Chapter 1

Introduction

1.1 Introduction

In recent time the chemical pesticides are drawing much attention globally due to their effects on human health and the environment [1]. The continued increase in the world population that resulted in a substantial increase in food demand has made crop protection a primary area of interest and concern for legislators and scientists [2]. Due to which a sheer volume of pesticide usage occurs and many of them affect non-target organisms and become a potential hazard to human health [3]. Concern about the adverse impacts of pesticides on the environment and on human health started being voiced in the early 1960s. Rachel Carson's book "*Silent Spring*", published in 1962, first drew attention to the hazard of the widespread extensive use of pesticides for the environment (namely birds) and also for human health [4]. Since then, debate on the risks and benefits of pesticides has not ceased and a huge amount of research has been conducted into the impact of pesticides on the environment [5]. Pesticide exposure is ubiquitous, due not only to agricultural pesticides use and contamination of foods but also to the extensive use of these products in and around households [6]. Thus the widespread global use of chemical pesticides for crop protection and other household uses have posed a grave threat to human health and the environment. The problem is steadily growing and becoming more serious, especially, in the developing countries [7]. The scientific community working in food science is nowadays continuously asked to give adequate answers to the consumers, whose perception about food safety and quality is constantly increasing. Besides, there is an overall tendency to connect food and health with the main purpose of boosting wellbeing and preventing future diseases [8]. Real-time detection of these toxic agents can offer significant advantages in monitoring the uses and controlled releases of pesticides for pest control purposes, ensuring compliance to pesticide use regulations and protection of public health from accidental or intentional releases of hazardous pesticides [9].

1.2 A brief introduction to different classes of pesticides

1.2.1 Organochlorine pesticides

Organochlorine pesticides (OCPs) were extensively used in agriculture from the 1950s to

the 1970s [10]. But concern about their potential carcinogenicity and environmental harmfulness led to restricting their use or banning them in many countries; as a result, OCP residue levels in foodstuffs have declined over the recent years [11]. Though many persistent OCPs are restricted or banned in developed countries, they are still produced and used to a large extent in developing countries [12]. Only a few active substances, essentially DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] is still used to control malaria vectors through indoor residual spraying and lindane for treatment of lice and scabies. So, most exposures derive from past uses and, in certain cases, are still significant because of the long persistence and biomagnifications of these compounds.

Organochlorinated pesticides are structurally heterogeneous class of organic compounds composed primarily of carbon, hydrogen and several chlorine atoms per molecule [13]. The mode of action of DDT-type insecticides is to invade primarily the peripheral nervous system and produces toxic effects in nervous tissues and enzyme systems. For several decades the mechanism of action of DDT-type insecticides has been investigated and the currently accepted hypothesis was essentially put forth in its present form by Holan *et al*. [14]. At the sodium gates of the axon, DDT exerts its toxic action by preventing the deactivation or closing of the gate after activation and membrane depolarization. The result is a lingering leakage of Na^+ ions through the nerve membrane, creating a destabilizing negative after-potential. The hyperexcitability of the nerve results in repetitive discharges in the neuron after a single stimulus and/or occur spontaneously [15].

The basic structure of the DDT class of insecticide and its analogues is shown in Figure 1.1. As illustrated, the fundamental structure of active compounds consists of p-substituents X, which may be either halogens, or short-chain alkyl or alkoxy groups, Y is always hydrogen, and Z may be CCl_3, $CHCl_2$, $CH(NO_2)CH_3$ or $C(CH_3)_3$. In the case of DDT especially X is Cl, Y is hydrogen and Z is trichloromethyl. It was found that in a given series with fixed X and Y substituents, successive substitution of the Z substituents by the groups from CCl_3 to $C(CH_3)_3$ was accompanied by a progressive decline in insecticidal potency [16].

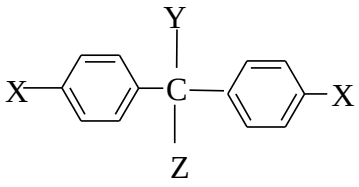


Figure 1.1. General structure of DDT and its analogues.

Chemically stable and strongly lipophilic, organochlorine compounds have slow degradation rates and tend to bioaccumulate in lipid-rich tissues [17]. Organochlorine pesticides are known to resist biodegradation and therefore, they can be recycled through food chains and produce a significant magnification of the original concentration at the end of the chain [18]. Because of their ability to concentrate up the food web, their continuous detection in the food supply and drinking water is highly necessary. They also seem to accumulate in organisms and then can cause endocrine disruption at environmentally realistic exposure levels [19].

1.2.2 Organophosphate pesticides

The organophosphate (OP) compounds are a group of most widely used insecticides that were first discovered in 1938 by a group of German chemists. Organophosphates were introduced as nerve poisons or chemical warfare agents during World War II and later they were developed into less potent chemicals. Organophosphates have gained popularity worldwide in an effort to substitute persistent organochlorine pesticides. However, organophosphate pesticides are generally much more toxic to vertebrates compared to other classes of insecticides even though they rapidly degrade in the environment [20-22]. As they are degraded, these chemicals slowly leave behind residues in food and water sources, which can become more concentrated as they move up the food chain and also some products formed during the transformation process are even more toxic than the parent compounds. These organic toxins enter animal and human bodies directly or indirectly via inhalation, absorption into the skin and ingestion through food chain or drinking water and threat human health [23].

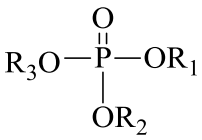


Figure 1.2. General chemical structure of organophosphate functional group.

The acute toxicity of an exposure to these OP chemicals stems from the fact that they target a number of important proteins, including a group of hydrolytic enzymes, such as acetylcholinesterase (AChE), which is particularly critical for central and peripheral nervous-system functions. The OP agents irreversibly phosphorylate AChE serine residue, required for its catalytic activity, forming a stable complex that inhibits enzyme activity [24,25]. The nerve agents covalently block the active site serine residue of AChE by the process of nucleophilic attack to produce a serine-phosphoester adduct. This irreversible inactivation leads to accumulation of acetylcholine causing cholinergic over stimulation, culminating in death caused by respiratory failure [26]. Infants and children may be especially sensitive to health risks posed by these pesticides [27]. The usage of these pesticides has brought about great concern in the scientific community on the possible toxic effects of these pesticide contaminations to both aquatic flora and fauna as well as to humans. Because of the potential for widespread toxicity of OPs, the development of a rapid, sensitive and inexpensive method to detect OP pesticides and nerve agents is truly necessary.

1.2.3 Carbamate pesticides

Carbamate pesticides have been synthesized and sold commercially since the 1950s [28]. They were used extensively in pest control due to their effectiveness and broad spectrum of biological activity (insecticides, fungicides, and herbicides) [29]. The proven persistence and toxicity of organochlorine pesticides justify their replacement by more easily degradable, polar, labile and less persistent carbamate pesticides [30]. Currently, carbamates are one of the major classes of synthetic organic pesticides and are used annually on a large scale worldwide. Carbamates are mainly used in agriculture against insects, fungi and weeds [31]. In addition, they are used as biocides for industrial and other applications as well as in household products. Most of the carbamates have high melting points and low vapor pressures. They are usually distributed in aqueous

environments because of their high solubility in water. Studies have shown that carbamates and their degradation products are potential contaminants of the environment and food resources. There is increasing evidence indicating that carbamates may spread throughout ecosystems by leaching and runoff from soil into the ground and surface water [32].

