Progress in Enzyme Inhibition Based Detection of Pesticides

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Abbreviations:

acetylcholinesterase (AChE)

limit of detection (LOD)

Ion Sensitive Field Effect Transistor [ISFET]

Cyanuric Chloride (CyC)

Practical application: Considerable efforts have been made to construct a cholinesterase biosensor with improved sensitivity and selectivity. The sensitivity of this biosensor was improved by incorporating efficient immobilization methods such as self assembled monolayer, thin polymer films. Additionally carbon nanotubes, nano metal particles helped to enhance the sensitivity and detection limit of the biosensor. Genetic engineering of cholinesterase enzyme helps in discriminative detection of pesticides in the real sample, which will lead to the development of multienzyme biosensors.

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Abstract

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The previous few decades have seen the development of biosensors and their use in monitoring of pesticides in food and environmental samples. Although inhibition based biosensors have been subject of several recent research works, their performance characteristics greatly depend on the type of immobilization, and the presence of interfering compounds in the samples. Moreover, sensitivity, detection limits and rapidity of the response are few of the other major features which need to be investigated further if they are to become operationally user-friendly. This review will highlight research carried out in the past on biosensors that are based on enzyme inhibition for determination of organophosphorus compounds and carbamate pesticides.

Introduction

Food-borne contaminants and environmental pollutants come in a variety of sizes ranging from simple chemical compounds to bacterial cells. Owing to health concerns, the food and environmental sector requires a sensitive, specific and rapid method to monitor the presence of toxic substances, which occur due to natural or deliberate contamination. Conventional methods [1,2] are good enough to detect the pesticides, but they are very much time consuming, need chemicals, are expensive, require skilled persons to operate and not suitable for real time analysis. The specificity, portability, simplicity, high sensitivity, potential ability for real-time and on-site analysis in food analysis, environmental control, clinical detection and agricultural industries has made the use of biosensors attractive for such applications. Biosensors are devices which convert information about the chemical substance in the system into measurable signals like ions, electro active species, heat release, absorption and mass change of wave propagation using a bio-sensing element. They comprise of two components: the bio-sensing element and a transducing element (electrochemical, optical and or piezoelectric) coupled through a suitable immobilization method.

The response of an enzyme-inhibition-based biosensor depends on the concentration of the enzyme on its surface and its interaction with the substrate. Substrates play a vital role in determining the activity of the enzyme; hence the choice of the substrate is an important criterion for the development of a biosensor. Selection of a suitable substrate helps in fixing the transducing element. Acetylcholine and butyrylcholine salts are specific and natural substrates for cholinesterase in both acetyl and butyryl form of the enzyme. When they are used as substrates, potentiometric detection is preferred because of the formation of H^+ ions after enzymatic reaction. Some of the other substrates used for measuring the activity of cholinesterase are acetylthiocholine, butyrylthiocholine salts and salts of acetate (3-indoxyl acetate, 4-aminophenyl acetate, nitrophenyl acetate, indophenyl acetate). Amperometric and piezoelectric transducers are the preferred detection techniques when thiocholine and acetate salts are used as substrates. Their sensing strategy is based on the formation of electro active species and change in mass owing to precipitation of 3-indoxyl, 4-aminophenyl, nitrophenyl and indophenyl acetate after the hydrolysis. The choice of enzyme plays a significant role in the construction of a biosensor. Although butyrylcholinesterase has been used as a biosensing element for detection of organophosphorus compounds, higher sensitivity and low detection limits have been achieved using acetylcholinesterase (AChE) when compared to

butyrylcholinesterase. The sensitivity of a biosensor depends on the amount of enzyme loading and purity of enzymes used. High enzyme concentration (80 mUA per electrode) have been used [3] to obtain low detection limits for pesticide presence owing to the low specific activity of acetylcholinesterase (bovine erythrocytes). This established that sensitivity is dependent on the purity of enzyme. Attainment of maximum signals from enzymatic reaction helps in inhibition studies. Reports on high sensitivity using low enzyme concentration are available in literature [4]. Different inhibitors give different degrees of inhibition, which are proportional to the concentration of inhibitor. Quantitative analysis of enzyme activity depends on the response of the sensor before and after the incubation with inhibitor. Biosensor characterization is dependent on factors like enzyme loading, pH, range, sensitivity and reproducibility. These parameters have to be determined and the most suitable conditions for the detection have to be adopted for carrying out the detection studies using biosensors.

Principle of Enzyme-Based Biosensor

Detection of pesticides in the food and environmental samples using biosensors is based on the measurement of enzyme activity. The enzyme is immobilized to transducing element either directly or indirectly and the activity of immobilized enzyme is measured in terms of current, voltage, total ions present in the solution (conductivity), any change in optical properties reflectance and or refractance. The percentage inhibition is calculated as the ratio of the difference in the activity before and after the inhibition to the original activity of the enzyme. The enzymatic reaction can be diffusion controlled or kinetically-controlled. Most biosensors are developed based on the kinetically-controlled reaction. The percentage inhibition is calculated as the ratio of difference in the activity before and after inhibition to the activity of immobilized enzyme. The selection of the immobilization method depends on the cost of enzyme and the support (organic, inorganic, other reagents) and the effectiveness of immobilized enzyme for the purposes of commercialization. New factors such as buffering capacity of the solution and membrane are used for entrapment of biosensor resulting in a high sensitivity and low detection limits for organophosphorus pesticides. Immobilization of acetylcholinesterase on membranes and screen-printed electrode are advantageous because they lessen the cost of material and provide miniaturization and in situ measurements.

Acetylcholinesterase and butyrylcholinesterase were immobilized with glutaraldehyde and bovine serum albumin using cross linking method for the detection of diisopropyl flurophosphates, trichlorofon, paraoxon-methyl and paraoxon ethyl. Both enzymes had different sensitivity towards these pesticides. This provided a way for the development for multibiosensors to detect pesticides in the real samples [5]. Potentiometric biosensor has been reported [6] for the detection of trichlorofon using co-immobilization method. A trienzyme electrode was developed by employing highly teflonized carbon black as an electrode material. The immobilization procedure was based on physical adsorption of peroxidase and co-immobilization of choline oxidase and cholinesterase using glutaraldehyde as a binding agent. The electrode retained 95% of its activity even after one month of storage at 4^{o}C. This work demonstrated the potential for application of potentiometric enzyme

electrodes based on mediatorless enzyme electrocatalysis for a fast and sensitive assay of organophosphorus pesticides. Acetylcholinesterase was immobilized around pH-electrode using gelatin and chitosan membrane. The parameters affecting the biosensor response were investigated and optimized and the operational stability of biosensor was improved. Cholinesterase from different sources was immobilized on the surface of antimony electrode and commercially available membrane (nylon and cellulose nitrate) with glutaraldehyde vapours and aqueous solutions. Cholinesterase biosensors were developed with bovine serum albumin (BSA) and compared for the response with different immobilization procedures [7]. Silicagel was used as a supporting material to immobilize acetycholinesterase and reduce the amount of sample volume. The use of this simple solution allowed operation of the biosensor at a low cost [8]. In this work, the time taken for analyzing the pesticide was more than half an hour. Higher amounts of enzyme (200 U and 150 U for potentiometric and conductometric system) were used. Tyrosinase was immobilized on the sensitive part of the conductometric electrode using glutaraldehyde vapour for the detection of alachor, diazion and carbaryl. The reproducibility of biosensor was 5% [9]. This novel immobilization strategy was adopted to immobilize the acetylcholinesterase on carbon nanotubes in flow injection system. The developed biosensor was sensitive to paraoxon of 0.4 pM [10]. The determination of phosphorothionate belonging to the organophosphate group in orange juice did not show inhibition with acetycholinesterase in non metabolized form [11] as the metabolized form of phosphorothionate was toxic. The challenge of detecting the pesticide was overcome by activation of phosphorothionate using chloroperoxidase in the sample. Biosensor based on 7,7,8,8-tetracyanoquino dimethane modified screen printed electrode with acetylcholinesterase immobilized on it was produced. The activity was measured before and after incubation of biosensor in the activated juice sample. This novel enzymatic activation and detection of phosphorothionate was developed and used in the food samples without any tedious pretreatment methods. This method was applied to chlorpyrifos, fenitrothion, methidathion, parathion methyl, and triazophos. Chlorpyrifos and triazophos exhibited complete oxidation, whereas others showed conversion rates 54% and 61% only. The detection limit of chlorpyrifos was 5μg/L. The method required 2h for analysis of the pesticide.

Immobilization of Other Biological System

A microbial biosensor using recombinant *E.coli* cells containing plasmid pJK33 was developed [12] for detection of paraoxon, parathion, methyl parathion and diazion. This biosensor comprised of immobilized *E. coli* cells on polycarbonate membrane and potentiometric transducer based on pH electrode. *E.coli* expressed organophosphorus hydrolase which hydrolysed organophosphorus compounds and released protons. The signal response was based on electrode potential of microbial response. The response was non-specific and metabolized glucose, sucrose, and fructose simultaneously. Non-specific response of the microbial sensor was dependent on the cell age. The sensor had short response time with wide operational span. The screening of chlorophenols, chlorobenzoates and their putative compounds was examined using microbial biosensor [13]. This amperometric biosensor was developed by immobilizing the induced microbial cells on the

