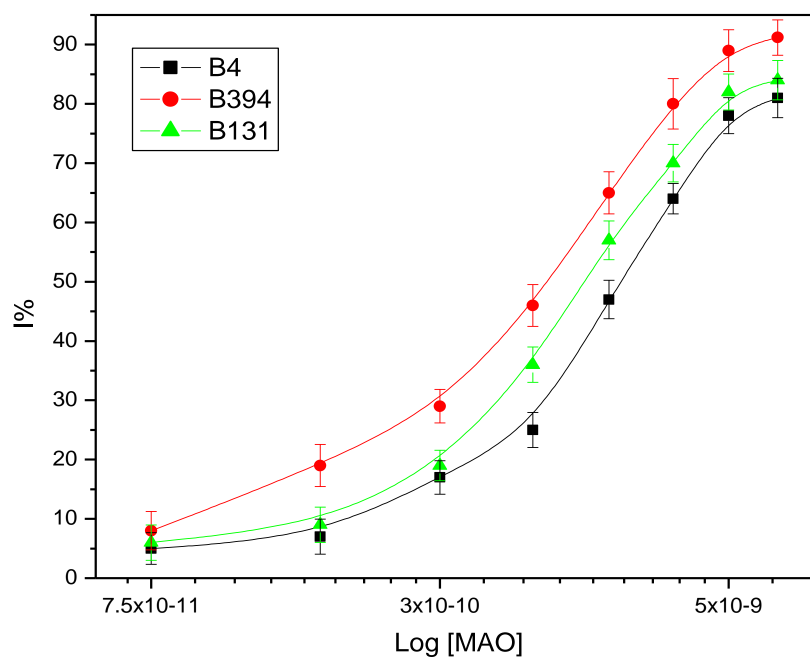



**Figure 6.6(c)** Calibration curves of MAO in PBS obtained using biosensors B4, B394 and B131 with optimized experimental conditions.

It was observed that biosensor B394 exhibited more affinity towards MAO compared to the other two biosensors. Biosensor B4 exhibited the best $IC_{50}$ value with MAO and showed 10 and 100 times more affinity as against CPO and EPOx. The obtained $IC_{50}$ values for MAO, CPO and EPOx are less than $1.25\times10^{-9}$, $2.5\times10^{-8}$ and $1.2\times10^{-7}$ M respectively. The calculated $IC_{50}$ values for MAO, CPO and EPOx are less than $1.25\times10^{-9}$, $5\times10^{-10}$ and $6\times10^{-8}$ M respectively using biosensor B394. In this case, CPO exhibited more affinity towards tested biosensor. Biosensor B131 showed highest affinity towards MAO similar to the B4 biosensor. The analytical figures of merit for developed biosensors are summarized in Table 6.3.

**Table 6.3** Analytical figures of merit for biosensors (B394, B131 and B4) in PB

| Pesticide | Biosensor | Linear range | LOD | $r^2$ | S.D. (N=3) |
|---|---|---|---|---|---|
| CPO | B394 | $5\times10^{-7}$-$5\times10^{-11}$ | $5\times10^{-12}$ | 0.99767 | 0.55433 |
| | B131 | $5\times10^{-7}$- $5\times10^{-10}$ | $5\times10^{-11}$ | 0.98612 | 1.63570 |
| | B4 | $5\times10^{-7}$-$5\times10^{-9}$ | $5\times10^{-10}$ | 0.99104 | 1.41620 |
| EPOx | B394 | $5\times10^{-7}$-$3\times10^{-8}$ | $7.5\times10^{-9}$ | 0.99835 | 0.39645 |
| | B131 | $5\times10^{-7}$-$6\times10^{-8}$ | $3\times10^{-8}$ | 0.99953 | 0.22903 |
| | B4 | $5\times10^{-7}$-$6\times10^{-8}$ | $1.5\times10^{-8}$ | 0.99970 | 0.18765 |
| MAO | B394 | $5\times10^{-9}$-$3\times10^{-10}$ | $1.5\times10^{-10}$ | 0.99387 | 0.95561 |
| | B131 | $5\times10^{-9}$-$3\times10^{-10}$ | $3\times10^{-10}$ | 0.99509 | 1.02806 |
| | B4 | $5\times10^{-9}$-$6\times10^{-10}$ | $3\times10^{-10}$ | 0.99486 | 0.95414 |

The calculated $IC_{50}$ for MAO, CPO and EPOx are $1.25\times10^{-9}$, $5\times10^{-9}$ and $2.5\times10^{-7}$ M respectively. MAO is predominantly showed more affinity towards B131with $IC_{50}$ $1.25\times10^{-9}$ M. In summary, MAO showed as a strong inhibitor for B4 and B131 whereas for B394 CPO turned to be the most potent inhibitor among tested OPs in PB matrix.

### 6.3.3 Calibration curve in milk

During the OPs analysis in PBS, it was observed that biosensor B394 was the most sensitive among the three biosensors. Therefore, biosensor B394 was used for the pesticide analysis in milk. To produce calibration data, milk (without fat) was used to determine the biosensor response. The wide dynamic range, from $5\times10^{-12}$ to $5\times10^{-6}$ M, was chosen to analyse spiked milk samples. Using B394, the LODs obtained for EPOx, CPO and MAO were $5\times10^{-9}$, $5\times10^{-12}$ and $5\times10^{-10}$ M, respectively. It is clearly visible in Figure 6.7(a) that the linear range of the tested pesticides was in a similar range, $3\times10^{-10}$ to $5\times10^{-9}$ M with S.D. of 3.59% and $r^2 = 0.9926$.