Structures of carbamates are based on N-substituted carbamic acid esters ($R_1OCONR_2R_3$) (Figure 1.3). Thiocarbamates ($R_1SCONR_2R_3$) are also included into this group [33]. The R_1 group is typically a phenyl or a heterocyclic aromatic ring, the R_2 group is usually a methyl substituent and the R_3 is either a hydrogen, methyl, or a more complex group [34].

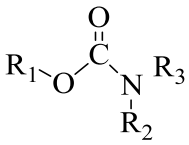


Figure 1.3. General chemical structure of carbamates.

Carbamates are increasingly used in agriculture due to their broad biological activity, low bioaccumulation potentials and relatively low mammalian toxicities. However, these pesticides have high acute toxicity. They act by a mechanism of reversible inhibition of acetylcholine, which is less toxic to mammals than organophosphorus pesticides. Since they are acetylcholinesterase inhibitors, carbamate pesticides are suspected carcinogens and mutagens [35]. These compounds are considered hazardous to the environment and human health and they have been listed by the United States Environmental Protection Agency (EPA) as common mechanism group (CMG) which induces a common toxic effect by a common mechanism of toxicity [36-38].

1.2.4 Pyrethroid pesticides

Pyrethroids are comparatively new insecticides used worldwide with a broad spectrum of application. They are synthetic derivatives of pyrethrins, the natural toxic constituents of the flowers of *Chrysanthemum cinerarieafolium* [39]. Pyrethrins are potent insecticides with relatively low mammalian toxicity; however, they have devastating effects on aquatic invertebrates, fishes and honeybees [40]. Due to high

sensitivity towards air and light, the use of pyrethrins for crop protection and to control disease-carrying insects is limited. With an altered structure, the pyrethroids are more photostable while retaining the insecticidal activity of the pyrethrins [41]. The basic structure of this class of pesticides consists of an acid and alcohol moiety. They can have one to three chiral centers resulting in two to eight enantiomers. The centers of chirality are in the cyclopropane ring of the acid moiety (Figure 1.4) and the α-carbon of the alcohol moiety. Each enantiomer may differ in insecticidal potency, mammalian toxicity and metabolic pathway. Pyrethroids can be classified as Type I or Type II compounds. Type I pyrethroids are esters of primary or secondary alcohols. Type II pyrethroids are esters of secondary alcohols with a cyano group at the α-carbon of the alcohol moiety [42].

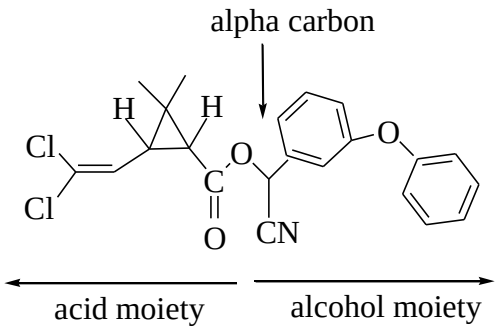


Figure 1.4. General structure of pyrethroid pesticides.

Their mode of action is primarily to impair the functioning of nervous system. Initial symptoms of poisoning include incoordination and locomotor instability, followed by hyperexcitation, tremors and convulsions [43]. Over the last decades, traditional organophosphate, organonitrogen and organochlorine pesticides have been increasingly replaced by synthetic pyrethroid pesticides; this pesticide family has been gaining popularity worldwide for the control of agricultural pests because of their relatively low mammalian toxicity, selective insecticide activity and low environmental persistence [44].

1.2.5 Benzimidazole pesticides

Compounds based on a benzimidazole structure are systemic pesticides and playing an ever increasing part in the control of fungal diseases [45]. They are widely used in

agriculture for pre- and post- harvest treatment for control of a wide range of pathogens. These substances are applied directly to the soil or sprayed over crop fields and hence are released to the environment. Benzimidazole pesticides can readily penetrate into the plants through the roots and leaves and can directly enter natural waters by drainage from agricultural land. Most of these compounds show persistance in the environment after application; some even remain for years. Two of the main compounds in the benzimidazole family are carbendazim and thiabendazole. Carbendazim exhibits both protective and curative activity against a wide range of fungal diseases. It is toxic to humans, animals and plants and also is very persistent in water, wastewater, soil, crop and food. Likewise, thiabendazole is used to control fruit and vegetable diseases such as mold, rot and blight and is used as a veterinary drug to treat worms. [46]

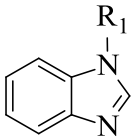


Figure 1.5. General structure of benzimidazole group.

These pesticides, act by inhibiting microtubule formation by binding to free β-tubulin monomers at the colchicine-binding site. In target organisms, the intended effect is cytotoxicity which occurs through disruption of microtubuli. At lower, non-cytotoxic concentrations, the impairment of the microtubuli of the spindle apparatus can disturb the alignment of chromosomes during mitosis and lead to the formation of micronuclei (MN) [47].

1.2.6 Phenolic pesticides

Phenolic pesticides are applied as herbicides all over the world as post-emergent herbicides mainly in crops, vegetables and cotton cultivations. Improper handling of these pesticides, even with correct agricultural practice can lead to the pollution of surface and ground water within the treated and adjacent areas [48]. Their detection has been increasing in recent years because of their toxicity, even at low concentrations [49]. These herbicides have a twofold mode of action: (i) they inhibit the functioning of photosystem II; all of them efficiently inhibit electron transfer out of the primary plastoquinone QA, putting a stop to photosynthesis, although binding experiments have

indicated that these herbicides may bind to different proteins of the PS II reaction center [50] (ii) they are uncouplers of oxidative phosphorylation and photophosphorylation as well, due to their protonophoric properties [51].

1.3 Conventional methods of pesticide detection

1.3.1 Gas chromatography (GC)

GC is an analytical technique for separating compounds based primarily on their volatilities. The determination of pesticides in environmental and food samples is usually carried out by this method. In general, many pesticides are thermally stable, less polar and volatile compounds, therefore, GC should be a good technique for pesticide analysis [52]. Since the introduction of GC in the late 1960s and the inherent remarkable feature to perform on a packed column multi-residue analysis, the technique became rapidly adopted. Further important developments such as capillary columns and sensitive and selective detectors significantly enlarged the number of pesticides efficiently analyzed in one run [53].

1.3.1.1 Electron capture detector (ECD)

GC-ECD is the favoured technique for the determination of majority of pesticides. The ECD is used for detecting electron-absorbing components (highly electronegative) such as halogenated compounds in the output stream of a gas chromatograph [54,55]. Organochlorine pesticides are primarily detected using this technique [56,57].

1.3.1.2 Nitrogen and phosphorus detector (NPD)

The NPD (sometimes called the thermionic detector) is a very sensitive and specific detector that responds to nitrogen and phosphorous compounds. It is based on the flame ionization detector (FID), but differs in that it contains a rubidium or cesium silicate (glass) bead situated in a heater coil, at a little distance from the hydrogen flame. Amvrazi *et al*. [58] developed a gas chromatographic method coupled with NPD for determination of 35 different pesticides belonging to diverse class of pesticides. Yagüe *et al*. [59] used GC-NPD detector for determination of OP, OC and polychlorinated biphenyls (PCBs).