polyethylene membrane on the clark type oxygen electrode. A dipstick assay for the detection of 2,4, dichlorophenoxyacetic acid was developed [14] using monoclonal antibody. This membrane coated with monoclonal antibody test trip was washed three times in the buffer and the membrane was blocked with casein and bovine serum albumin (BSA) solution. The membrane was then placed over the polystyrene strip and incubated at 4ºC for weeks. Dipstick was dipped in the sample or standard solution and in the enzyme tracer solution. Change in the color was measured in the portable reflectometer and color dye was precipitated on the membrane. Dipstick assay for pesticide provided a recovery of 100% when compared to that of ELISA test. Ultra bound membrane showed good stability over monoclonal antibody and allowed the color dye to present on the membrane. This newly developed dipstick assay was tested with water and urine samples. Investigations on enzyme based activation and detection of phosphorothionate in the food samples were carried out by [15]. The pesticide was non-toxic in non-metabolized form and toxic in metabolized form. Phosphorothionate was activated using cytochrome p450 BM-3 (mutant) into its oxon form. This detection method was applied to chloropyrifos, chloropyrifos methyl, methidathion, parathion in various fruits and vegetables. Chlorpyrifos (33%), carbendazim (39%), methidathion (11%), chloropyrifos methyl (6%) and parathion (1%) were detected in the monitoring program of pesticides in domestic and imported fruits and vegetables. This method had the potential ability to screen for all pesticides. The drawback of the enzyme based activation method was the requirement of expensive NADH as cofactor. An optical microbial biosensor was designed [16] for the detection of methyl parathion pesticide using *Flavobacterium* sp. This microorganism had organophosphorus hydrolase enzyme which hydrolysed methyl parathion and produced p-nitrophenol, with absorbance at 410 nm. The whole cells were immobilized on glass fiber filter and stored at 4ºC till further use. This microbial biosensor had limit of detection (LOD) 0.3 µM methyl parathion. The proposed device was simple, fast and disposable. The applicability of biosensor for spiked samples could be performed. An amperometric microbial biosensor was developed [17] for the detection of paraoxon, parathion and methylparathion to p-nitrophenol. The sensor was based on the carbon paste containing genetically engineered *Moraxella* sp. expressing organophosphorus hydrolase on the surface of the cell. The sensitivity of the sensor was based on the amount of whole cell immobilized as well as the concentration of pesticide. The sensor signal directly measured the concentration of pesticides. The sensor had limit of detection (LOD) of 0.2 µM paraoxon and 1 µM methyl parathion. The microbial biosensor showed 100% activity when stored at 4ºC for 45 days. It was used to measure organophosphorus compounds in lake water. A dipstick method [18] to detect parathion-methyl using immunoassay methods was developed. Polyclonal antibodies against parathion–methyl were spotted on the membrane and the residual sites of the membrane were covered with protein A or BSA. The antibody coated membrane was placed on the polystyrene strip and washed with buffer solution before use. This dipstick ELISA using membrane allowed quick visual detection at concentration of 10µg/L and reflection detection at concentration of 8.8 µg/L. The recovery of the assay was found to be 89%.

Enzyme-Inhibitor System

In order to develop a biosensor based on inhibition, information on the inhibition kinetics of free and immobilized enzyme is very important--. Enzyme inhibitor systems are very complex with irreversible and reversible inhibition mechanisms. Inhibition rate constants (K_i) are different for every inhibitor. K_i value is useful in the determination of lowest detection limit. Non-competitive inhibition of pesticide was illustrated by [19] when enzyme was preincubated with the pesticide. Inhibition is a function of both substrate and inhibitor concentrations. The degree of inhibition depends on its incubation time. The relationship between inhibition and preincubation time is described by the Aldridge equation log (100/%I) = K_2 [I] t., where K_2 is a biomolecular rate constant. The inhibition mechanism of biosensor was studied by [20] with carbaryl. They pre-incubated the biosensor with pesticide for different time and then added the substrate to measure the activity of inhibited enzyme. The inhibition was found to be non-competitive. Selection of substrate concentration [S] is important while carrying out study on the inhibition and determining the percentage inhibition. At high [S], higher inhibition was observed, but at low substrate concentration, detection of pesticide could not be achieved. The dynamic response of biosensor using tyrosinase was investigated by [21] for carbaryl determination. They developed a model with two independent parameters to detect carbaryl with respect to the given permissible value in less than 10 minutes. Detection of pesticide using this method was simple, quick and easy rather than steady state method of analysis, in which detection method is time consuming. An acetycholinesterase biosensor was developed by [22] for the detection of serine. From the inhibition kinetic studies, it was found that serine was a reversible competitive inhibitor which was detected in the range of 1.0 μM to 100 μM.

Limit of Detection

The term limit of detection (LOD), defined by the IUPAC as LOD= $k\varepsilon_r/SEN$, in which k is an integer that defines the number of standard deviations of separation that constitutes 'different'. Usually k is equal to 3, which is nearly 99% probability that the two measurements differ. This method of calculation was used by [23] to determine the lowest detection limit of carbaryl concentration by performing ten current-time measurements of the blank solutions. They detected 2.0×10^{-6} mol/L in the pure sample. This value was close to the one established for carbaryl in tomatoes (5.0×10^{-7} mol/L for 0.1 mg/Kg). Similar methodology was used by [24] to estimate the lowest detection limit of parathion and carbaryl in different water samples and fruits. Detection limits were established by [25] by measuring the response after injection of 20 μL, 10nM dichlorvos into the flow system. An apparent inhibition signal three times higher than the noise with inhibition degree about 6.81% was observed in the simulated seawater. Lowest detection limit of 2.0 μg/L were obtained by [26] for chlorpyrifos-oxon with application of 10 mU of acetylcholinesterase on the electrodes. Lesser enzyme loading on the electrode gave lower detection limit, which was suitable for sensing smaller concentrations of the pesticide. Acetylcholinesterase immobilized on carbon nanotubes by [27] was used to detect carbaryl in water to the extent of 10^{-12} g/L. [28] achieved smallest detection limit for genetically modified enzymes. They also found that there was a direct correlation between the LOD and the K_i of the enzymes. The higher the K_i, the better the theoretically achievable LOD. Detection limits of paraoxon and chlorpyrifos

ethyloxon were established based on 20% inhibition of acetylcholinesterase and bienzymatic system consists of acetylcholinesterase and tyrosinase [29]. The sensitivity towards inhibitors was tested. A detection limit of 10^{-7} M was established by [30] for the determination of trichlorofon using potentiometric based biosensor. Low detection limit for the determination of paraoxon by modifying the carbon surface with dialdehydes, glutaraldehyde, terephthaldicarboxaldehyde and then polyethyleneimine have been reported by [31]. They found that lowest detection limit obtained for non-covalent immobilization of acetycholinesterase onto polyethyleneimine modified carbon electrode was 10^{-10} M for paraoxon. Studies based on fibre optic monitoring of carbamate pesticides using porous glass with covalently bound chlorophenol red were carried out by [32]. Propoxur was detected at 0.4 ng/L when compared to the carbaryl 25 ng/L. Detection limit were estimated for carbaryl and parathion using the relation I_{LOD} % = 2×SD (standard deviations of blank solution) for three repetitive measurements in the calibration graph for every pesticide. These values were found to be 35.00 ng/ml for parathion and carbaryl [33]. The reuse of biosensor during inhibition studies can be carried out by using reactivators. Pyridine 2-aldoxime methliodide (2-PAM) was used to reactivate the biosensor inhibited by organophosphorus compounds and atropine was used for reactivation of biosensor inhibited by carbamate pesticides by [34] who investigated the reactivation of acetylcholinesterase using 5mM of 2-PAM in a flow injection system with different times of incubation. They found 15 minutes incubation of sulfotep 5 nM, 50 nM and 80 nM with 97, 95 and 94% reactivation of enzyme activity respectively. A conductometric biosensor was developed by [35] for the detection of organophosphorus compounds. They used pyridine 2- aldoximine methiodide for the reactivation of biosensor after the subjection with paraoxon with 5×10^{-6} M and 5×10^{-5} M with 10 and 80 minutes incubation. It was found that there was a complete recovery of biosensor with paraoxon concentration 5×10^{-6} M for 10 minutes incubation and higher concentrations than this led to reduced reactivation of enzyme.

Parameters Influencing the Performance of Biosensors

Effect of pH

pH is an important parameter in the characterization and optimization of free and immobilized enzyme. The characteristics of ionizable groups of amino acid depend on the pH, hence the activity of free enzyme and enzyme attached to any immobilization matrix or electrode surface (biosensor) plays vital role in the performance of biosensor. The effect of pH based system has not been studied much in the case of amperometric biosensors, particularly those made up using screen printed electrodes. Most studies have been conducted on immobilization systems based on membrane strategies because the movement of ions caused a change in the response of the sensor. A potentiometric butyrylcholine sensor for organophosphate pesticides was constructed by [36] in which they studied the performance of the biosensor by varying the pH from 2.0 to 10.5. Stability was observed in the pH range from 4.0 to 8.0. [37] developed an amperometric bi-enzymatic biosensor for the detection of paraoxon and studied the effect of pH on the pesticide detection by varying pH from 4.0 to 10.0. They obtained maximum response between 6.5 and 7.5. It was found that biosensor started losing its activity below pH 6.0 and pesticide underwent hydrolysis reaction above pH

8.0. The detection of organophosphorus insecticides was carried out by [29] with two enzyme sensors (acetylcholinesterase and tyrosinase). Investigations were done at varying pH for the amperometric system and good response was obtained while using phosphate buffer of pH from 5 to 9. The optimum pH for tyrosinase was observed at 6 and 6.5, whereas for acetylcholinesterase it was at 8.0. The detection of organophosphate and carbamate pesticides in vegetables was studied by [38] using a photothermal biosensor. Comparative studies using two buffer solutions Tris, pH 7.4 and phosphate buffer, pH 8.0 for maximum activity of cholinestersae enzyme were conducted. Phosphate buffer was found to be better than Tris HCl. A nanostructured polyacrilonitrile membrane for immobilization of acetylcholinesterase was developed by [39] and studied for the effect of pH ranges from 6.0 to 9.0. The optimum pH obtained for free enzyme was 8.0 whereas it was different for immobilized acetylcholinesterase.

Effect of Substrate Concentration

Detection techniques using biosensors are highly dependent on the substrate concentration. A conductometric biosensor was developed for the detection of organophosphorus compounds [5]. The effect of acetylcholine concentration during the inhibition process was studied and saturation substrate concentration of 8 mM was selected for detection of pesticides with incubation time of 10-30 minutes. The influence of substrate concentration was considered to be small for biosensor made up of carbon nano-tubes. A flow based biosensor system was developed for the detection of carbofuran and carbaryl [8]. They studied the effect of substrate concentration by varying acetylcholine from 0.5 mM to 7.0 mM and selected an optimum substrate concentration of 2.5 mM with response time for sensing the pesticides in potentiometric and conductometric system.