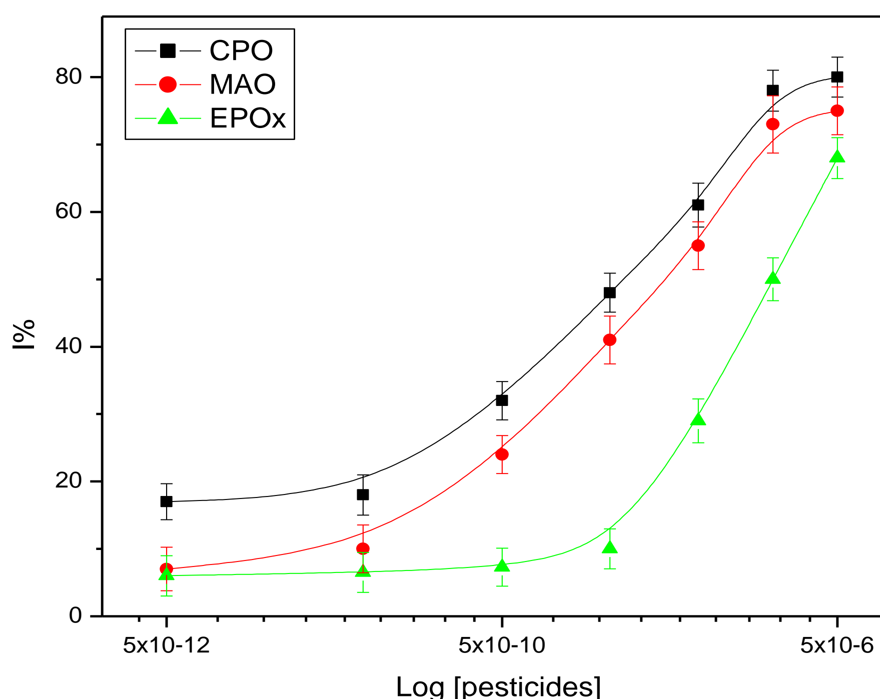



**Figure 6.7 (a)** Calibration curves for CPO, MAO and EPOx in milk samples using biosensor B394 with optimized experimental conditions.

The detection limits obtained in this study are the most sensitive LODs in milk compared to reported methods (Zhang et al., 2005). Figure 6.7 (b) shows the real peaks obtained during the analysis of MAO in milk. It can be seen that there is no significant variation in three peaks before and after incubation but the I% can be easily noticed. The flow-based biosensor system

exhibited very stable signals during the analysis of the OPs. The inhibition values obtained from the milk samples correlated well with the calibration curve in PBS. There was no unspecific inhibition caused by the milk samples. The LODs obtained using biosensor B394 in milk was higher than those obtained in PBS for each tested analyte (signal to noise ratio $\geq 3$).

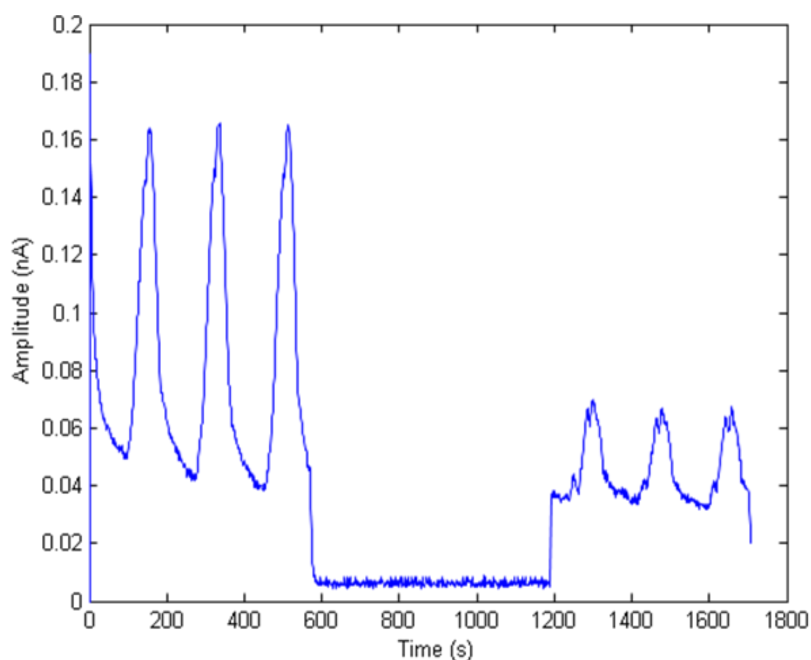



**Figure 6.7 (b)** Real peaks obtained during the analysis of MAO ($5\times10^{-9}$ M) in a milk sample using biosensor B394 with optimized experimental conditions.