1.3.1.3 Flame photometric detector (FPD)

Flame photometric detection (FPD), due to its very good selectivity and preferably sensitive response for phosphorus and sulfur, has been specifically employed in the determination of phosphorus or sulfur containing compounds [60]. FPD is based on element specific luminescence produced when sulfur or phosphorus compounds are burnt in a hydrogen-rich flame [61]. Jia *et al.* [62] developed a procedure for determination of OP pesticides using GC-FPD. Yu *et al.* [63] also studied concentrations of OPs in real sample using GC-FPD.

1.3.1.4 Mass spectrometry detector (MSD)

Gas chromatography mass spectrometry (GC/MS) is irreplaceable in pesticide analysis. It is the universal and non-specific detector that allows not only to detect and to quantify the analytes present in the sample, but also to identify these compounds on basis of their structure [64]. The mass spectrometer is without doubt the most specific detector available in multiresidue analysis. The specificity is based on the fact that molecules when bombarded with electrons of particular energy under vacuum conditions fragmentation occurs and the resulting fragmentation pattern reflects the individual molecular structure in a mass spectrum that is often considered as the fingerprint of the substance. These mass spectra show such specific characteristics that it is possible to differentiate among thousands of compounds. It is of great importance that these mass spectra do not depend on the instrument used for measuring them but only on the ionization conditions applied. Standardized ionization conditions can easily be reproduced. Therefore, it is possible to compile all the mass spectra recorded all over the world in libraries. Such documented spectra are used to identify unknown compounds by comparing their spectra with those already well established. The formerly laborious task of comparing mass spectra is now performed with the help of computers and sophisticated software programs. Within a few seconds such a computer program can perform a search of more than 40,000 documented mass spectra and draw up a list of a few mass spectra ranked according to their strongest resemblance to the one just recorded. In this way, it is possible to identify an unknown peak in a gas chromatogram without having the corresponding test substance available [65]. Thus the key characteristics of GC/MS in pesticide analysis are its selectivity and sensitivity. Major

limitations in the use of GC/MS depend on GC limits. Therefore, very polar and thermally labile pesticides are not suitable for GC separation and therefore cannot be analyzed by GC/MS [66].

1.3.1.5 Gas chromatography tandem mass spectrometry (GC/MS/MS)

When a second phase of mass fragmentation is added, for example using a second quadrupole in a quadrupole instrument, it is called tandem MS (MS/MS). MS/MS can sometimes be used to quantitate low levels of target compounds in the presence of a high sample matrix background. Due to the high selectivity provided by MS/MS detection, simple extraction techniques with little cleanup are employed [67].

These detectors are primarily composed of a collision cell, also called interface, connected to two mass analysers. The ions, entering the MS/MS from the chromatography column, pass through the first quadrupole Q1. The Q1 then selects an ion of a specific mass to enter Q2 where collision gas (N_2 or Ar) split the ions into product ions. The product ions of all masses enter the third quadrupole, Q3, where only one size of ions is let through for the conclusive detection. One advantage with MS/MS is its selectivity as the background noise is reduced when specific compound-dependent fragments of ions are formed. The high specificity of these kinds of detectors is an important feature in the aspect of identification, as it functions even for pesticides with similar retention time. These detectors are of choice for pesticide analysis because of their high performance; however, they come with the disadvantages of high cost as well as maintenance.

1.3.2 High performance liquid chromatography (HPLC)

High performance liquid chromatographic (HPLC) methods for pesticide residue analysis were firstly developed for non-volatile or thermally labile compounds such as carbamate insecticides. As HPLC can offer a simpler and/or faster approach to analyse a wide number of other compounds, HPLC methods are continually increasing in acceptance and applications [68]. HPLC methods are generally preferred over GC methods, because HPLC is applicable to thermally labile and polar compounds [69]. It typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column and a detector that provides a characteristic

retention time for the analyte. Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used and the flow rate of the mobile phase. With HPLC, a pump (rather than gravity) provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This in practice allows for a better separation on columns of shorter length when compared to ordinary column chromatography [70].

1.3.2.1 UV, DAD, fluorescence and chemiluminescence detector

HPLC methods for the determination of pesticide residues employ a variety of detection methods including UV absorption, UV diode array, fluorescence (FL) and chemiluminescence (CL) etc [71]. Liquid chromatography (LC) with a diode array detector is a non-destructive method that offers many advantages. Apart from the possibility of monitoring at several wavelengths simultaneously, this detector allows us to compare the UV spectrum of a peak of interest with a library of UV spectra in order to confirm or help with identification. One problem with the UV detection is that pesticides absorb appreciably at wavelengths below 250 nm, the same spectral region where many reactive and matrix-derived interferences absorb. For this reason, LC–UV analysis is generally more applicable in high-concentration formulations or very clean environmental substrates [72].

1.3.2.2 Mass spectrometry detector

The powerful features of LC-MS, such as high efficient separation, identification and quantification of polar analytes, make this technique very attractive to the field of pesticide residue analysis. It is highly selective in selected ion monitoring and in multiple reactions monitoring mode. Only the signal of interest is registered, leaving out the information about the occurrence of all the other compounds [73]. Thus, these LC-MS instruments are well suited to solve difficult problems of identification, quantification and confirmation of pesticides and their metabolites. The selection of the mass analyzer, apart from its accessibility, is determined by the required sensitivity and selectivity [74].

Although chromatographic techniques have very high sensitivity, these methods suffer from many disadvantages. Such analysis is generally performed at centralized

laboratories, requiring extensive labor and analytical resources. Being complex, costly and time consuming, chromatographic methods are primarily limited to laboratory settings and their use is prohibited for rapid analyses under field conditions [75,76].

1.3.3 Immunoassay (IA)

Immunoassays are analytical methods based on the interaction of an analyte with an antibody that recognizes it with high affinity and specificity. They are rapid, sensitive, cost effective and field-portable, do not require sophisticated instrumentation, and are able to accurately and precisely analyze a large number of samples of single analyte simultaneously. All these features make immunoassays very favourable analytical tools in pesticide monitoring programs, particularly for those chemicals which are difficult to determine by conventional chromatographic techniques, or for specific pesticides that deserve special attention because of their toxicity, extensive use or high frequency of appearance in foodstuffs. The usefulness of this technique is experienced during screening analyses when a large number of samples have to be analyzed in parallel for a single analyte within a short time [77]. However, immunoassays are prone to a common problem "matrix interference" which could reduce the sensitivity and reliability of the immunoassays and cause false positives by lowering the color development [78]. The most commonly used immunoassays for pesticide analysis are immunosensors (IS), immunolabeling and enzyme-linked immunosorbent assay (ELISA). ELISA encompasses ~90% of IA used for pesticide analysis. ELISA is very specific, which could reduce matrix effects and the need for extensive sample clean-up and concentration [79]. Enzyme-linked immunosorbent assay is a solid-phase assay. One of the reactants, mainly the antigen, is adsorbed onto the surface of a test tube or microtiter well. To determine the quantity of antibody in a sample, an aliquot of antiserum is reacted with the adsorbed antigen and the unreacted molecules are washed away. In the next step, an enzyme-linked anti-immunoglobulin (anti-Ig) is added. Finally, the substrate is added and the amount of developed color is determined. The content of antibody present can be quantified from standard curves, because the amount of color is directly proportional to the amount of the reacted enzyme-linked second antibody.