Effect of Enzyme Concentration

Varying the concentration of enzyme helps in arriving at the right quantity of enzyme to develop the biosensor. However, loading smaller concentration of enzyme in the reaction system will give a maximum inhibition percentage, without possibility of reuse [40]. A potentiometric biosensor was fabricated by using polyaniline membrane [41]. Different types of enzymes, active acetylcholinesterase, butyrylcholinesterase and low active butyryl cholinesterase were used on the pan membrane with BSA and crosslinked with glutaraldehyde. The use of 1 µl of enzyme solution was found to give good sensitivity for active acetylcholinesterase immobilized using glutaraldehyde.200 and 150 units of enzyme for potentiometric and conductometric system have been used by [8] in screen printed sensors. 1 U/µl of enzyme stabilized with BSA concentration with 1 µl of enzyme solution on the sensor was used [42]. The activity in different immobilization procedure such as precipitation, solgel, interception in gelatin membrane and capture on graphite microparticle was investigated [42]. Good inhibition response was found for immobilization with precipitation for organophosphorus compound. An amperometric based biosensor made up of Prussian blue–chitosan electropolymerised on glassy carbon electrode for the detection of carbaryl was developed by [43]. The effect of enzyme concentration on the electrode response was studied. It was inferred that as the enzyme concentration increased, the amperometric response increased till 25 U/ml, after that response started to decrease as

excess application of enzyme on the surface interfered with its interaction with Prussian blue, thereby causing decreased transfer rate at the electrode.

Effect of Organic Solvents

Inhibition determination in organic phase is a useful method for development of biosensors for detecting pesticides. Before biosensor is applied for use with real samples, pesticides have to be extracted from the source using organic solvents. Each pesticide has different solubilization with different organic solvents. When enzyme based biosensor is subjected to the pesticides in organic solution, the effect of solvents on the immobilized enzyme as well as free enzyme solution must be taken into account. But the use of solvents will also affect the enzyme, substrate and immobilized matrix. Hence, it is important to study the effect of polar and non-polar organic solvent on enzyme based biosensor. A bienzymatic system was developed consisting of butyryl cholinesterase and choline oxidase for the detection of paraoxon and aldicarb in the 50% water saturated chloroform- hexane mixture [44]. The biosensor showed good response in the 50% water saturated chloroform-hexane system. The detection limit for paraoxon and aldicarb was obtained as 4.5µg/L with a long lifetime. The effect of hexane on the bienzyme system using substrate phenyl acetate was studied by [45]. Experimental results showed zero inactivation of enzyme activity to 100% hexane. Hexane did not induce any inhibition effect on acetylcholinesterase and tyrosinase system and it was confirmed that biosensor could be used in the presence of hexane extracted pesticides. A disposable biosensor for the determination of paraoxon in the orange juice sample was developed by [46]. In this work, iso-octane was used as extraction solvent in the orange juice sample. The effect of iso-octane on the biosensor activity before inhibition experiments was investigated. After incubation, within 30 minutes activity of immobilized enzyme was reduced only by 3%. An amperometric biosensor for the detection of carbofuran, dichlorvos, carbaryl and methylparaoxon in 5% acetonitrile was developed [28]. The biosensor was checked for its residual activity after exposure to 20%, 10%, 5% and 1 % of acetonitrile with phosphate buffer. The residual activity of biosensor was low for 20% and 10% of acetonitrile. It was same for 5% and 1% acetonitrile in the phosphate buffer. 5% acetonitrile was chosen for further investigations to study the effect of substrate in the organic solvents. There was no significant change in activity. An amperometric biosensor consisting of acetylcholinesterase immobilized on graphite electrodes was investigated [47] and electron properties were enhanced by adding Prussian blue. This biosensor was characterized in the presence of ethanol, cyclohexanone, benzene and propanol. The activity of immobilized enzyme in the presence of 10% water-ethanol system showed good activity than in pure organic solvent system. The quantity of water-polar system lowered the dielectric constant of enzyme active site microenvironment, which could be varied to obtain the enhanced electron communication in the biosensor system.

Pesticide-Inhibition Investigation Using Enzyme-Based Biosensor

An amperometric based biosensor was prepared [48] for the detection of aldicarb, methomyl, carbaryl, carbofuran and propoxur. The strategy for the development of sensor was based on using cholinesterase of different specific activity from various sources such as bovine erythrocytes, electric eel, human erythrocytes, human serum and horse serum. Amperometric measurements were made by using two electrode system consisting of reference (Ag/AgCl)

and working electrode (platinum paste). The working electrode was modified by coating graphite paste with Cobalt Pthalocyanine [CoPC] and acetyl cellulose as a binder. Cholinesterases were immobilized using 1% glutaraldehyde solution with bovine serum albumin. The response of the biosensor from different specific activity was obtained. The results were found to be good for acetylcholinesterase from bovine erythrocytes and electric eel, however it was poor for acetylcholinesterase from human erythrocytes and butyrylcholinesterase from horse and human serum. The biosensor had a linear range of $5x10^{-5}$ to 50 mg/Kg. The response of the biosensor was tested in the real samples especially in vegetables. Amperometric based biosensors with improved performance were developed [3] for the detection of pesticides by varying the enzymatic charge, substrate concentration, sensitivity and stability. Acetylcholinesterase from electric eel and butyrycholinesterase from horse serum were used for immobilization using glutaraldehyde solution (1% v/v) with bovine serum albumin. The stability study of the cholinesterase inhibitors in reference water samples was performed at different conditions: (a) at -4ºC, (b) room temperature and light in a colorless vial, (c) light protected and stored at room temperature in a well closed vial. A nonlinear mathematical model was proposed to extend the working range of carbofuran using chronoamperometric method. The stability of biosensor was affected in reconstituted water at different conditions. Relatively high percentage inhibition was observed in the water stored at -4ºC with light and room temperature with exposed light. This biosensor was applied in the real sample analysis in different fruit and vegetables. Fibre-optic biosensor was developed for the monitoring of carbamate pesticides [32]. Chlorophenol red was used as optical transducer. The reflectance signal measured gave concentration of propoxur and carbaryl. The linear range for propoxur and carbaryl was found to be 0.03 – 0.5 mg/l and 0.8 – 3 mg/l respectively. A portable fiber-optic pesticide biosensor for in-field use was developed by [49]. The cholinesterase enzyme was immobilized over the three layer sandwiched membrane. The membrane was in contact with sol-gel indicator (bromophenol blue), which was then deposited on the glass disk. Sensor life time was for 3 weeks. The response of the biosensor was measured in terms of absorbance which was correlated with concentration of pesticide in the analysis. The working range for carbaryl and dichlorovos was 0.11-8 mg/l and 5 – 30 µg/l respectively. The reproducibility was in the order of 3-5% with an accuracy of 94.9%. A new method was developed by [50] for the detection and discrimination of neurotoxins such as organophosphate and carbamate pesticides in the real samples. The major drawback for the inhibition based biosensor was unpredictable cross interaction of multiple pollutants in the real sample. This was overcome by the joint action of organophosphorus hydrolase (OPH) and acetylcholinesterase (AChE). The former enzyme was used to hydrolyse organophosphates and the latter one was used to measure carbamate pesticide by means of inhibition. The combined inhibition effects in mixtures of organophosphates and carbamates were investigated and it was found to be different than what was expected from additive effects of single neurotoxins. Presence of more than one pesticide in the samples led to the competition for acetylcholinesterase binding sites. It was found to be useful in detection of non organophosphorus neurotoxins on the acetylcholinesterase and determination of its concentration. The detection ranges of these integrated biosensors were 10^{-9} to 10^{-5} M for paraoxon or di-isopropyl fluorophosphate, and 5×10^{-8} to 10^{-5} M for carbaryl. It was applied for detection of carbamate pesticides in the real sample. Investigations on comparison of different methods of immobilizing acetylcholinesterase enzyme have been conducted [29]. Immobilization of acetylcholinesterase and histidine modified acetylcholinesterase was studied using polyvinylalcohol bearing styryl pyridinium group polymers, sol-gel, and metal chelate affinity methods. Enzyme was entrapped inside the polymer matrix where it was encapsulated inside sol-gel matrix. In this method, silica-nitriloaceticacid-nickel had affinity to the proteins. Nickel in the metal chelate bonded with

peptide and protein containing histidine. Enzyme entrapment in polyvinyl alcohol bearing styryl pyridinium group polymers allowed stable sensors for more than 8 months at 4^{o}C, while the sensors with acetylcholinesterase encapsulated in sol-gel kept the initial activity for six months when stored at -20^{o}C under vacuum. The sensors with metal-chelate affinity had a lower life time.

Amperometric based monoenzymatic and bienzymatic system were developed [45] for the detection of parathion and chlorpyrifos ethy oxon. Monoenzymatic system was fabricated by immobilizing acetylcholinesterase in 30% polyvinyl alcohol bearing styryl pyridinium group polymers. Bienzymatic system was prepared by tyrosinase in carbon paste and acetycholinesterase immobilized in 50% polyvinyl alcohol bearing styryl pyridinium group polymers. P-aminophenylacetae was used as a substrate for the monoenzymatic system. The calibration plot was linear and showed sensitivity of 33.2 μA/M. Phenyl acetate was used as substrate for bienzymatic system. Acetycholinesterase was hydrolysed with phenyl acetate and phenol as a product of first reaction. Phenol was converted into quinines and increase in current was recorded in screen-printed electrode against Ag/AgCl as a reference electrode. The LOD for parathion in monoenzymatic and bienzymatic systems were 1.9×10^{-8}M and 1.82×10^{-8}M. The LOD for chlorpyrifos ethy oxon in monoenzymatic and bienzymatic systems were 1.76×10^{-9} M and 2.96×10^{-9} M. The response of the biosensor was improved in both systems in the presence of hexane and applied for real sample in agriculture and environment monitoring. A portable light addressable potentiometric sensor capable of measuring four different ions by different sensing surface incorporated in the transducer was developed by [51]. An easy and efficient immobilization of acetylcholinesterase was prepared by [52] on screen printed electrodes based on adsorption method. Despite the disadvantages such as involvement of high amount of enzyme and a gradual leaching while working, it was used for its simplicity in immobilization. It showed better operational stability without diffusional limitation. A potentiometric biosensor was developed by [53] using electroactive polymer polyaniline for the detection of trichlorofon, coumaphos, methiocarb and aldicarb. Electrochemically generated self-doped polyaniline exhibited good sensitivity of 70 mV/pH unit. Presence of chemically doped polyaniline membrane showed improved sensitivity of 89mV/pH. This was first time study on the influence of polyaniline on the inhibition measurements of pesticides. Acetycholinesterase and butyrylcholinesterase were immobilized on polyaniline based glassy carbon electrode using cross linking method. Acetylcholinesterase showed the best sensitivity of 120±5 mV/pH whereas others were 95±5 mV/pH for acetylcholine determination. The same systems were tested for inhibition measurements. The polyaniline based biosensor provided better sensitivities for coumaphos (69±6% inhibition per decade of concentration) and trichlorfon (69±5% inhibition per decade of concentration). The sensitivity for methiocarb and aldicarb was in the 40% inhibition per decade of concentration. This biosensor exhibited good performance characteristics for organophosphorus compounds. It was stable for ten days both in dry and wet conditions. Reports of modification of screen-printed electrodes with dialdehyde, glutaraldehyde, terepthaldicarboxaldehyde and then with polyethyleneimine are available in literature [37]. Modified screen-printed electrode activity was improved by electrochemical reduction of 4-nitrobenzene to aryl radicals. This aryl radical activated the carbon surface. It increased the binding of enzyme to carbon surface. Monitoring of environmental pesticides was proposed