The biosensor also distinguishes the $IC_{50}$ values in PB and milk because of matrix effect. Calculated $IC_{50}$ for PB using B394 are $1.25\times10^{-9}$ M, $5\times10^{-10}$ M and $6\times10^{-8}$ M for MAO, CPO and EPOx respectively whereas in milk, the obtained $IC_{50}$ values are below $5\times10^{-8}$ M, about $5\times10^{-9}$ M and $5\times10^{-7}$ M for MAO, CPO and EPOx respectively.

### 6.3.4 Applicability of biosensor in water and milk

The water and milk samples were spiked with CPO ($5\times10^{-11}$ and $5\times10^{-7}$ M), EPOx ($5\times10^{-9}$ M and $5\times10^{-7}$ M) and MAO ($5\times10^{-10}$ M and $5\times10^{-7}$ M). The spiked concentrations were chosen from biosensor B394's linear range of the calibration curve constructed for PB and milk. Water

samples were analyzed after filtration using 2 and 5 µm filter papers sequentially. In each spiked concentration, the percentage of the recovery obtained was smaller than 100%. The recoveries obtained in water samples were 95.7-99.2%. Table 6.4 shows the calculated % recovery in real water samples.

**Table 6.4** Recovery studies of CPO, EPOx and MAO in real water samples using biosensor B394 under optimum conditions

| Pesticides | Added [M] | Found [M] | % recovery | S.D. (N=3) |
|---|---|---|---|---|
| CPO | $5\times10^{-11}$ | $4.94\times10^{-11}$ | 98.8 | 2.40 |
| | $5\times10^{-7}$ | $4.96\times10^{-7}$ | 99.2 | 2.2 |
| EPOx | $5\times10^{-9}$ | $4.85\times10^{-9}$ | 97.0 | 2.53 |
| | $5\times10^{-7}$ | $4.78\times10^{-7}$ | 95.7 | 2.72 |
| MAO | $5\times10^{-10}$ | $4.87\times10^{-10}$ | 97.5 | 1.93 |
| | $5\times10^{-7}$ | $4.84\times10^{-7}$ | 96.8 | 2.0 |

Two milk samples of the same brand that differed only in their fat content were also analyzed after filtration using 5 µm filter paper. As expected, no significant inhibitions were observed. This finding leads to the conclusion that after filtration, the matrices did not affect the analysis. The percentage was in expected values as milk is a complex matrix. The recoveries were not less than 95% for lower fat milk (15.5%) samples but were less than 90% in higher fat containing milk (30%) samples. The higher percentage milk could have a small effect on the biosensor.
The obtained recoveries are presented in Table 6.5. Though direct incubation in the milk samples would be advantageous, the low recovery rates and matrix effects would impede sample analysis. The biosensor food screening test was fast compared to chromatographic methods. The AChE biosensor test is suitable for milk analysis, but more suited to low fat milk samples. The recovery rate of OPs in milk was in the range of the legislative regulations that require a recovery rate between 70 and 110% (European Communities, 1997).

**Table 6.5** Recovery studies of CPO, EPOx and MAO in two different fat-containing milk samples using biosensor B394 under optimum conditions

| Pesticides | Added [M] | Pesticide Found [M] | % recovery (15% fat milk) | S.D. (N=3) | Found [M] | % recovery (30% fat milk) | S.D. (N=3) |
|---|---|---|---|---|---|---|---|
| **CPO** | $5\times10^{-11}$ | $4.92\times10^{-11}$ | 98.5 | 2.70 | $4.75\times10^{-11}$ | 95.0 | 3.03 |
| | $5\times10^{-7}$ | $4.91\times10^{-7}$ | 98.2 | 2.40 | $4.50\times10^{-7}$ | 90.0 | 3.25 |
| **EPOx** | $5\times10^{-9}$ | $4.80\times10^{-9}$ | 96.0 | 2.63 | $4.45\times10^{-9}$ | 89.0 | 3.21 |
| | $5\times10^{-7}$ | $4.75\times10^{-7}$ | 95.0 | 2.82 | $4.35\times10^{-7}$ | 87.0 | 3.11 |
| **MAO** | $5\times10^{-10}$ | $4.85\times10^{-10}$ | 97.0 | 2.03 | $4.63\times10^{-10}$ | 92.60 | 3.34 |
| | $5\times10^{-7}$ | $4.82\times10^{-7}$ | 96.5 | 2.32 | $4.67\times10^{-7}$ | 93.40 | 3.46 |

### 6.3.5 Influence of flow rate on OPs detoxification

The effect of contact time on the hydrolysis of a $1\times10^{-6}$M EPOx solution was studied by changing the flow rate (0.5 mL $min^{-1}$ and 1.0 mL $min^{-1}$). The results revealed the relationship between OPs degradation and flow rate as a feed solution through an Omnifit column. Three EPOx concentration levels ($1\times10^{-4}$ M to $1\times10^{-6}$ M) were tested. For all experiments, the feed solution was prepared in 0.1M PB, pH 8. Degradation of OPs by the PTE reached almost 100% with the flow rate 0.5 mL $min^{-1}$. The time required to reach the maximum degradation percent was found to be inversely proportional to the flow rate at each pesticide concentration. An optimum flow rate of 0.5 mL $min^{-1}$ was selected, corresponding to a contact time of 6 min.