An inhibition method can also be used in ELISA assays. In these type of assays, varying

amounts of soluble antigen are added to the antiserum, this soluble antigen binds to the antigen-binding sites on the antibody and thus competes with the immobilized antigen. A reduction in binding of the antiserum is used to determine the amount of antigen or antibody present in the sample.

ELISA assays can also be used to quantify the amount of antigen in a sample by adsorbing unlabeled antibody to a solid surface followed by antigen. The antigen–antibody (Ag–Ab) complex is quantified by adding enzyme-linked antibody directed against the antigen, i.e., labeled antibody of the same specificity as the unlabeled antibody already adsorbed to the surface, rather than an anti-immunoglobulin. This variation, called a `sandwich' ELISA, requires the antigen to have at least two accessible binding sites for antibody because two antibody molecules must be bound to the same antigen molecule. This kind of antibody-binding immunoassay depends mainly on two factors: the target compound and the immune reactives used. Figure 1.6 shows a scheme of a sandwich ELISA format containing a coated antigen competing by the binding sites of related antibody. This format has been extensively used for pesticide analysis [80].

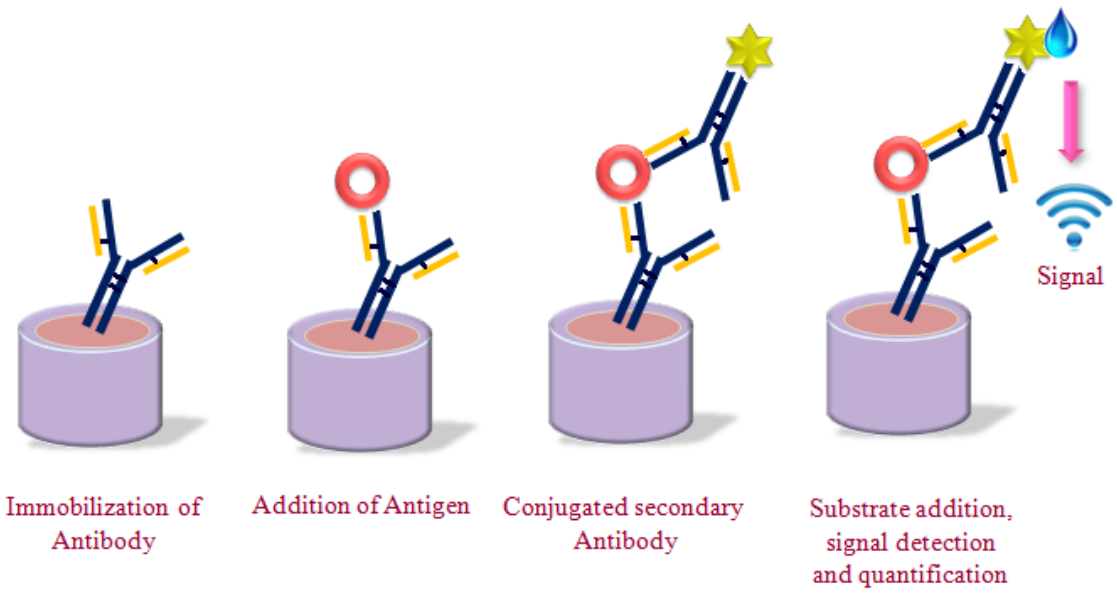


Figure 1.6. Schematic representation of a sandwich ELISA.

Despite the promise of immunoassay techniques, since these methods require long analysis time (1–2 h) and extensive sample handling (large number of washing steps), they are unsuitable for on-line monitoring of detoxification processes.

1.4 Biosensors

Over the past two decades, an explosive growth has been noticed in the development of biosensors for the detection of pesticides as a promising alternative to conventional methods. A biosensor is a self-contained device that integrates an immobilized biological element (e.g. enzyme, DNA probe, antibody) that recognizes the analyte (e.g. enzyme substrate, complementary DNA, antigen) and a transduction element used to convert the (bio)chemical signal resulting from the interaction of the analyte with the bioreceptor into an electronic one. According to the signal transduction technique, biosensors are classified into electrochemical, optical, piezoelectric and mechanical biosensors. Electrochemical transducers have been widely used in biosensors for pesticides detection due to their high sensitivity. Additionally, their low cost, simple design and small size, make them excellent candidates for the development of portable biosensors. Based on biorecognition element, enzymatic, whole cell, immunochemical and DNA biosensors have been developed for pesticides detection [81].

1.5 Components of Biosensor

1.5.1 The Transducer element

The transducer part of the sensor serves to transfer the signal from the output domain of the recognition system, mostly to the electrical domain. The transducer part of a sensor is also called a detector, sensor or electrode, but the term transducer is preferred to avoid confusion [82]. The sensitivity of the biosensor is greatly influenced by the transducer. The transducer of an electrical device responds in a way that a signal can be electronically amplified, stored and displayed. Suitable transducing system can be adapted in a sensor assembly depending on the nature of the biochemical interaction with the species of interest [83]. Transducers include change in pH, heat, conductivity, light, mass change and electroactive substances. Suitable transducing system can be adapted in

a sensor assembly depending on the nature of the biochemical interaction with the species of interest.

There are two types of transduction methods [84]:

- Chemical transduction including amperometry, potentiometry, conductometry and optical detection.
- Physical transduction including micro gravimetric detection and colorimetry.

1.5.2 The sensing element

The sensing element used in the construction of sensors can vary from entire sections of tissue to individual molecules such as enzymes. However, lack of selectivity and slow response time of tissues and living cells restricts their applicability in most of the cases which is overcome by use of enzymes which not surprisingly represent the most commonly used sensing agents [85]. The biological recognition element i.e., the enzyme of a biosensor has a specific binding affinity to the desired molecule, assuring the selectivity of the sensors.

Enzymes are biocatalysts which are characterized by two fundamental properties. First, they increase the rate of chemical reactions without themselves being consumed or permanently altered by the reaction. Second, they increase reaction rates without altering the chemical equilibrium between reactants and products. Generally, the pesticides are not substrates of the enzymes but act as specific inhibitors. Consequently, their detection is not based on the enzymatic catalysis of the pesticide but on the inhibiting capacity of a specific enzyme activity.

1.5.2.1 Advantages of using enzyme as sensor element

- Enzyme provides high specificity and selectivity towards the analyte.
- They are more biocompatible than most catalysts and have more potential for biological applications.
- It also shows short response time.
- When enzymes were immobilized on transducer surfaces, an improvement in enzyme stability was found.

- The driving force, the redox potential that is needed to achieve enzymatic biocatalysis, is often very close to that of the substrate of the enzyme. Therefore, biosensors can operate at moderate potentials.