by [54] using sono-chemically-fabricated enzyme microelectrode array. Enzyme-microelectrode arrays were prepared by electropolymerizing polydiaminobenzene, a non-conductive polymer on carbon paste containing cobalt pthalocyanine and sonicating at frequency of 25 Hz. Sonochemical ablation of polydiaminobenzene modified screen printed electrode led to microelectrode pores. Enzyme was then applied electrochemically on the microelectrode array in the presence of aniline, which polymerized electrochemically and acetycholinesterase was entrapped inside the polymer matrix. Paroxon was determined using amperometric sensor. The working range was found to be 1×10^{-17} to 1×10^{-8} M and lowest detection of limit was 1×10^{-17} M. This method was highly applicable for flow systems with rate changes like in river or stream. A rapid and disposable amperometric biosensor was developed by [58] for the detection of organophosphorus (parathion) and carbamate pesticide (carbaryl). Screen-printed thin film working electrode was prepared and various mutants of acetycholinesterase were used for immobilization to improve the sensitivity of biosensor towards the pesticides. Double mutant acetylcholinesterase showed low detection limit (1ug/ml) and high sensitivity with a high K_i value (1.8×10^{-6} $M^{-1}min^{-1}$). The effect of various parameters on the activity of the free and immobilized enzyme were investigated by [55]. A novel matrix was prepared using sodium alginate beads and sodium alginate-carrageenan polymatrix. Advantages of this strategy were low cost, ease of enzyme accessibility and hydrophilic character. It was found that for both free and immobilized enzyme, the optimum pH was 7 at temperature of 30^0C. Kinetic parameters were evaluated for both free and immobilized enzyme with V_{max} as 50mM/min, 8.68mM/min, 12.7 mM/min, and K_M as 6.35mM, 39.7 mM, and 52.9mM. The values of Michalis Menten parameters were high due to diffusional limitations. Thermal stability of immobilized enzyme was found to be better than free enzyme. The main disadvantage of entrapment method was leakage of enzyme, when it was stored for longer days. The stability of biosensor also was affected. Improved stability for the sensor in dry and wet state was reported by [53]. Screen-printed electrode was electrochemically modified using nitrobenzene. Later this aminated screen printed electrode was modified covalently and non-covalently by dialdehyde and polyethylenimine. Stabilizers of different ranges were chosen and it was demonstrated that polyethyeneimine modified electrodes increased dry and wet stability. Stabilizer compositions such as 5% (w/v) sucrose and 1% (w/v) polygalacturonic acid and 5% (w/v) sucrose and 0.1% dextran sulphate showed improved stability. Another method adopted in the same work showed less satisfactory stability at room temperature and it had to be used immediately after preparation. Biosensor based on flow injection analysis was developed [8] using potentiometric and conductometric tranducers for the detection of carbaryl and carbofuran. Acetycholinesterase was immobilized on silica gel column. The performance characteristics of potentiometric system for carbaryl and carbofuran detection were evaluated. Carbofuran showed good reproducibility and analysis time of 35-45 minutes. The performance characteristics for conductometric biosensor for carbaryl and carbofuran showed good reproducibility and analysis time of 31-37 minutes. Potentiometric system seemed to be better than the conductometric type in terms of reproducibility of the biosensor and vice versa in terms of analysis time. A method to improve the stability of acetylcholinesterase using nanoporus carbon matrix was proposed by [56]. The enzyme was immobilized onto the porous carbon

matrix by adsorption methods. The stability of immobilized and free enzyme was compared at 25^{o}C. Immobilized enzyme showed good storage stability for one month, whereas free enzyme activity decreased in five days. Biosensors were proposed for the first time by incorporating hydrophobic ionic liquids on the carbon paste [57]. Carbon paste electrodes were constructed by entrapping heme protein in carbon and ionic liquid mixed with paraffin oil as a binder. Covalent immobilization of acetylcholinesterase on the gate surface of the Ion Sensitive Field Effect Transistor [ISFET] using cyanuric-chloride were reported by [24]. This ion sensitive field effect transistor based biosensor showed better and faster response than the electrode based enzyme biosensor. Cyanuric Chloride (CyC) was incubated at 70^{0}C in vacuum. One of the three cyanuric-chloride groups condensed with the free hydroxyl groups on the aluminum oxide gate surface. HCl was produced as a result of condensation reaction and was eliminated. Later on acetylcholinesterase immobilized on CyC monolayer at room temperature. Biosensors for the detection of carbaryl in fruit, vegetable juices without any pretreatment were developed by [23]. The working electrode was prepared with nujol oil on carbon paste electrode. Then, acetylcholinesterase was immobilized using 1% glutaraldehyde on the modified carbon electrode. Amperometric response was obtained using square wave voltammetry methods. The LOD for the biosensor was 0.4×10^{-3} g/L.with working range of 5×10^{-5} to 75×10^{-5}M. It provided good recovery 83.4% and variation coefficient is 2.3 %. Reproducibility of the biosensor was obtained by conducting five consecutive tests and the variation of coefficient was 5%. A biosensor was developed by designing the potentiostat and graphite modified screen printed electrode [26]. The instrument linearity was checked within the range of 0-16 µg/L of chlorpyrifos-oxon. The working range of the biosensor was from 2 to 8 µg/L Concentration of pesticide for enzyme concentration of 1.0 mU. Reproducibility of biosensor for 5 days was 5.1%. This biosensor was used for field analysis. They validated a system with the results from chromatography methods. In another work [27] developed amperometric-based biosensor for the detection of carbaryl in water. Single walled carbon nanotubes dispersed in poly diallyl dimethyl ammonium chloride solution was adsorbed on the surface of glassy carbon electrode owing to the positive charge present in poly diallyl dimethyl ammonium chloride. The poly diallyl dimethyl ammonium chloride-single walled carbon nanotube modified electrode was then immersed in acetylcholinesterase (10 U/ml) and enzyme was immobilized using layer-by-layer method. This biosensor showed good detection limit 10^{-12} g/l. It provided good recovery of 99.8±2.7% which was obtained from seven times detection of carbaryl (10^{-10} g/l). High sensitive biosensor was developed by [58] for the detection of carbofuran with low detection limit (10^{-11}g/L). An amperometric based biosensor was proposed by [59] for the detection of organophosphorus compounds such as phoxim (0.05-10 µg/L, 10ng/L), dichlorvos (0.01-10.00 ng/L, 2.5 ng/L), omethoate (detection range: 0.05-10 µg/L, 15 ng/L), trichlorfon (detection range : 0.03 – 5.00 µg/L, 5 ng/L). The biosensor was developed on glassy carbon surface with electrodeposited Prussian blue. Prussian blue was used to improve the electrocatalytic activity and to enhance the electron transfer rate on the surface of the electrode at low potentials. Chitosan membrane was used to improve the biostability of the enzyme, so it prevented the loss of enzyme activity. The measurements of biosensor were tested using voltammogram method. The operational parameters of the biosensor such as pH, substrate concentration and time were optimized and

the values were found to be 8, 10 mM and 10 minutes. Under these conditions, inhibition measurements of the biosensor were performed. The calibration curve was linear for all pesticides tested. An amperometric biosensor was developed by [60] for the detection of chlorphenvinphos. The carbon nanotube was mixed with nujol oil and formed into a paste. Acetycholinesterase was added to this paste. This wet paste was packed into a glass tube and used as the working electrode. The response of biosensor was measured using square wave voltammetry method. The physical characterization of AChE-carbon nanotube was performed by scanning electron microscopy. The electrical property of the electrode with carbon nanotube and AChE-carbon nanotube was performed. Nyquist plot were used to give the electrical properties of the electrode such as resistance and capacitance. The charge transfer resistance value was higher for AChE-carbon nanotube coated electrode whereas it was small for carbon nanotube electrode. Capacitance was higher for carbon nanotube electrode and small for AChE-carbon nanotube electrode. The stability of biosensor was checked by preserving them at -4^0C and measurements were taken each day for 20 days. There was considerable change in the activity under optimized condition. Calibration plot for pesticide determination was made. It was linear and working in the range of 4.90×10^{-7} – 4.76×10^{-6}M. The LOD of the biosensor developed was 1.15×10^{-7}M. [42] based on screen printed electrode using amperometric techniques. Simple sorption, precipitation with glutaraldehyde, entrapment in gelatin, sol-gel, and graphite microparticles were the different methods used to immobilize the acetylcholinesterase. The response of the biosensor was evaluated using square wave voltammograms and cyclic voltammetry. The peaks of square wave voltammogram are sharper, thinner than the cyclic voltammetry. So, square wave voltammetric way of detecting the pesticides was chosen for further analysis of pesticide di isopropyl flurophosphate. Of these different immobilization methods, enzyme immobilized on simple sorption and precipitation with glutaraldehyde showed better activity than other methods. The efficacy of immobilization depended on the distribution of substrates, stability and conformation of immobilized enzyme. Since they provided long term stability, less diffusion effects of substrates and prevented the washing out of enzyme from the membrane. Precipitation with glutaraldehyde provided lots of pores so that enzyme could be immobilized sufficiently within the pores and hence it provides good linear range of detection, where-as, solgel and other methods, showed less effective activity with respect to detection limits. Multielectrode biosensor was developed to detect as well as to discriminate the pesticides in real sample at the same time using artificial neural networks model [61]. Zhaozhu et al., 2011 developed a biosensor with satisfactory accuracy and reproducibility for quick determination of low concentrations of organophosphorus compounds in real vegetable and fruit samples by integrating optical transducer of semiconductor quantum dots with acetylcholinesterase enzyme by the layer-by-layer assembly technique. The detection limits of the proposed biosensors were as low as 1.05×10^{-11}M for paraoxon and 4.47×10^{-12} M for parathion, which were significantly better than those of the conventional detection methods. An automated flow-based biosensor employing genetically modified acetylcholinesterase enzymes B394, B4 and wild type B131 was developed by Rupesh et al., 2012. The biosensor was based on a screen printed carbon electrode that was integrated into a flow cell. The enzymes were immobilised on cobalt (II) phthalocyanine modified electrodes by entrapment