### 6.3.6 Effect of storage on column efficiency

The stability of the Omnifit-PTE based column over the 30 days following immobilization on Sepharose 4B was examined by measuring the capacity for degradation of OPs. The stability with time of the detoxification column was studied using different concentrations of EPOx ($1\times10^{-6}$ M -$1\times10^{-9}$ M). The column exhibited excellent stability in water matrix over the milk

matrix. For first 10 days, no significant loss is activity was observed in water. When Omnifit column immobilized PTE was used for detoxification of OPs in water over 3 weeks, the column retained about 75% activity. An intra-assay coefficient of variation (CV) of 2.2% was observed. In case of milk, the column retained about 50% activity after 7 days of operation.

### 6.3.7 Optimization of column conditions for detoxification

To check the performance of the prepared PTE based Omnifit column, $1\times10^{-6}$M solution of EPOx was first spiked in water and checked as a reference to test the column and efficiency of PTE, then spiked in milk and passed through the column. For each samples, weather it is water or milk, the pH was adjusted to 8 because PTE works optimum at this point. All experiments were done in 0.1 M PB, pH 8, at 25 C°. Each experiment was repeated at least three times. In first attempts, the control of the pesticide solution, flow rate was checked using a peristaltic pump fitted with polytetrafluorethylene (Teflon) tubes. The efficiency of the developed detoxification column was studied using EPOx, CPO and MAO. The effect of contact time on the hydrolysis of a $1\times10^{-6}$ M EPOx solution was studied by varying the flow rate (0.5 mL $min^{-1}$ and 1.0 mL $min^{-1}$). The detoxification efficiency was controlled using an AChE biosensor B394, which allows detecting pesticide lower down to $1\times10^{-11}$M. As expected, decreasing the flow rate allows increasing the contact time thus the percentage of detoxification. An optimum flow rate of 0.5 mL $min^{-1}$ was selected, corresponding to a contact time of 6 min. In these conditions, PTE based column showed consistency in the performance and allow us to assume that the EPOx was completely hydrolyzed.

### 6.3.8 Detoxification of CPO, EPOx and MAO spiked in water and milk

Detoxification of CPO, EPOx and MAO was performed using a detoxification Omnifit column filled with 4 mL of Sepharose Gel 4B coupled with 335 IU of PTE. Table 6.6 presents the brief summary of the detoxification of OPs in milk. The column was connected via peristaltic pump fitted with Teflon tubes and the flow rate was set to 0.5 mL $min^{-1}$. Because of the high activity of PTE for EPOx, the detoxification of EPOx was more as compared with other two analytes CPO and MAO. Different concentrations of EPOx, CPO and MAO ranging from $1\times10^{-6}$ M to $1\times10^{-9}$ M were tested in water and milk.

**Table 6.6** Summary of % detoxification of individual OPs spiked in milk measured using PTE column (CPO: Chlorpyriphos oxon, EPOx: Ethyl paraoxon, MAO: Malaoxon)

| **OPs** | **Before treatment- flow rate 0.5mL $min^{-1}$** | | **After treatment- flow rate 0.5 mL $min^{-1}$** | | |
|---|---|---|---|---|---|
| | **Actual Conc. (M)** | **Inhibition (%)** | **Inhibition (%)** | **Calculated Conc. (M)** | **% Detoxification** |
| **CPO** | $1\times10^{-6}$ | 80 | 12 | $<1\times10^{-12}$ | 85.00 |
| | $1\times10^{-7}$ | 78 | 14 | $<1\times10^{-12}$ | 82.05 |
| | $1\times10^{-8}$ | 61 | 12 | $< 1\times10^{-12}$ | 80.32 |
| | $1\times10^{-9}$ | 48 | 6.5 | $< 1\times10^{-12}$ | 86.45 |
| **EPOx** | $1\times10^{-6}$ | 68 | 16.0 | $\leq 5\times10^{-9}$ | 76.47 |
| | $1\times10^{-7}$ | 50 | 13.6 | $< 5\times10^{-10}$ | 72.80 |
| | $1\times10^{-8}$ | 29 | 7.5 | $< 5\times10^{-11}$ | 74.13 |
| | $1\times10^{-9}$ | 10 | 2.6 | $< 5\times10^{-12}$ | 74.00 |
| **MAO** | $1\times10^{-6}$ | 75 | 23.7 | $\leq5\times10^{-10}$ | 68.40 |
| | $1\times10^{-7}$ | 73 | 21.9 | $<5\times10^{-10}$ | 70.54 |
| | $1\times10^{-8}$ | 55 | 18.0 | $<5\times10^{-10}$ | 67.27 |
| | $1\times10^{-9}$ | 41 | 12.3 | $<5\times10^{-10}$ | 70.00 |

PTE can hydrolyze EPOx and CPO completely (100%) whereas MAO hydrolysis is 88% in water. Significant detoxification of these pesticides was obtained in milk. EPOx was found to be the best substrate for PTE as it degraded EPOx by 86% in milk whereas PTE showed 76% and 70% degradation for CPO and MAO respectively. To ensure the detoxification of OPs in the milk, the end products were passed through the flow based biosensor and compared with the calibration of the original solution.