1.5.2.2 Disadvantages of using enzyme as sensor element

- Limited numbers of substrates are available for which enzymes have been evolved, which in turn shows limited interaction between environmental pollutants and specific enzymes [86].
- Enzymes are very expensive. The cost of extraction; purification and isolation of enzymes are relatively high. Sometimes the cost of source of the enzyme is high [87].

1.6 Enzyme Immobilization matrix

Immobilization is defined as the physical confinement or localization of enzyme in a certain defined region of space with retention of their catalytic activities and which can be used repeatedly and continuously. The support or matrix on which the enzyme is immobilized, allows the exchange of medium containing substrate or effector or inhibitor molecules.

1.6.1 Advantages of immobilized enzyme

- Increases functional efficiency of the enzyme.
- Enhances the stability of the enzyme under both storage and operational conditions.
- Reuse of enzyme is possible.
- Remarkably reduces the cost of the enzyme and the enzymatic product.
- Reduces the chance of contamination.
- Easy separation of the enzyme from the reaction mixture.
- Helps in efficient recovery and reuse of expensive enzymes.

1.6.2 Disadvantages

- Immobilization requires additional time, equipment and materials so is more expensive to set up.
- Immobilized enzymes may be less active as they cannot mix freely with the substrate.
- Any contamination is costly to deal with because the whole system would need to be stopped.

Various immobilization supports have been developed in order to improve enzyme activity. The selection of support material can be a rather complex matter since an appropriate immobilization matrix should be biocompatible, nontoxic to biorecognition element, durable and should possess appropriate functional groups for immobilization along with low cost and mass production. In addition, the immobilization chemistry should not use any toxic or denaturing reagents that could affect enzyme activity and also after the immobilization procedure, the enzyme molecules should strongly attach onto the electrode surface, maintaining its native catalytic activity and random orientation [88,89]. Some of the most commonly used support matrices are discussed below:

1.6.3 Conducting polymers (CPs)

CPs with an extended π-electron system have properties such as electrical conductivity, low energy optical transitions, low ionization potential and high electron affinity etc [90]. This extended conjugated system of the conducting polymers have single and double bonds alternating along the polymer chain are an important class of material for biomolecule-based technology since they provide homogeneous and manageable film character, stability, biocompatibility, reproducibility and ease of production. The use of CPs in biosensor construction permits creative opportunities for the fabrication of biosensors. Especially, electrochemical methods for film formation lead to precise control of polymer formation on the surface of the transducer. Transducers fabricated via these methods provide extensive stability for the enzyme immobilization [88]. Mostly, CPs such as polyacetylene, polythiophene, polyaniline, polyindole and polypyrrole etc. are used as biosensor material [91].

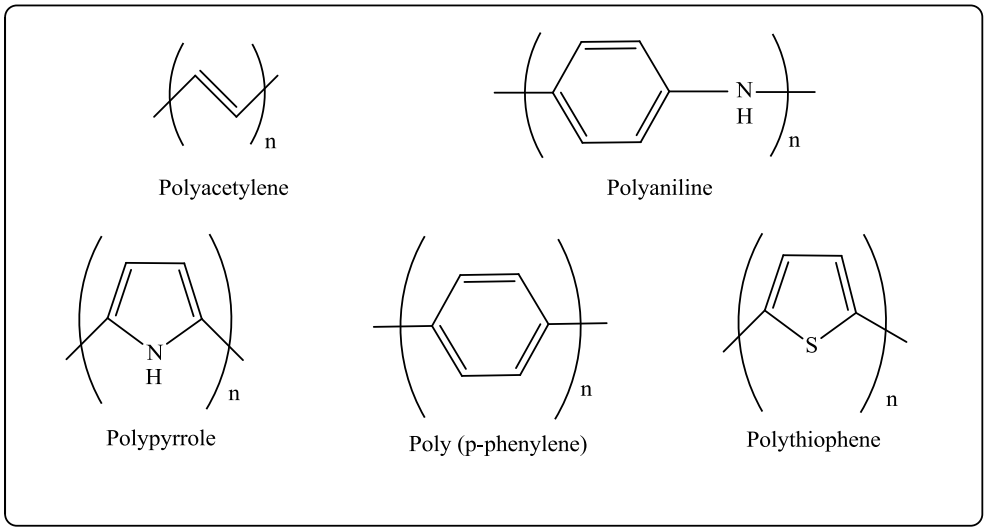


Figure 1.7. Structure of polymers used for biomolecule immobilization.

1.6.4 Silica

Another attractive method that ensures stability of the enzymatic layer is to entrap the enzyme in a silica material prepared by low temperature sol–gel processing. Silica sol–gel technology is a technique of synthesizing oxide glasses and ceramics starting from alkoxysilanes or aqueous silicate precursors such as sodium silicate or colloidal silica in a two-step process of hydrolysis and polycondensation. A variety of enzymes, have been immobilized in silica sol-gel glasses with retention of enzymatic activity. Many workers have demonstrated the possibility of combining sol-gel technology with screen-printing protocols for biosensor fabrication. Some others have indicated the potential of the sol-gel method as a versatile and efficient technique for conserving enzyme activity in organic solvents. Reports have been found indicating that immobilization in a sol-gel material requires lower enzyme loading as compared to covalent binding via glutaraldehyde to obtain comparable current values. This happens because of the absence of chemical bond formation, thus preserving the enzymatic activity during the immobilization process. However, there are several limitations of using silica gels for enzyme immobilization, such as brittleness and an aggressive chemical environment that can lead to enzyme denaturation and negatively impact the sensor performance. Therefore, alternative non-silica materials such as vanadium pentoxide, zirconium dioxide, alumina or titania with increased biocompatibility have more recently been investigated [92].

1.6.5 Nanoparticles

Nanoparticles act as very efficient support materials for enzyme immobilization, because of their ideal characteristics for balancing the key factors that determine efficiency of biocatalysts including high specific surface area, electrical conductivity, mass transfer resistance and effective enzyme loading. Enzyme immobilized on the nanomaterials can offer many advantages such as it increases the enzyme activity, stability and biosensor performance. Therefore, various nanomaterials have been coated or deposited on the electrode to fabricate enzyme biosensors which open a new avenue to amplify the signal of biosensors [93]. Moreover, the enzyme bound nanoparticles show Brownian movement when dispersed in aqueous solutions indicating that the enzymatic activities are comparatively better than that of the unbound enzyme [94].

Carbon-based nanomaterials, such as carbon nanotubes (CNTs) and graphene have attracted considerable interest among nanostructured materials for their unique mechanical, thermal and electrical properties as well as for their biocompatibility. These characteristics facilitated their use in electronic devices, fuel cells, as carriers for drug delivery, or as supports for biomacromolecules immobilization. Graphene is a single layer of carbon atoms in a honeycomb like two-dimensional lattice which has a high specific surface area and can be fabricated from graphite. CNTs are one-dimensional nanomaterials which can be considered as concentric rolled graphene sheets with a diameter up to 100 nm and length up to a few micrometers; in addition to multi-walled CNTs, single-wall nanotubes can also be prepared. The difference in graphene and CNTs curvature results in different properties, such as the higher water dispersability of graphene oxide derivatives compared to the corresponding functionalized CNTs [95].