in a photocrosslinkable polymer. The automated flow-based biosensor was successfully used to quantify chlorpyriphos-oxon, ethyl paraoxon and malaoxon. The total analysis time for the assay was less than 15 min. Based on the initial biosensor performance using the enzymes in phosphate buffer solution, calibration data in milk with the three organophosphate pesticides in the concentration range of 5×10^{-6}M to 5×10^{-12}M was generated. The limit of detection obtained in milk for chlorpyriphos-oxon, ethyl paraoxon and malaoxon were 5×10^{-12}M, 5×10^{-9}M and 5×10^{-10}M, respectively. This study showed that this method was inexpensive, sensitive, portable, and non-invasive as the automated flow-based biosensor successfully quantified the pesticides in different fat-containing milk samples. Oliviera et al., 2013 developed a novel enzymatic biosensor for carbamate pesticides detection by the direct immobilization of Trametes versicolor laccase on graphene doped carbon paste electrode functionalized with Prussian blue films. The Prussian blue film electrodeposited onto graphene doped carbon paste electrode allowed considerable reduction of the charge transfer resistance and of the capacitance of the device. The combined effects of pH, enzyme concentration and incubation time on biosensor response were optimized using a 23 full-factorial statistical design and response surface methodology. Based on the inhibition of laccase activity and using 4-aminophenol as redox mediator at pH 5.0, the device exhibited suitable characteristics in terms of sensitivity, intra- and inter-day repeatability, reproducibility, selectivity, accuracy and stability for quantification of five carbamates widely applied on tomato and potato crops. The attained detection limits enabled testing pesticide levels in food samples to fit with regulations and food inspections. Weiying et al 2014 carried out a review which gave an overview of recent advances and new trends in nanomaterial-based biosensors for environmental and biological monitoring of organophosphorus pesticides and nerve agents. They provided background information and a general overview of electrochemical and immunoassay detection techniques. Since nanomaterials function as signal transducers to mediate current flow or as recognition agents, their incorporation in biosensor fabrication improved sensitivity, selectivity and response time. In addition, they also discussed future considerations and opportunities for advancing the use of biosensors for environmental and health applications. Gregory et al., 2015, constructed a biosensor by physical adsorption of the isolated endophytic fungus *Eupenicillium shearii* FREI-39 esterase on halloysite, using graphite powder, multi-walled carbon nanotubes and mineral oil for the determination of carbofuran pesticide by inhibition of the esterase using square-wave voltammetry. Specific esterase activities were determined every two days over a period of 15 days of growth in four different inoculation media. The highest specific activity was found on 6th day, with 33.08 U. The biosensor showed good repeatability and reproducibility and remained stable for a period of 20 weeks. Haiyan et al 2015 developed a novel sensing platform based on electrodeposition of electrochemical reduced grapheneoxide-Au nanoparticles-β-cyclodextrin and Prussian blue-chitosan on glass carbon electrode for efficiently fixed acetylcholinesterase to fabricate organophosphorus pesticides (OPs) biosensor. The Prussian blue-chitosan not only effectively catalyzed the oxidation of thiocholine, but also shifted its oxidation potential from 0.68 to 0.2V, resulting in improved sensitivity of the biosensor. The synergistic effect between grapheneoxide and gold nanoparticles promoted the electron transfer between leading to enhanced electrochemical

oxidation of thiocholine. Besides, β-cyclodextrin could interact with substrate by reversible bonding, which further contributed to increased selectivity and sensitivity of the biosensor. Their sensor showed wide linear ranges of 7.98-2.00 x10^3 pg mL^{-1} and 4.3-1.00 x10^3 pg mL^{-1} with low detection limits of 4.14 pg mL^{-1} and 1.15 pg mL^{-1} for malathion and carbaryl, respectively. Everlyne and Jonathan, 2016 reviewed the recent advances in the fabrication of enzyme biosensors for organophosphorus pesticides and their applications for toxicity monitoring of organophosphorus pesticide residues in real samples. They focussed on the various strategies for the biosensor construction, their analytical performance and the advantages and disadvantages of the biosensing methods. They discussed about the recent works done to improve the analytical performance, sensitivity and selectivity of these biosensors. Rajni et al., 2016, demonstrated a novel sensing strategy for malathion, employing unmodified gold nanoparticles, aptamer and a positively charged, water-soluble polyelectrolyte. The Polydiallyl dimethyl ammonium chloride when associated with the aptamer prevented the aggregation of the gold-nanoparticles while no such inhibition was observed when the aptamer specific pesticide was added to the solution, thereby changing the color of the solution from red to blue. The proposed method was linear in the concentration range of 0.5–1000 pM with 0.06 pM as the limit of detection. Moreover, the proposed assay selectively recognized malathion in the presence of other interfering substances and thus, can be applied to real samples for the simple and rapid screening of malathion.

Conclusion

Considerable efforts have been made to construct a cholinesterase biosensor with improved sensitivity and selectivity. The sensitivity of this biosensor was improved by incorporating efficient immobilization methods such as self assembled monolayer and thin polymer films. Additionally, carbon nanotubes and nano metal particles helped to enhance the sensitivity and detection limit of the biosensor. Genetic engineering of cholinesterase enzyme helps in discriminative detection of pesticides in the real sample, which will lead to the development of multienzyme biosensors. When we see the performance of biosensor, the right choice of transducer plays a vital role in enhancing the sensitivity. Amperometric transducer without mediator increases the sensitivity of biosensor with mediator. It serves as a disposable biosensor and is fast in response. In case of potentiometric biosensor, novel polymer membrane synthesized were sensitive towards proton and ion sensitive. Field effect transducer based sensors are easy to handle, providing an easy way of developing multienzyme biosensor with simple immobilization method. In addition to this, the computational model from observed data based on different inhibition mechanism with different inhibitors helped to discriminate and detect the pesticides in the real samples at the same time.

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References:

[1] Bruce, C. L., James, C. M., Robert, C. H., Glenn, H. F., Determination of carbosulfan and carbofuran residues in plants, soil, and water by gas chromatography. *J. Agr. Food Chem.*1983,*31*, 220-223.

[2] Jose, F., Huertas-Perez, Ana Maria Garcia-Campana, Determination of N-methyl carbamate pesticides in water and vegetable samples by HPLC with post-column chemiluminescence detection using the luminol reaction. *Anal. Chim. Acta.* 2008,*630 (*2), 194-204.

[3] Gilvanda, S. N., Damia, B., Bozidar, S. G., Jose, M. D., Maria, L. R., Evaluation of highly sensitive amperometric biosensor with low cholinesterase charge immobilized on a chemically modified carbon paste electrode for trace determination of carbamates in fruit , vegetable and water sample. *Anal. Chim. Acta.* 1999, *399*, 37-49.

[4] Anton, A. C., Carmela, N., Richard, P. B., Detection of pesticides using a amperometric biosensor based on ferropthalocyanine chemically modified carbon paste electrode and immobilized bienzymatic system. *Biosens. Bioelectron.*2003,*18,* 303-310.

[5] Dzyadevych, S. V., Shul'ga, A. A., Soldatkin, A. P., Nyamsi Hendji Andre' M. et al., Conductometric biosensors based on cholinesterases for sensitive detection of pesticides. *Electroanalysis,* 1994, *6,* 752-758.

[6] Diehl – Faxon, J., Ghindilis, A. L., Atanasov, P., Wilkins, E., Direct electron transfer based tri-enzyme electrode for monitoring of organophosphorus pesticides. *Sens. Actuators, B.* 1996, *35(1-3),* 448-457.

[7] Ivanov, A. N., Evtugyn, G. A., Gyurcsanyi, R. E., Toth, K., Budnikov, H. C., Comparative investigation of electrochemical cholinesterase biosensors for pesticide determination. *Anal. Chim. Acta.* 2000,*404,* 55-65.

[8] Siriwuan, S., Proespichaya, K., Punnee, A., Chusak, I. et al., Semidisposable reactor biosensor for detecting carbamate pesticides in water. *Biosens. Bioelectron.* 2005, *21*, 445-454.

[9] Xuejiang, W., Ling, C., Siqing, X., Zhiliang, Z. et al., Tyrosinase biosensor based on interdigited electrodes for herbicides determination. *Int. J. Electrochem Sci.*2006, *1*, 55-61.

[10] Guodong, L., Yuehe, L., Biosensor based on self-assembling acetylcholinesterase on carbon nanotubes for flow injection amperometric detection of organophosphate pesticides and nerve agents. *Anal. Chem.* 2006, *78,* 835 – 843.

[11] Clarisse, B. R., Susanne, B. M., Schulze, H., Bachmann T. T. et al., Analysis of phosphorothionate pesticides using a choroperoxidase pretreatment and acetylcholinesterase biosensor detection. *J. Agric. Food Chem.* 2010, *58,* 8748-8756.

[12] Ashok, M., Priti, M., Wilfred, C., Enzyme biosensor for determination of organophosphates. *Field Anal. Chem. Tech.* 1998, *2(6),* 363-369.

[13] Beyersdorf-Radeck, B., Riedel, K., Karlson, U., Bachmann, T. T., Schmid, R. D., Screening of xenobiotic compounds degrading microorganisms using biosensor techniques. *Microbiol. Res.*1998, *153(3),* 239 – 245.

[14] Cuong, N.V., Bachmann, T. T., Schmid, R. D., Development of a dipstick immunoassay for quantitative determination of 2, 4-dichlorophenoxyacetic acid in water, fruit and urine samples. *J. Anal. Chem.*1999, *364,* 584 – 589.

[15] Schulze, H., Schmid R. D., Bachmann T. T., Activation of phosphorothionate pesticides based on a cytochrome P450 BM-3 (CYP102 A1) mutant for expanded neurotoxin detection in food using acetylcholinesterase biosensor. *Anal. Chem.,*2004,*76,* 1720-1725.

[16] Jitendra, K., Sandeep, K. J., D' Souza, S. F., Optical microbial biosensor for detection of methyl parathion pesticides using *Flavobacterium* sp. whole cells adsorbed on glass fibre filters as disposable biocomponent. *Biosens. Bioelectron.* 2006, *21*, 2100 – 2105.