### 6.3.9 Detoxification of mixtures of OPs in milk (EPOx, MAO and CPO)

After individual detoxification of three OPs, mixtures of these pesticides in milk were also tested using constructed detoxification column. Combination of pesticides was degraded using PTE enzyme. The pesticide concentration from $1\times10^{-6}$ M to $1\times10^{-9}$ M was used for degradation studies. The compiled results are depicted in figure using bar graph between % detoxification and concentration of OPs mixture. As known to us, with increase in concentration, % detoxification is observed to be more for mixture of pesticides. Figure 6.8 represents detoxification of mixture of pesticides at four different concentrations in milk.

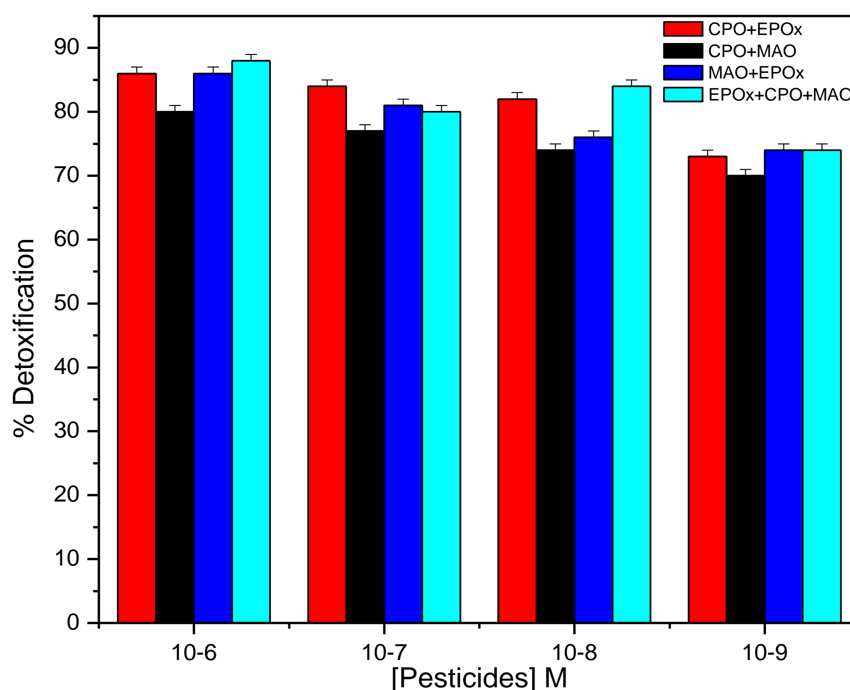



**Figure 6.8** Analysis of mixture of OPs (EPOx, MAO and CPO) in milk using PTE based detoxification column. Error bars represent mean and standard deviation of 4 values (N=4).

When mixture of two pesticides is degraded, maximum degradation was observed for binary mixture CPO + EPOx at each concentration. The observed trends are as follows: EPOx + CPO > EPOx + MAO > CPO + MAO. The mixture which contains EPOx, showed always higher detoxification as compare to other mixtures. When mixture of 3 OPs (EPOx + MAO + CPO) was analyzed, cumulative/or additive detoxification effect was observed. Hence,

obviously the degradation was observed to be more than binary mixture composition. It is also observed from the obtained data that the decrease in concentration also causes the reduction in % detoxification. The analytical figures of merit for detoxification of OPs are shown in Table 6.7.

**Table 6.7** Analytical figures of merit obtained for detoxification of OPs using PTE column

| Analytical features | Results achieved |
|---|---|
| Matrix tested | Water and milk |
| Temperature | 25°C |
| Response time | 5 min |
| Analysis time | 15 min |
| Range of detoxification | $1\times10^{-6}$ to $1\times10^{-9}$ M |
| Minimum % detoxification | $1\times10^{-9}$ M |
| Maximum % detoxification achieved | Water 100% and milk 86% |
| % CV | 1.20 |

**6.4. Conclusions**

The development of an automated flow-based biosensor test for the detection and detoxification of OPs in milk was described. The biosensor met the requirements set by EU regulations. Biosensor B394 could determine EPOx, CPO and MAO lower down to $5\times10^{-9}$, $5\times10^{-12}$ and $5\times10^{-10}$ M, respectively, in milk samples. The developed system could successfully determine the presence of OPs. The test could be completed in less than 15 min with good reproducibility. The proposed system can be applied successfully in online monitoring of OPs in milk processing

units and collection centres. Investigation also showed the potential of PTE as a detoxification tool for OPs in water and milk. Three most toxic insecticides (EPOx, MAO and CPO) were tested to check the cumulative detoxification effect in the presence of PTE. Under optimum conditions, a column containing 335 IU of PTE was shown to immediately detoxify EPOx, MAO and CPO in the concentration down to $1\times10^{-9}$M. In the case of MAO, the detoxification was lower, due to the very slow hydrolysis of this pesticide by PTE. In case of EPOx and CPO, the detoxification was found 86% and 76%, when the detoxified product was evaluated using flow based biosensor. OPs mixtures were also significantly detoxified in milk up to 86%. The method was shown to be adapted for the detoxification of OPs containing water and milk.