1.7 Different methods of immobilization of enzyme

A critical step in development of biosensor is effective enzyme immobilization while maintaining free diffusion of substrates and products into and out of the enzyme layer. Enzyme immobilization appears as a key factor to develop efficient biosensors with appropriate performances such as good operational and storage stability, high sensitivity, high selectivity, short response time and high reproducibility. Immobilized biomolecules have to maintain their structure, their function, to retain their biological activity after

immobilization, to remain tightly bound to the surface and not to be desorbed during the use of the biosensor. Moreover, an ideal biosensor has to be stable for long-term application. The type of immobilization method affects activity and stability of enzymatic biosensors. Factors such as accuracy of measurements, the sensor-to-sensor reproducibility and operational lifetimes are drastically influenced by enzyme stability. Since the analytical performances of a biosensor are strongly affected by the immobilization process, intensive efforts have been made to develop successful immobilization strategies in order to assure greater sensitivity and stability of biosensors [96].

Table 1.1. Advantages and drawbacks of the five basic immobilization methods:

Method	Binding nature	Advantage	Disadvantage
Adsorption	Weak bonds e.g. by van der Waals forces, ionic binding or hydrophobic forces.	Simple, mild conditions, less disruptive to enzyme protein.	Enzyme linkages are highly dependent on pH, solvent and temperature; desorption may occur.
Covalent bonding	Chemical binding between functional groups of the enzyme and those on the support.	Stable enzyme-support complex, leakage of the biomolecule is very unlikely, short response time.	Complicated and time-consuming: possibility of activity losses is due to the reaction involving groups essential for the biological activity, matrix not regenerable.
Entrapment	Within a gel or polymer.	Mild procedure, no chemical reaction between the monomer and the enzyme that	Large diffusional barriers, loss of enzyme activity by leakage, possible denaturation of the enzyme molecules as a

		could affect the activity; Several types of enzymes can be immobilized within the same polymer.	result of free radicals, High concentrations of monomer and enzyme needed for electropolymerization.
Crosslinking	Bond between enzyme/cross-linker (e.g. glutaraldehyde)/inert molecule (e.g. BSA).	Simple procedure, strong chemical binding of the biomolecules; widely used in stabilizing physically adsorbed enzymes or proteins that are covalently bound onto a support.	Difficult to control the reaction, requires a large amount of enzyme, the protein layer has a gelatinous nature (lack of rigidity), relatively low enzyme activity [97,98].
Encapsulation	Wrapping of the biological components inside different forms of semi permeable membranes.	Best means of avoiding any negative influence on the structure of the enzyme, co immobilization is possible.	Limited space is available for free movement of the enzyme, biocatalysis of large substrates are limited as they are susceptible to mass transfer limitations [99-101].

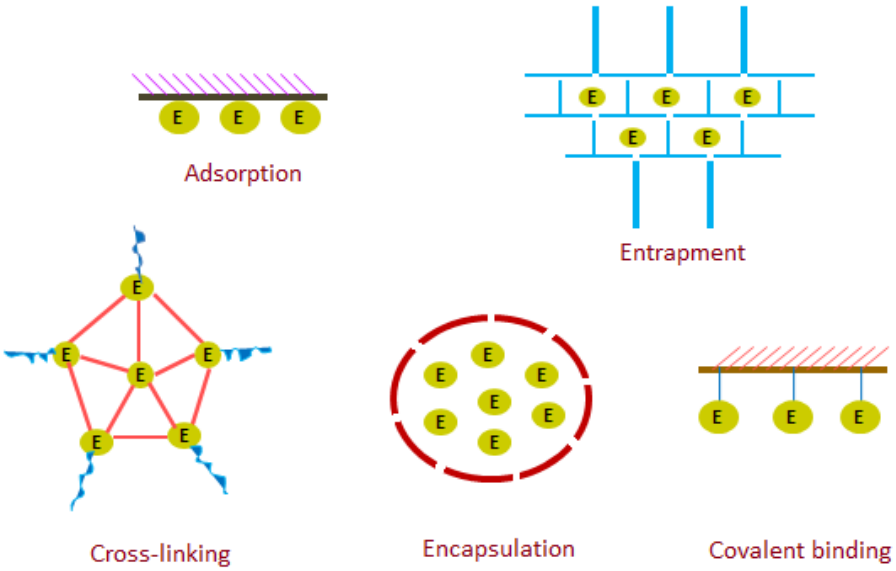


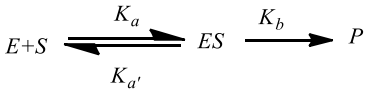
Figure 1.8. Schematic representation of five different immobilization techniques.

1.8 Enzyme kinetics

Experimental studies of enzyme kinetics are typically performed by monitoring the initial rate of product formation in a solution where enzyme is present at very low concentration. The principal features of many enzyme-catalyzed reactions are as follows:

- For a given initial concentration of substrate, $[S]_{0,}$ the initial rate of product formation is proportional to the total concentration of the enzyme $[E]_{0.}$
- For a given $[E]_0$ and low values of $[S]_{0,}$ the rate of product formation is proportional to $[S]_0$.
- For a given $[E]_0$ and high values of $[S]_0$, the rate of product formation becomes independent of $[S]_0$, reaching a maximum value known as maximum velocity, $V_{max.}$

Michaelis-Menten mechanism is in charge for these characteristics. According to this mechanism, an enzyme-substrate complex is formed in the initial step and either the substrate is released unchanged to form product.



Scheme 1.1. Michaelis-Menten mechanism.

The above mechanism leads to the Michaelis-Menten equation for the product formation rate as follows:

$$V = \frac{k_b[E]_0}{1 + K_m/[S]_0} \quad (1.1)$$

$$K_m = (K_{a'} + K_b)/K_a$$

Where, $K_m = (K_{a'} + K_b)/K_a$ is the Michaelis constant, characteristic of a given enzyme working on a given substrate. Michaelis constant is a reflection of how well a substrate complexes with a given enzyme, otherwise known as its binding affinity. An equation with a low K_m value indicates a strong binding affinity, as the reaction will approach V_{max} more rapidly. An equation with a high K_m indicates that the enzyme does not bind as efficiently with the substrate and V_{max} will only be reached if the substrate concentration is high enough to saturate the enzyme. An enzyme that catalyzes a reaction between two or more different substrates has different K_m value for each of the substrate. Further simplification of equation 1.1 can be made. When all the enzyme has reacted with substrate at high concentration, $[S]_0 \gg K_m$ the reaction will go at maximum rate and independent of $[S]_0$,

$$V = V_{max} = k_b[E]_0 \quad (1.2)$$

We can rearrange equation 1.1 using 1.2 in to a form that is responsive to data analysis by linear regression:

$$\frac{1}{V} = \frac{1}{V_{max}} + \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]_0} \quad (1.3)$$

A Lineweaver-Burk plot (double reciprocal plot) is a plot of $\frac{1}{V}$ against $\frac{1}{[S]_0}$ and it should yield a straight line with slope of $\frac{K_m}{V_{max}}$, a y-intercept at $\frac{1}{V_{max}}$ and x intercept at $-\frac{1}{K_m}$.