[17] Priti, M., Wilfred, C., Ashok, M., Microbial biosensor for direct determination of nitrophenyl – substituted organophosphate nerve agents using genetically engineered *Moraxella* sp. *Anal. Chim. Acta.* 2006, *568,* 217 - 221.

[18] Min, J. K., Jee, Y., Yong, T. L., Lee, H. S., Development of a dipstick enzyme-linked immunosorbent assay for the organophosphorus insecticide parathion-methyl. *Int. J. Food Sci. Tech.* 2006, *41*, 927 – 932.

[19] Rosa, L. C., Pariente, F., Hernandoz, L., Lorenzo, E., Amperometric flow through biosensor for the determination of pesticides. *Anal. Chim. Acta.*1995, *308,* 129-136.

[20] Kok, F. N., Bozoglu, F., Hasirci, V., Construction of an acetylcholinetsterase – choline oxidase biosensor for aldicarb determination. *Biosens. Bioelectron.* 2002, *17,* 531-539.

[21] Kuusk, E., Rinken, T., Transient phase calibration of tyrosinase based carbaryl biosensor. *Enzym. Microb. Technol.* 2004, *34,* 657-661.

[22] Aviad, H., Deborah, B., Nina, K., Ariel, C. et al., Acetylcholinesterase ISFET based system for the detection of acetylcholine and acetylcholinesterase inhibitors. *Biosens. Bioelectron.* 2006, *22*, 605 – 612.

[23] Josiane, C., Sergio, A. S., Determination of carbaryl in tomato "in natura" using an amperometric biosensor based on the inhibition of acetylcholinesterase activity. *Sens. Actuators B.* 2008, *129*, 40-46.

[24] Valber, A. P., Josiane, C., Sergio, A. S., Mauro, B., Determination of parathion and carbaryl pesticides in water and food samples using a self-assembled monolayer/acetylcholinesterase electrochemical biosensor. *Sensors,* 2008, *8*, 4600 -4610.

[25] Mianhong, S., Jingjing, X., Song, Z., Baohong, L., Jilie, K., A mediator free screen printed amperometric biosensor for screening of organophosphorus pesticides with flow injection analysis (FIA) system. *Talanta,* 2006, *68,* 1089-1095.

[26] Hildebrandt, A., Bragos, R., Lacorte, S., Marty, J. L., Performance of a portable biosensor for the analysis of organophosphorus and carbamate insecticides in water and food. *Sens. Actuators, B: Chem.*, 2008, *133*, 195-201.

[27] Shaik, F., Fang, M., Xiuli, Y., Zhifei, D. et al., A novel amperometric biosensor based on single walled carbon nanotubes with acetylcholinesterase for the detection of carbaryl pesticide in water. *Talanta,* 2010, *83 (1),* 269-273.

[28] Gabriela, V. R., Montserrat, C., Maria, T. R., Jean, L. M., Acetylcholinesterase-based biosensors for quantification of carbofuran, carbaryl, methylparaoxon, and dichlorvos in 5% acetonitrile. *Anal. Bioanal. Chem.* 2008, 392*(1)*, 699-707.

[29] Andreescu, S., Avramescu, A., Bala, C., Magearu, V., Marty, J. L., Detection of organophosphorus insecticides with immobilized acetlcholinesterase-comparative study of two enzyme sensors, *Anal. Bioanal. Chem. ,* 2002a, *374*, 39-45.

[30] Karine, R., Sofiane, Z., Nicole, J. R., Bernard, F., The use of polyethyleneimine for fabrication of potentiometric cholinesterase biosensors. *Talanta.* 2002, *56*, 1015-1020.

[31] Vakurov, A., Simpson, C. E., Daly, C. L., Gibson, T. D., Millner, P. A., Acetylcholinesterase –based biosensor electrode for organophosphate pesticide detection. I. Modification of carbon surface for immobilization of acetylcholinesterase. *Biosens. Bioelectron.* 2004, *20,* 1118-1125.

[32] Maria, P. X., Begona, V., Maria, D. M., Maria, C. B. et al., Fibre optic monitoring of carbamate pesticides using porous glass with covalently bound chlorophenol red. *Biosens. Bioelectron ,* 2000, *14*, 895-905.

[33] Del Carlo, M., Masicini, M., Pepe Alessia, Diletti, G., Compagnone, D., Screening of food samples for carbamate and organophosphate pesticides using an electrochemical assay. *Food Chem.* 2003, *84,* 651-656.

[34] Vivek, B. K., Huangxian, J., Binding of acetylcholinesterase to multiwall carbon nanotube- cross linked chitosan composite for flow-injection amperometric detection of an organophosphorus insecticide. *Chem. Euro. J.* 2006, *12,* 1074-1080.

[35] Dzyadevych, S. V., Soldatkin, A. P., Arkhypova, V. N., El'skaya, A. V. et al., Early warning electrochemical biosensor system for environmental monitoring based on enzyme inhibition. *Sens. Actuators, B.* 2005,*105,* 81-87.

[36] Toshihiko, I., Nobuhiko, I., Potentiometric butyrylcholine sensor for organophosphate pesticides. *Biosens. Bioelectron.* 1995, *10,* 435-441.

[37] Zhang, S., Zhao, H., John, R., Development of a quantitative relationship between inhibition percentage and both incubation time and inhibitor concentration for inhibition biosensors – theoretical and practical considerations. *Biosens. Bioelectron.* 2001, *16,* 1119-1126.

[38] Lea, P., Mladen, F., Detection of organophosphate and carbamate pesticides in vegetable samples by a photothermal biosensor. *Biosens.Bioelectron.* 2003, *18,* 1-9.

[39] Ivanov, M., Katya, G., Jana, V., Tzonka, G., Immobilization of acetylcholinesterase on nanostructure polyacrylonitrile membranes. *Int. J. Biol. Macromol.* 2009, *44,* 338-345.

[40] Gaberlein, S., Knoll, M., Spener, F., Zaborosch, C., Disposable potentiometric enzyme sensor for direct determination of organophosphorus insecticides. *The Analyst.* 2000, *125,* 2274-2279.

[41] Ivanov, A. N., Evtugyn, G. A. , Lilia, V. L., Elena, E. K. et al., New polyaniline-based potentiometric biosensor for pesticides detection, *IEEE Sensors Journal.* 2003, *3,* 333-340.

[42] Mirslov, P., Oto, D., Zuzana, K., Jana, Z. K. et al., Voltammetric biosensor based on acetylcholinesterase and different immobilization protocols: A simple tool for toxic organophosphate assay. *Anal. Lett.* 2011, *44,* 1-11.

[43] Song, Y., Zhang, M., Wang, L., A novel biosensor based on acetylecholinesterase/prussian blue-chitosan modified electrode for detection of carbaryl pesticides. *Electrochim. Acta.* 2011, *56,* 7267-71.

[44] Luigi, C., DeLuca, S., Sammartino, M. P., Mauro, T., A new organic phase enzyme electrode for the analysis of organophosphorus pesticides and carbamates. *Anal. Chim. Acta.* 1999, *385,* 59-71.

[45] Andreescu, S., Barthelmebs, L., Marty, J. L., Immobilization of acetycholinesterase on screen printed electrodes: Comparative study between three immobilization methods and applications to the detection of organophosphorus pesticides. *Anal. Chim. Acta.* 2002b, *464,* 171-180.

[46] Schulze, H., Schmid, R. D., Bachmann T. T., Rapid detection of neurotoxic insecticides in food using disposable acetylcholinesterase-biosensors and simple solvent extraction. *Anal .Bioanal. Chem.* 2002, *372,* 268-272.

[47] Wilkins, E., Carter, M., Voss, J., Ivnitski, D., A quantitative determination of organophosphate pesticides in organic solvents. *Electrochem. Commun.* 2000, *2,* 786-790.

[48] Gilvanda, S. N., Petrskladal, Hideko, Y., Damia, B., Determination of carbamate residues in crop samples by cholinesterase-based biosensors and chromatographhic techniques. *Anal. Chim. Acta.* 1998, *362,* 59-68.

[49] Vangelis, G. A., Yannis, D. C., A portable fibre-optic pesticides biosensor based on immobilized cholinesterase and sol-gel entrapped bromocresol purple for in-field use. *Biosens. Bioelectron.* 2002, *17,* 61-69.

[50] Simonian, A. L., Flounders, A. W., Wild, J. R., FET- based biosensors for the direct detection of organophosphate neurotoxins. *Electroanalysis,* 2004, *22 (16),* 1896-1906.

[51] Yoshinobu, T., Schoning, M. J., Otto, R., Furuichi, K. et al., Portable light-addressable potentiometric sensor (LAPS) for multisensory applications. *Sens. Actuators.* 2003, *95(1-3),* 352-356.

[52] Bonnet, C., Andreescu, S., Marty, J. L., Adsorption: an easy and efficient immobilization of acetylcholinesterase on screen printed electrodes. *Anal. Chim. Acta.* 2003, *481,* 209-211.

[53] Vakurov, A., Simpson, C. E., Daly, C. L., Gibson, T. D., Millner, P. A., Acetylcholinesterase –based biosensor electrode for organophosphate pesticide detection. II. Immobilization and stabilisation of acetylcholinesterase, *Biosens. Bioelectron.* 2005, *20,* 2324-2329.

[54] Jeanette, P., Karen, L., Alexander, V., Paul, M., Seamus, P. J. H., Sonochemically fabricated enzyme microelectrode arrays for the environmental monitoring of pesticides. *Biosens. Bioelectron.* 2004, *20(4),* 765-772.

[55] Ferat, S., Gokhan, D., Hayrettin, T., A novel matrix for the immobilization of acetylcholinesterase. *Int. J. Biol. Macromol, Structure, Function and Interactions.* 2005, *37,* 148-153

[56] Sotiropoulou, S., Vamvakaki, V., Chaniotakis, N. A., Stabilization of enzymes in nanoporous materials for biosensor applications. *Biosens. Bioelectron.* 2005, *20(8),* 1674–1679.

[57] Wang, S. F., Xiang, H., Xiang, Q., Design of carbon paste biosensors based on the mixture of ionic liquid and paraffin oil as a binder for high performance and stabilization. *Electrochem. Commun.* 2007, *9,* 807-812.

[58] Zhang, S., Shan, L., Zhing, Y., Yishi, L., Study of enzyme biosensor for monitoring carbamate pesticides in sea water. *IFMBE Proc.* 2008, *19,* 323-325.