# Chapter 7

# Conclusions

This thesis has been focused on the development of novel biosensing techniques using optical and electrochemical methods based on enzyme inhibition. There is an urgent need for novel high-throughput techniques for monitoring the contamination of water and daily consumed food commodity such as milk. The significance of the research lies in the facilitation of high-throughput, sensitive biosensors for determination of OPs. In this work, native and stabilized BuChE, native as well as genetically engineered AChE and native ALP enzymes were deployed as prime bio-component for the quantification of OPs (individual as well as mixture of pesticides) and CM. Determination of OPs at pg $mL^{-1}$ is a major outcome of developed biosensor. The developed biosensors are found to be highly sensitive.

Extensive experimental work was carried out for optimizing reaction parameters, evaluation of the biosensor performance, interferences of the similarly potent analytes and analysis of real samples. During the course of study, significant observations were noted. The list of observations and conclusions are:

1. Enzymes are observed to be sensitive biomolecules in its native form. Several different approaches were studied for enzyme stabilization to achieve sensitive bioassay. A novel enzyme stabilizing protocol for long term stability (up to 6 weeks) of BuChE in micro-well plate was achieved (chapter 2).

2. A high sensitivity, high throughput, enzyme inhibition assay is demonstrated using 384, 1536 well plates. The developed optical bioassay was proved to be specific, can quantify analytes up to pg $mL^{-1}$ level. Analysis of OPs in 1536 wellplate format facilitates tremendous capacity of high-throughput analysis of milk samples at milk collection centres and milk parlors. Analysis of mixture of OPs was also studied to evaluate cumulative toxicity of analytes (chapter 2).

3. The key parameters to be followed to evaluate the assay performance with miniaturized plate assays were the LOD, low toxic waste generation and less analysis time. Based on these results, we can claim that 384 wellplate can significantly enhance the assay performance even in absence

of automation (considering all aspects reagent and material consumption, performance etc.). For higher well plate such as 1536, automation is preferred (chapter 2).

4. Micro and nano fabrication technologies offer small size chip devise. The small size chip has ability to use very small quantities of samples and reagents and to carry out separations and detections with high resolution and sensitivity; low cost; short times for analysis; and small footprints for the analytical devices. The miniaturized assay on Au chip using AChE was successfully demonstrated for screening low level OPs. The study demonstrates one of the rare attempts for high throughput CL based detection for MPOx in water/milk using biochip devise wherein up to 40 samples can be analyzed simultaneously. The biochip could be stable at room temperature for more than 3 months without significant loss in enzymatic activity. A major achievement of the work is miniaturization of assay and point of care testing at milk collection centres with ease of operations. The developed biochip can determine OPs concentration lower down to 0.001 µg $L^{-1}$ in milk which is at par the EU standard. This work signifies application of biochip platforms as an ideal vehicle for enzymatic assays because of their versatility, efficiency, and ability to handle small volumes. The present invention has multifold applications in dairy industries and milk collection centers with ease and use of microwell chip device as a screening technology. The biochip based results has been cross validated and showed an excellent co-relation with standard chromatographical techniques LC-MS/MS (chapter 3).

5. A novel on chip inhibition assay for heavy metals was demonstrated in water using $Hg^{2+}$ as a model analyte. Aluminium nanostructures were used for GOx immobilization. On chip measurement for $Hg^{2+}$ was found highly sensitive with LOD 0.25 µg $L^{-1}$. The device facilitates high-throughput analysis of $Hg^{2+}$ in 20 minutes using CL technique. Further improvements can be done in assay using more selective enzymes with other HMs such as $Cd^{2+}$ and $Pb^{2+}$ (chapter 4).

6. $SiO_2$, a low cost material was successfully demonstrated for ALP immobilization in micro column. The analysis of pesticide residues visually as well as colorimetrically has been demonstrated successfully using $SiO_2$ packed micro column. The presented work utilizes immobilized ALP on a disposable micro column with the following merits; (a) The technique is affordable and simple to perform and interpret the results visually (b) The assay is optimized to

meet the drinking water quality criteria for safe drinking water in fields and (c) ALP coupled micro-silica column as a disposable bio-component for analysis of pesticides in water. Thus, the proposed bioassay can be useful to provide safe drinking water to the common population. Interference of HMs; such as $Hg^{2+}$, $Cd^{2+}$ and $Pb^{2+}$ were investigated. No obvious interferences were noticed with the presence of these selected metal ions for determination of OPs (chapter 5).

7. A good intra-batch and inter-batch reproducibility was obtained for developed biosensors (% RSD 3.2). Studies for the matrix effect and recoveries in spiked distilled and drinking water samples suggest that method can be extended for determination of large number of real samples containing OPs and CM in drinking water (chapter 5).

8. AChE proved to be a perfect choice for OPs analysis due to its high sensitivity and high specificity towards MPOx, EPOx and CPO as compare to MAO and CF in the same matrix. Genitically modified enzymes such as B394 immobilized on PVA showed excellent stability over the period of 12 weeks. An application of automated flow based electrochemical biosensor for determination of OPs in milk has been successfully demonstrated using highly sensitive genetically modified enzymes B394 (chapter 6).

9. Detoxification studies were also demonstrated successfully for removal of MPOx from water and milk. About 90% of EPOx detoxification was successfully demonstrated using PTE as detoxifying agent. The detoxification product was evaluated using developed highly sensitive flow based biosensor B394. Thus work relates the practical application of the biosensors to determine and detoxify the environmental pollutants. Such attempts have been rarely reported in the literature (chapter 6).