Thus this plot provides a useful graphical method for calculation of enzyme kinetics parameters.

1.8.1 The catalytic efficiency of enzymes

The turnover frequency or catalytic constant, $k_{cat,}$ is the number of catalytic cycle (turnovers) performed by the active site of an enzyme in a given interval divided by the duration of the interval. This quantity has units of a first order rate constant and in terms of the Michaelis-Menten mechanism, is numerically equivalent to k_b, the rate constant for release of product from the enzyme-substrate complex:

$$k_{cat} = k_b = \frac{V_{max}}{[E]_0} \qquad (1.4)$$

The catalytic efficiency, ε (epsilon), of an enzyme is the ratio of k_{cat}/K_m. The higher the value of ε, the more efficient is the enzyme in converting the substrate into the product [102]. Unit for catalytic efficiency is $M^{-1}sec^{-1}$.

$$\varepsilon = \frac{k_{cat}}{K_m} \qquad (1.5)$$

1.9 Types of Enzyme Inhibition

Enzyme inhibition is a science of enzyme-substrate reaction influenced by the presence of an inhibitor due to their covalent or non-covalent interaction with the enzyme's active site. Different types of inhibition can be observed based on mode of interaction with the enzyme's active site. Presence of an inhibitor can cause a reduction in the rate of an enzyme catalyzed reaction. This loss of activity caused by an inhibitor can occur either reversibly or irreversibly. If it is reversible, activity may be restored by the removal of the inhibitor, while if it's irreversible the loss of activity is time dependent and cannot be recovered during the timescale of interest during the reaction. There occurs different types of inhibition and for each mode of inhibition; it is possible to calculate a dissociation constant, K_i, for the inhibitor that reflects the potential interaction between the enzyme and the inhibitor. It can be said that K_i for an inhibitor is analogous to K_m for a substrate; a small K_i value exhibits tight binding of an inhibitor to an enzyme, whereas a larger K_i value shows weaker binding.

1.9.1 Competitive Inhibitors

This type of inhibitor refers to a compound that bears a close structural and chemical similarity to the substrate of the enzyme, and this similarity causes the inhibitor to bind reversibly to the enzyme's active site and occupies it in a mutually exclusive manner with the substrate. Thus, the competitive inhibitor competes with the substrate for enzyme active site. Since the substrate and inhibitor are not identical, therefore, the enzyme will not be able to convert the inhibitor into product and it will simply block the active site of the enzyme as illustrated in Figure 1.9. Also, if the substrate binds to the active site before the inhibitor, the inhibitor is incapable of binding to the enzyme.

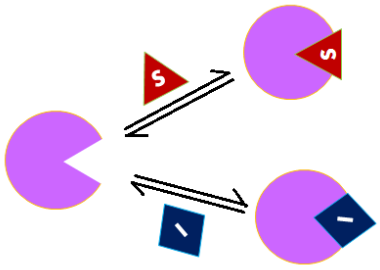


Figure 1.9. Schematic representation of a competitive inhibitor blocking the active site of an enzyme for its substrate.

Since both the substrate and inhibitor compete for binding to the active site of the enzyme, the inhibition is most noticeable at low substrate concentration but can be overcome at sufficiently high substrate concentration as the value of V_{max} remains unaffected. The rate equation can then be expressed as:

$$V = \frac{V_{max}\ [S]}{K_m^{app} + [S]} \qquad (1.6)$$

Where, K_m^{app} represents the apparent K_m for the reaction, which can be expressed as:

$$K_m^{app} = K_m\ [1 + \frac{[I]}{K_{ia}}] \qquad (1.7)$$

Here, K_{ia} is the enzyme-inhibitor binding constant. It should be noted that the competitive inhibitor bears some structural similarity to the substrate and is often a reaction product. In this case there is product inhibition that can cause a substantial loss

of productivity when high degrees of conversion are required. Using equations (1.6) and (1.7) the rate equation for product inhibition can then be derived as:

$$V = \frac{V_{max}[S]}{K_m\left[1+\frac{[I]}{K_{ia}}\right]+[S]} \qquad (1.8)$$

1.9.2 Non-competitive Inhibitors

A non-competitive inhibitor is able to bind at a location other than the active site of an enzyme and partially block the active site or alter the shape of the enzyme completely. In some cases the inhibitor may bind so firmly to the enzyme that it becomes difficult to remove the inhibitor and in this case it permanently disrupt the functioning of the enzyme molecule and acts as a poison. While in some other cases, it is observed that the inhibitor attaches to the enzyme for a brief period only and temporarily blocks the enzyme's activity which can be restored once the inhibitor gets detached from the enzyme. The V'_{max} for an enzyme in the presence of a non-competitive inhibitor will be less than the one observed under uninhibited conditions V_{max}. The magnitude of this decrease will reflect the strength of the interaction between the enzyme and the inhibitor. However, there will be no change in the K_m. It should be noted that this inhibition is most commonly encountered in multi-substrate reactions where the inhibitor is competitive with respect to one substrate but uncompetitive with respect to another [103].

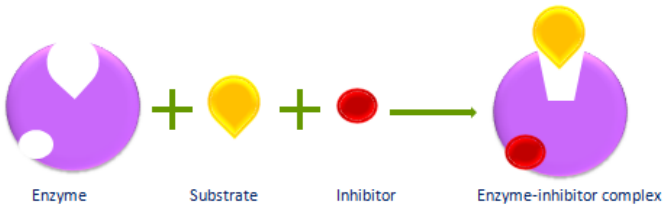
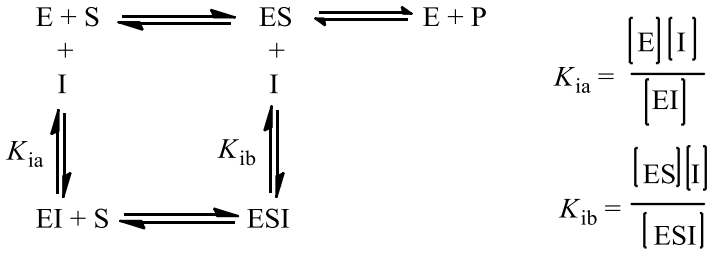


Figure 1.10. Schematic representation of non-competitive inhibition.

1.9.3 Mixed type of inhibition

The mixed type of inhibitors does not have structural similarity to the substrate but it binds both the free enzyme and the enzyme-substrate complex. It is not possible to calculate a single K_i value for this type of inhibition as dissociation constant for binding the free enzyme may differ from the dissociation constant for binding the enzyme-

substrate complex. Mixed inhibition may result in either a decrease in the apparent affinity of the enzyme for the substrate (K_m value appears to increase; $K_m^{app} > K_m$) or an increase in the apparent affinity of the enzyme for the substrate (K_m value appears to decrease; $K_m^{app} < K_m$).



Scheme 1.2. Mechanism for mixed type of inhibition.

The result of the inhibitor binding is a slight alteration to the Michaelis-Menten equation:

$$v = \frac{V_{max}\ [S]}{K_m\left(1+\frac{[I]}{K_{ib}}\right)+ [S]\left(1+\frac{[I]}{K_{ib}}\right)} \qquad (1.9)$$

Here, K_{ib} is the enzyme-substrate binding constant.