[59] Xia, S., Xiangyou, W., Acetylcholinesterase biosensor based on prussian-blue-modified electrode for detecting organophosphorus pesticide. *Biosens. Bioelectron.* 2010, *25,* 2611-2614.

[60] Oliveira, A. C., Mascaro, L. H., Evaluation of acetylcholinesterase biosensor based on carbon nanotube paste on the determination of chlorphenvinphos. *Int. J. Anal. Chem.* 2011,*6*.

[61] Bachmann, T. T., Baetrice, L., Francois, V., Jean, L. et al., Improved multianalyte detection of organophosphates and carbamates with disposable multielectrode biosensors using recombinant mutants of *Drosophila* acetylcholinesterase and artificial neural networks. *Biosens. Bioelectron.* 2000, *15,* 193-201.

[62] Luigi, C., Achilli, M., Sammartino, M. P., Mauro, T., Butyrylcholine enzyme sensor for determining organophosphorus inhibitors. *Bioelectroch. Bioener.* 1991, *26,* 237-249.

[63] Andrea, C., Haria, P., Ilaria, L., Marco, M. et al., Disposable ruthenised screen-printed biosensors for pesticides monitoring. *Sens. Actuators B: Chem.* 1995, *24(1-3)*,85-89.

[64] Cremisini. C., Di Sario, S., Mela, J., Pilloton, R., Palleschi, G., Evaluation of the use of free and immobilized acetylcholinesterase for paraoxon detection with an amperometric choline oxidase based biosensor. *Anal. Chim.Acta.* 1995, *311,* 273-280.

[65] Abad, J. M., Pariente, F., Hernandez, L., Abruna, H. D., Lorenzo, E., Determination of organophosphorus and carbamate pesticides using a piezoelectric biosensor. *Anal. Chem.*1998, *70,* 2848-2855.

[66] Collier, W. A., Clear, W., Hart, H. L., Convenient and rapid detection of pesticides in extracts of sheep wool, *Biosens.Bioelectron.* 2002, *17,* 815-819.

[67] Dimitrios, P. N., Maria, G. S., Christiana, G. S., Klara, T., Flow injection analysis of carbofuran in foods using air stable lipid film based acetylcholinesterase biosensor. *Anal. Chim. Acta.* 2005, *537,* 169-177.

[68] Guodong, L., Shawn, L. R., Maria, C. M., Yuehe, L., Sensitive electrochemical detection of enzymatically generated thiocholine at carbon nanotube modified glassy carbon electrode. *Electrochem. Commun.* 2005, *7 (11),* 1163-1169.

[69] Bogdan, B., Didier, F., Andrei, D., Jean-L. M., Biosensors based on highly sensitive acetylcholinesterases for enhanced carbamates insecticides detection. *Anal. Chim. Acta.* 2006, *562 (1),* 115-121.

[70] Dipankar, G., Kakoli, D., Dipankar, B., Priyabrata, S., Amperometric detection of pesticides using polymer electrodes. *Environmental Monitoring and Assessment,* 2006, *119,* 481-489.

[71] Ming – Q. Z., Rui, Y., Da – Ning, W., Jin, F., Qin, H., A novel immobilized cholinesterase for onsite screening of organophosphate and carbamate compounds. *Pestic. Biochem. Phys.* 2006, *86,* 162 -166.

[72] Hwa-Young No, Young Ah Kim, Yong Tae Lee, Hye-Sung Lee., Cholinesterase –based dipstick assay for the detection of organophosphate and carbamate pesticides. *Anal. Chim. Acta.* 2007, *594,* 37-43.

[73] Jinzhong, Z., Aimin, L., Ping, L., Shaping, W. et al., Detection of organophosphorus pesticides using potentiometric enzymatic membrane biosensor based on methyl cellulose immobilization. *Anal. Sci.* 2009, *25,* 511-515.

[74] Gan, N., Yang, X., Xie, D., Wu, Y., Wen, W., A disposable organophosphorus pesticides enzyme biosensor based on magnetic composite nano-particles modified screen printed carbon electrode. *Sensors.* 2010, *10,* 625-638.

[75] Zhaozhu, Z., Yunlong, Z., Xinyu, L., Shaoqin, L., Zhiyong, T., Highly-sensitive organophosphorous pesticide biosensors based on nanostructured films of acetylcholinesterase and CdTe quantum dots. *Biosens. Bioelectron.*2011, *26*, 3081–3085.

[76] Rupesh, K. M., Rocio, B. D., Sunil, B., Roberto, M., Jean-Louis, M., A novel automated flow-based biosensor for the determination of organophosphate pesticides in milk. *Biosens. Bioelectron.* 2012, *32*, 56–61.

[77] Oliveira , T.M.B.F., Fátima, M. B, Simone, M., Mariana, A., Laccase–Prussian blue film–graphene doped carbon paste modified electrode for carbamate pesticides quantification. *Biosens. Bioelectron.* 2013, *47*, 292–299.

[78] Weiying, Z., Abdullah, M. A., Deli, L., Dan, D., Yuehe, L., Nanomaterial-based biosensors for environmental and biological monitoring of organophosphorus pesticides and nerve agents. *Trends Anal.Chem.*, 2014, *54*, 1-10.

[79] Gregory, F. G., Tássia, R. O., Esther, A. N. , Sally, K. M. et al., Electrochemical biosensor for carbofuran pesticide based on esterases from *Eupenicillium shearii* FREI-39 endophytic fungus. *Biosens. Bioelectron.* 2015, *63,* 407–413.

[80] Haiyan, Z., Xueping, J., Beibei, W., Na, W., et al., An ultra-sensitive acetylcholinesterase biosensor based on reduced graphene oxide-Au nanoparticles- β- cyclodextrin/Prussian blue- chitosan nanocomposites for organophosphorus pesticides detection, *Biosens. Bioelectron.*, 2015, 65,23-30.

[81] Everlyne, A. S., Jonathan, O. O., Recent approaches to improving selectivity and sensitivity of enzyme-based biosensors for organophosphorus pesticides: A review, *Talanta,* 2016, *155*, 289-304.

[82] Rajni, B., Munish, K., Kavita, B., Rohit, K. S., Nishima, W., Ultrasensitive aptamer biosensor for malathion detection based on cationic polymer and gold nanoparticles *Biosens. Bioelectron*. *85* (2016) 445–449.

Table1: Enzyme inhibition based biosensor research work carried out for the detection of organophosphorus compounds and carbamate pesticides

Inhibitor	Enzymes	Immobilization Method	Transducing element	Sample	Working range/ detection limit	Comments	Reference
Paraoxon, Aldicarb	Butryl cholinesterase	Kappa Carrageenan gel and Dialysis membrane	Amperometric	50% water saturated chloroform and hexane mixture	4.5 µ g/l	Long life time	[62]
Paraoxon methyl, Paraozon ethyl, Trichlorfon, DFP	Butryl cholinesterase (Horse serum)/ Acetyl cholinesterase (Electric eel)	Using saturated glutaraldehyde vapour	Conductometric	Liquid sample	10^{-6}M. Incubation time: 10 minutes	Disposable, No need for reference electrode, Ability to miniaturization	[5]
Carbofuran, Propoxur	Acetyl cholinesterase (Electric eel) Choline oxidase (Alcaligenes sp.)	Adsorption and Electro polymerization method	Amperometric	Pesticides prepared in the standard solution	1 nM	Single use, High sensitivity, In situ measurements possible	[63]
Paraxon	Acetyl cholinesterase, Choline oxidase	Physical entrapment, Covalent immobilization on membranes	Amperometric	Standard solution	1-1.5 ppb	Good sensitivity, Precision	[64]
2,2 Dichloro vinyldi methyl phosphate,DDVP, Profenofos	Butryl cholinesterase, Acetyl cholinesterase	Entrapped on plasticized poly vinyl chloride	Potentiometric	Standard solution	10^{-6} – 10^{-8} M (profenofos), 10^{-5} – 10^{-6} M DDVP)	Improved selectivity	[36]
Trichlorofon	Acetyl cholinesterase, choline-oxidase, peroxidases	Coimmobilisation with electrode using glutaraldehyde	Potentiometric	Standard solution	Detection limit: 5nM	Disposable type, Shelf life: 1 month	[6]
Paraoxon, Carbaryl	Acetyl cholinesterase	Direct adsorption on the gold surface, Covalently immobilized using cystamine functionalized gold electrode with aqueous glutaraldehyde	Piezoelectric	Standard solution prepared in ethanol	$5x10^{-8}$ – $1x10^{-5}$ M $1x10^{-7}$ – $5x10^{-5}$ M	Reproducibility: ±10% Preincubation Time: 5 minutes	[65]
Aldicarb, Carbaryl Carbofuran, Methomyl, Propoxur	Cholinesterase (Bovine Erythrocytes, Electric Eel, Human Erythrocytes, Horse serum, Human serum)	Immobilized with 1 % Glutaraldehyde Cobalt (II) Phthalocyanine	Amperometric	Vegetables	$5x10^{-5}$ to 50 mg/kg	Disposable sensor, Cost effective method, No sample pretreatment	[48]