10. The following advantages are shown by the proposed system; it is automated, the analytical signal is practically unaffected by matrix, and it does not require separation of the reaction products. Thus a sensitive, robust method applicable for continuous online monitoring of OPs is successfully shown in lab scale. The results reported in this thesis are among the most sensitive values for OPs using stabilized BuChE and genetically modified AChE (chapter 6).

## Future Scope of the work

- Pesticide biosensor probe can be further extended to monitor food commodity such as vegetables and fruits juices.
- The developed biosensors can be tested for online monitoring and remediation of pesticide contaminated sites.
- Miniaturized biochip can be extended for the other biomarkers for study of human exposure in biological fluids.
- ALP based colorimetric probe can be extended for onsite OPs analysis in drinking water for common population.
- Aluminium nanostructure can be tested for others heavy metals with selective enzymes.

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# Primary Water and Milk Standards

## Primary Drinking Water Standards

| Quality monitoring Agencies | Pesticide ( μg $mL^{-1}$) | Mercury ( μg $mL^{-1}$) | Reference |
|---|---|---|---|
| Bureau of Indian Standard | 0.001 | 0.001 | BIS report |
| European Union | 0.0001(individual) 0.0005 (total) | 0.001 | EU, Commission Directive1999/ 50/EC |

## Standards for Milk and Milk products

| Quality monitoring Agencies | Pesticides | References |
|---|---|---|
| Food Safety and Standards Authority of India | 0.001mg/kg | Food safety and standards, 2011 |
| European Union | individual pesticides 10 μg $kg^{-1}$ | EU, Commission Directive1999/50/EC |

# List of Publications

## Patent application

1. Co-inventor in Indian Patent Application No. 933/MUM/2012 (filed) and Co-inventor in Patent Co-operation Treaty (PCT) to be filed (Output from chapter 3).

## List of publications counted in Thesis

1. R. K. Mishra, G. Istamboulie, S. Bhand, J.-L. Marty. Detoxification of organophosphate residues using phosphotriesterase and their evaluation using flow based biosensor. *Analytica Chimica Acta.* 2012, 745, 64-69 (Output from chapter 6).

2. R. K. Mishra, R. B. Dominguez, S., Bhand, R. Muñoz, J.-L. Marty. A novel automated flow-based biosensor for the determination of organophosphate pesticides in milk. *Biosensors and Bioelectronics.* 2012, 32, 56-61 (Output from chapter 6).

3. R. K. Mishra, K. Deshpande, S. Bhand. A Highthroughput enzyme assay for organophosphate residues in milk. *Sensors.* 2010, 10, 11274-11286 (Output from chapter 2).

4. R. K. Mishra, G. K. Mishra, Dharma Teja V, B. Danielsson S. Bhand. A visual colorimetric dual readout bioassay for determination of pesticide residues in drinking water. Submitted to *Talanta* : TAL-D-12-03495 (Output from chapter 5).

5. R. K. Mishra, H. J. Pandya, S. Chandra, S Bhand. A novel micro biosensor for glucose and mercury detection. Manuscript under prepration. To be submitted (Output from chapter 4).

## Other publications

1. K. Deshpande, R. K. Mishra, S. Bhand, Determination of Methyl Parathion in Water and Its Removal on Zirconia Using Optical Enzyme Assay. *Applied Biochemistry and Biotechnology.* 2011, 64, 906-917.

2. G. K. Mishra, R. K. Mishra, S. Bhand, Flow injection analysis biosensor for urea analysis in adulterated milk using enzyme thermistor. *Biosensors and Bioelectronics.* 2010, 26, 1560-1564.

3. K. Deshpande, R. K. Mishra, S. Bhand, High sensitivity micro format chemiluminescence enzyme inhibition assay for determination of Hg (II). *Sensors.* 2010, 10*,* 6377-6394.

4. K. Deshpande, R.K. Mishra, S. Pal, B. Danielsson, M. Willander, S. Bhand. A novel on-chip analysis of dissolved Hg (II) in drinking water. NSTI-Nanotech. 2010, 3, 133-136.

## Conferences and workshop attended

1. Poster Presentation on National Symposium/Workshop on New Trends of Biosensor Technology *(*NSNTBT-2009*)* organized by Biosensor Society of India.

2. Oral presentation on International Conference and Exhibition on Total Engineering. Analysis and manufacturing Technologies. November 2009 in Bangalore (Chemical and Bio-sensors: Design / Modeling and Manufacturing).

3. Poster Presentation on “National Conference on Green & Sustainable Chemistry” organized by Chemistry Group BITS, Pilani, Pilani Campus February 19-21, 2010.

4. One Day Workshop on AFM for Biological Materials on 4$^{th}$ Feb, 2010 organized by National Center for Nanomaterials and Nanotechnology, University of Mumbai.

5. Workshop/ Training programme on Immobilization techniques for miniaturized microarray based biosensors. January 10-14, 2011 at Department of Biotechnology, Punjabi University Patiala, Punjab.

6. Poster presentation on Transfrontaliar Meeting on Sensors and Biosensors, organized by University of Paul Sabatier Toulouse, France (29-30 Nov 2011).