Paraoxon, Aldicarb, Carbaryl	Butryl cholinesterase (Horse serum) Choline oxidase (Alcaligenes sp.)	Entrapment in Kappa Carrageanan gel	Amperometric	Pesticides standard solution	4.8 µg/l 0.5 µg/l	Well developed for measurement in the organic phase	[44]
Trichlorofon, Coumphos, Methiocarb, Aldicarb	Acetyl cholinesterase, Butryl cholinesterase	Cross linked with polyaniline film	Potentiometric	River water and grape juice	1.5×10^{-7} mol/l 5.0×10^{-9} mol/l 8.0×10^{-7} mol/l 2.0×10^{-7} mol/l	Stable for 10 days in wet and dry	[7]
Propoxur, Carbarylvos	Acetyl cholinesterase (Electric eel)	Covalently bound to controlled pore glass	Optical	Chopped onion lettuce	0.03 – 0.5 mg/l, 0.8 – 3 mg/l	Good reproducibility, Stable for a month	[32]
Dichlorvos, Diazinon, Denthion, Fenthion	Acetyl cholinesterase (Electric eel)	Covalently immobilized on film on the graphite electrode	Amperometric	Organic solvents	Detection limit : $0.5x10^{-6}$ M, $0.8x10^{-6}$M, $1.0x10^{-6}$ M	60 days under dry condition	[47]
Paraoxon	Acetyl cholinesterase and choline oxidase	Polyurethane and Polyethylene oxide membrane	Amperometric	Incubation solution containing paraoxon	Detection limit:10 nM	Inhibition diffusion mechanism	[37]
Paraoxon, Chlorfen bimphos, Diazinon	Acetyl cholinesterase (Electric eel),Acetyl cholinesterase (Bovine erythrocytes), Butryl cholinesterase	Crosslinking with (0.05%) glutaraldehyde, (3%w/v) hydroxyl ethyl cellulose	Amperometric	Wool extract	Working range: 10^{-2} to 10^{-3} M	Incubation time: 15 minutes	[66]
Paraoxon	Acetyl cholinesterase	Cross linking immobilization	Amperometric	Orange juice, apple, peach with honey	1µg/ to 60µg/l	Reproducibility: 6%,Incubation Time:30 minutes	[46]
Aldicarb	Acetyl cholinesterase, Choline oxidase	Poly (2-hydroxy ethyl methacrylate) with Cibacron blue and Epichlorhydrin	Amperometric	Commercially available Aldicarb solution	10-100 ppb	Storage stability: months, Incubation time: 5 minutes	[20]
Trichlorfon, Methoicarb aldicarb	Butryl cholinesterase	Enzyme on film with glutaraldehyde	Potentiometric	Liquid	10^{-7}M	Response time: quick	[30]
Carbaryl, Dichlorovos	Butryl cholinesterase	Sol gel entrapment	Optical	In field use	0.11-8 mg/l, 5 – 30 µg/l	Reproducibility: 3-5%, Accuracy: 94.9%	[49]

Carbofuran, Aldicarb, Paraoxon	Acetyl cholinesterase (Electric eel), Acetyl cholinestrease (Bovine erythrocytes), Choline oxidase	Co-immobilization on the surface of membrane	Amperometric	Pesticides prepared in the buffer solution	10^{-5} M	Incubation time: 30 minutes	[4]
Carbaryl and Parathion methyl	Acetyl cholinestrease (Electric eel)	Entrapped on the nafion membrane fixed on chemically modified electrode	Amperometric	Egg, Bovine, honey, milk	2-90 ng/ml, 1-100 ng/ml	Assay time: 5 minutes	[33]
Carbofuran, Propamocarb Parathion ethyl, Oxydemeton – methyl	Acetyl cholinesterase (Electric eel), Butryl cholinesterase (Horse serum)	Cross linked with controlled poro glass beads	Photo thermal	Onion, lettuce, salad	0.005 mg/kg to 0.013 mg/kg	Cost effective, No sample pretreatment	[38]
Paraoxon	Acetyl cholinesterase (*Electrophorus electricus)*	Entrapment on Polymer matrix (Micro electrode Array)	Amperometric	River/ stream	$1x10^{-7}$M	High sensitivity	[54]
Carbaryl	Tyrosinase	Cylindrical membrane covered with oxygen sensor	Amperometric	Aqueous solution	1.5 mg/l detection limit	Soluble tyrosinase was used to avoid the suicide inhibition of enzymes by the products	[21]
Carbofuran	Acetyl cholinesterase	Polymerized into lipid film	Transient current measurements	Food	10^{-7} M to 10^{-9} M	No matrix effects	[67]
Paraoxon	Acetyl cholinesterase	Self assembled on carbon nano tubes modified glassy carbon electrode	Amperometric	Online moitoring	0.4pM	Inhibition time:6 minutes	[68]
Carbaryl, Carbofuran	Acetyl cholinesterase	Entrapped on silica gel column	Potentiometry and Conductometry	Water	0.3 -10 ppm 0.02-0.3 ppm	Analysis time : 31-37 minutes Reproducibility : 4.0+1.3 %	[8]
Eserine	Acetyl cholinesterase, Neurons	Covalently immobilized on the gate surface of ISFET using Cyanuric chloride	Ion sensitive Field Effect Transistor	Real time use in vitro and in vivo applications	0.1 – 100 µM	Hybrid system is useful for the real time detection , Good sensitivity	[22]
Methomyl, Carbofuran, Carboryl, Aldicarb	Entrapment on sol gel matrix covered on chemically modified electrodes	Entrapped in sol gel matrix	Amperometric	Potable water sample	8×10^{-10} M, 1×10^{-8} M, 2×10^{-8} M	Mutant enzymes showed increased sensitivity and low detection limit	[69]

Fenthion monocrotophos	Acetyl cholinesterase (Bovine erythrocytes)	Graphite electrode copolymerization of Acetyl cholinesterase and Choline oxidase on polymer network upon screen printed electrode	Amperometric	Prepared in distilled water	10^{-6} M 0.1-10 ppb	Cheap and reliable	[70]
Dichlorvos	Acetyl cholinesterase	Al_2O_3 sol gel	Amperometry	Sea water	0.1 – 80µM	Reactivation:<80% Inhibited enzyme Activity, reproducibility: 4.92% (n=9)	[25]
Fenthion, Malathion, Dichlorvos,Carbaryl, Chlorpynifos, Carbofuran	Acetyl cholinesterase	Physical adsorption on Polyvinyl pyrrolidone	Pesticide analyzer with biochemical assay	On-site	20µl of pesticides used	Stable for 20 days in room temperature	[71]
Sulfotep	Acetyl cholinesterase (Electric eel)	25% Glutaraldehyde cross linked with chitosan coated on carbon nanotubes	Amperometric	Tap water and river water	1.5 to 80 µM	High throughput sample screening, reactivation	[34]
Alachor, Diazinon, Carbaryl	Tyrosinase	Cross linking with bovine serum albumin using glutaraldehyde	Conductometric	Aqueous solution	1.5×10^{-7} M, 5.0×10^{-8} M, 2.0×10^{-7} M	30 minutes, Good storage stability	[9]
Organophosphorus and carbamate pesticides	Acetyl cholinesterase	Entrapment on to the membrane	Amperometric and Potentiometric	Lettuce	10^{-6} to 10^{2} µg/L, 10^{-6} to 10^{0} µg /L	Quick and good detection limit	[72]
Carbofuran, Carbaryl Dichlovos, Methylparaxon	Acetyl choline esterase (Electric eel), Genetically Engineered *Drosophila Melanogaster*	Photo polymerization with mediator in 5% Acetonitrile	Amperometric	Apple Extract	4.5×10^{-6} mol/ 1.6×10^{-7} mol/l 9.6×10^{-1}1mol/l 2.6×10^{-9}mol/l	Stable for 7 months	[28]
Chlorpyrifos, Chlorphyriphos-oxon chlorfenvinphos, Primiphos-methyl, malathion, Carbofuran,	Acetyl cholinesterase (*D. melanogaster*)	1mU and 10 mU of Acetyl cholinesterase entrapped in Poly vinyl acetate polymer sheet	Amperomeric	Bottled water, tap water, ground	2 µg/l	Repeatability : 7.6% A. (n=12)	[26]

Propoxur		with Tetra cyanoquino dimethane		water, sea water, tea, orange juice, milk champagne			
Carbaryl	Acetyl cholinesterase (Bovine erythrocytes)	1% Glutaraldehyde solution cross linked with Cobalt modified carbon paste	Amperometric	Tomato	0.8×10^{-5} – 10.0×10^{-5} mol/l Pathalocyanine	83.4% recovery No matrix effects	[23]
Carbary1 and Parathion	Acetyl cholinesterase (Bovine erythrocytes)	Confined immobilization by surface assembled monolayer	Amperometric	Tomato and apple	9 -10.3 µg/l	91.0%-98.0% recovery µg/l	[24]
Parathion, Carbaryl	Wild type acetyl cholinesterase combined with Nb Acetyl cholinesterase	Cross linking using glutaraldehyde vapor	Amperometric	Milk	Detection limit: 1µg/l	Disposable	[58]
Organophosphorus pesticide	Acetylcholinesterase (*Electrophorus electricus*)	Immobilized on chemically modified and non modified polyaniline membrane	Amperometric	Standard solution	10^{-10} to 10^{-7} g/l 7.39×10^{-11} g/l	Increased storage stability and operational stability, high sensitivity	[41]
Methamidophos, Phorate, Parathion, Chlorphyifos,	Acetyl cholinesterase	Entrapped in methyl cellulose membrane	Potentiometric	Potted lettuce	10 ng/l, 2.5 ng/l, 15 ng/l, 5 ng/l	Exhibit good reproducibility and storage stability for 20 days, Selective detection in the presence heavy metal ions	[73]
Dimethoate	Acetyl cholinesterase	Assembled on magnetic composite nanoparticles	Amperometry	Chinese cabbage	1.0×10^{-3} – 10 ng/ml, Detection limit: 5.6×10^{-4} ng/ml	High sensitivity, Disposable, Low consumption of sample	[74]
Carbaryl	Acetyl cholinesterase (Electric eel)	Layer by layer self assembled using carbon nanotubes	Amperometric	River water	10^{-6} – 10^{-11} g/l, Detection limit: 10^{-12} g/l	Average recovery is 99.8±2.7 % at 10^{-10} g/l, Good stability and	[27]

						specificity towards carbaryl	
Phoxim, Dichlorvos, Trichlorfon	Acetyl cholinesterase	Cross linked on the chitosan membrane using glutaraldehyde solution	Amperometric	Standard solution	Not available	Good sensitivity and selectivity towards OP compounds, Exhibit low detection limit, better reproducibility	[59]
Diisopropylfluorophosphate	Acetyl cholinesterase	Precipitation with glutaraldehyde, spherical micropar- ticles, sorption, interception into gelatin, sol gel	Amperometric	Laboratory condition5 % IPA to pesticide in organic solvents	(2.9 ±1.8) $\times 10^{-8}$mol/l (6.9±2.9)× 10^{-7} mol/l (3.5±1.5) × 10^{-8}mol/l (8.3±3.1) × 10^{-8} mol/l	Not available	[42]
Chlorphenvinophos	Acetyl cholinesterase (BE)	Added directly with MWC mixed with nujol oil	Amperometric	Insecticidal solution prepared in methanol	4.90×10^{-7} – 7.46×10^{-6}M	Incubation time : 10 minutes, fast response, durability	[60]
Carbaryl	Acetyl cholinesterase	Prussian blue- Chitosan hybrid film On GCE (electrostaric Interaction)	Amperometric	Water	Detection limit: 3 nM	Recovery: 96.3% to 106.0%	[43]

Figure 1. Biosensor detection mechanism