**Accepted abstract in conferences**

1. Rupesh K. Mishra, Gustavo A. Alonso, Georges Istamboulie, Roberto Muñoz, Sunil Bhand, Jean-Louis Marty. Automated flow based biosensor for determination and discrimination of pesticide mixtures using genetically modified enzymes. Biosensor 2012, 15-18 May, Cancun, Mexico.

2. Rupesh K. Mishra and Sunil Bhand. A colorimetric biosensor for determination of organophosphate pesticide residues in drinking water. 10-12 October 2011- Amsterdam, The Netherlands.

3. Rupesh K. Mishra and Sunil Bhand. Sensitive colorimetric enzyme assay for organophosphate residues in milk 10$^{th}$ Workshop on Biosensors and Bioanalytical micro techniques in environmental and clinical analysis. 19-22 June 2011, Weimar Germany.

4. Rupesh K Mishra, Kanchanmala Deshpande, Sudhir Chandra and Sunil Bhand. "Novel biochip for analysis of organophosphate residues" in first Biosensing Technology Conference held at Bristol. (November 10-12, 2010).

5. Rupesh K Mishra, Kanchanmala Deshpande and Sunil Bhand. "Enzymatic assay for determination of methyl paraoxon in low fat milk" in 20$^{th}$ World Biosensor Congress, Glasgow, UK (May 26-28, 2010).

# Brief Biography of the Candidate

| | |
|---|---|
| Name | Rupesh Kumar Mishra |
| Date of Birth | 02-04-1983 |
| Education | M. Sc. (Biotechnology) |
| | Pt. Ravishankar ShuklaUniversity Raipur, C.G. (2006) |
| | B. Sc (Chemistry, Botany, Zoology) |
| | Devi Ahilya Vishv Vidhyalay Indore, M.P. (2004) |
| Email ID | rupeshmishra02@gmail.com |

**Research Experience (4 years 9 months)**

**1.** Presently working as Research Associate in National Agricultural Innovation project (NAIP), ICAR (May 2011 to March 2013).

**2.** Worked as Senior Research Fellow in National Agricultural Innovation project (NAIP), ICAR (March 2008 to April 2011).

**Research Publications**

01 Patent, 07 publications in international journals

**Conferences/workshop attended**

06 (04 national and 02 international)

**Work Experience**

**1.** Worked as a Microbiologist in Prem Pharmaceuticals Pvt Ltd. Indore (Sep 2007 to March 2008).

**2.** Worked as a trainee chemist in Anusandhan Analytical Laboratories Pvt. Ltd. Indore (Sep 2006-Aug 2007).

**Honors and Awards**

Recipient of sandwich Ph. D. fellowship awarded by French Embassy in India, New Delhi, (May 2011-Nov 2011).

## Brief Biography of the Supervisor

| | |
|---|---|
| Name | Prof. Sunil Bhand |
| Date of Birth | 17.03.1969 |
| Present Position | Associate Professor & Head, Department of Chemistry |
| | Department of Chemistry, BITS, Pilani-KK Birla Goa Campus |
| Address | C-201 BITS, Pilani-KK Birla Goa Campus |
| | NH17B Bypass, Zuari Nagar Goa 403726 India |
| Email | sunilbhand@goa.bits-pilani.ac.in, sunil17_bhand@yahoo.com |
| Education | Ph.D. 1996 |
| | M.Sc. 1990 (First in University Merit) |

**Post-Doctoral Experience**

Department of Pure and Applied Biochemistry

Lund University Sweden 2001-2002,

Short term visits 2003, 2004, 2005, 2007, 2008

**No. of Sponsored Research Projects**

**(a)** Completed projects

**i.** Joint Indo-Swedish Project on Biosensors for Environmental analysis 2003-2005 funded by Swedish Research Council (Prof. B. Danielsson and Prof. Sunil Bhand as joint PIs) 35 lakhs.

**ii.** CSIR Project 2006-2009 on biosensors for analysis of pesiticides in sea water 14.6 lakhs.

**(b)** Ongoing project

**i.** Consortium PI for NAIP, ICAR New Delhi funded project on "Development of biosensors and micro techniques for analysis of pesticide residues, aflatoxin, heavy metals and bacterial contamination in milk.729 lakhs, in collaboraiton with IITD, NDRI and PU Patiala.

**ii.** Consortium Co-PI, NAIP project on "Detection and mitigation of dairy pathogens and detection of adulterants using chemical biology" 45 lakhs.

**Honors and awards**

i. Invited as Opponent to a Ph.D. Thesis for Linkoeping University Sept. 2011.

ii. Best Poster award "Biosensors for arsenic analysis" 7th Intl Conference on Biogeochemistry of trace elements 2003 Uppsala Sweden

iii. UV Rao memorial awards for young scientists by Indian Chemical Society 1998.

**Publications**

i. 2 Patents and 26 publications in internaitonal journals.

ii. Membership of societies: Affiliate member IUPAC since 2000 IAEAC Switzerland, AAAS, USA, 2012.

**Reviewer for international journals**

Biosensors and Bioelectronics, Analytical Letters, Int Journal of Env Anal Chemistry, Applied Biochemistry and Biotechnology, J Agri food Chemistry.

**No of Ph.D. Students**

Completed 01, Registered 05, total 06 scholars working in the biosensor group.

**No of Conferences organized:** 02, (01 upcoming in Feb 2013).