1.9.4 Uncompetitive Inhibition

This special case of inhibitor kinetics is occasionally observed, primarily with multi-substrate enzymes. This type of inhibition which is really another special case for mixed inhibition occurs when the inhibitor only binds to the ES complex. The altered Michaelis-Menten equation for this kind of inhibition is:

$$V = \frac{V_{max}\ [S]}{K_m\ +[S]\left(1+\frac{1}{K_{ib}}\right)} \qquad (1.10)$$

Table 1.2. The values of K_m^{app} and V_{max}^{app} for different types of inhibition are summarized here [104]:

Type of inhibition	K_m^{app}	V_{max}^{app}
None	K_m	V_{max}
Competitive	$K_m\left(1+\frac{[I]}{K_{ia}}\right)$	V_{max}
Non-competitive	K_m	$\frac{V_{max}}{\left(1+\frac{[I]}{K_{ib}}\right)}$
Mixed	$K_m\frac{\left(1+\frac{[I]}{K_{ia}}\right)}{\left(1+\frac{[I]}{K_{ib}}\right)}$	$\frac{V_{max}}{\left(1+\frac{[I]}{K_{ib}}\right)}$
Uncompetitive	$\frac{K_m}{\left(1+\frac{[I]}{K_{ib}}\right)}$	$\frac{V_{max}}{\left(1+\frac{[I]}{K_{ib}}\right)}$

1.10 Electrochemical biosensor

Electrochemical devices have traditionally received the major share of the attention in biosensor development. An electrochemical biosensor is a biosensor with an electrochemical transducer. It is considered a chemically modified electrode since electronic conducting, semi-conducting or ionic conducting material is coated with a biochemical film [105]. It is well known that the response of a biosensor to the addition of a substrate is determined by the concentration of the product (P) of the enzymatic reaction on the surface of the sensor [106].

Electrochemical biosensors are based on the electrochemical species consumed and/or generated during a biological and chemical interaction process of a biological active substance or substrates. In such a process, an electrochemical detector measures the electrochemical signal produced by the interaction. Thus, the differences among the three types of electrochemical biosensors (conductometric, potentiometric and amperometric detectors) include their technique of measuring biochemical changes in solution [107].

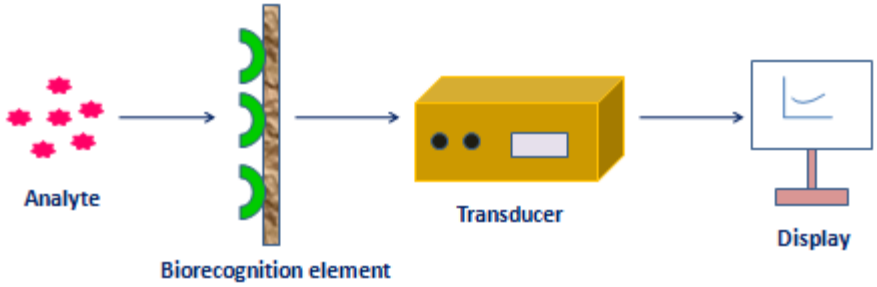


Figure 1.11. Basic principle of electrochemical biosensor.

1.10.1 Amperometry

Amperometry is characterized by measuring the current resulting from the electrochemical oxidation or reduction of an electroactive species. It is usually conducted by maintaining a constant potential at a Pt, Au or C based working electrode or an array of electrodes with respect to a reference electrode which may also act as auxiliary electrode, if the current is low (10^{-9} to 10^{-6} A). Advantage of using three electrode system is that the charge from electrolysis passes through the auxiliary electrode instead of the reference electrode which protects the reference electrode from changing its half-cell potential. In amperometry, changes in current generated by the electrochemical redox reactions are monitored directly with respect to time. Amperometric detection is commonly employed in biocatalytic and affinity sensors because of its simplicity and low limit of detection [108]. Immunosensors based on amperometry which is held at a fixed electrical potential can detect the concentration dependent current, generated when an electroactive species is either oxidized or reduced at the electrode surface to which Ab-Ag binds specifically. The current in this case is directly proportional to specific Ab-Ag binding and it is along with bulk concentration of the detecting species can be approximated as:

$$I = Z\ F\ k_m\ C^* \qquad (1.11)$$

Here, I is the current to be calculated, Z and F are constants, k_m is the mass transfer coefficient and C * is the bulk concentration of the detecting species [109].

Amperometric techniques are characterized by linear dependency on analyte concentration which gives a normal range in the measurement of current. Almost all microorganisms can now be sensed amperometrically by their enzyme-catalyzed electrooxidation or electroreduction or their involvement in a bioaffinity reaction. Amperometric biosensors have the advantage of being highly sensitive, rapid and inexpensive.

1.10.2 Potentiometry

Potentiometric measurements involve the determination of potential of an electrochemical cell while drawing negligible current. The most common examples of potentiometric devices are pH electrodes; ion selective electrodes for ions such as F^- , I^- , CN^- , Na^+ , K^+ , Ca^+ , NH_4 or gas- (CO_2, NH_3) selective electrodes [109]. Potentiometric biosensors are designed by coating them with a biological element such as an enzyme that catalyzes a reaction forming the ion that the underlying electrode is assigned to sense. Potentiometric immunoelectrodes are based on the electrocatalytic properties of redox enzymes. Attachment of the electrocatalytic active enzymes to the electrode surface in the presence of substrate initiates an electro-catalytic reaction generating a potential shift [110]. Potentiometric sensors manifest suitability for measuring low concentrations in tiny sample volumes since they ideally offer the benefit of not chemically influencing a sample [105]. Thus, these sensors are an ideal choice where speed, simplicity and accuracy are essential.

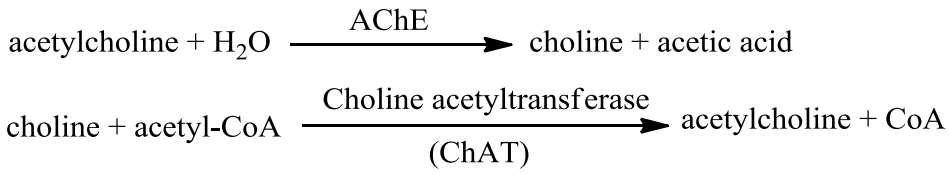
1.10.3 Conductometry

The principle of the conductometric detection is based on monitoring the changes in the electrical conductivity of the sample solution, or a medium such as nanowires, as the composition of the solution/medium changes in the course of the chemical reaction [108]. Conductance measurements involve the resistance (reciprocal conductance) determination of a sample solution between two parallel electrodes. Conductometric biosensors usually include enzymes whose charged products result in ionic strength changes and thereby increased conductivity. Conductometry has been often used as the detection mode in biosensors for environmental monitoring and clinical analysis. Many enzyme reactions, such as that of urea and many biological membrane receptors may be

recorded by ion conductometric or impedimetric devices, using interdigited microelectrode. In immunosensors, there is an overall electrical conductivity of the solution and capacity alteration due to the Ab-Ag interaction at the electrode surface [109].

1.11 Acetylcholinesterase based biosensor

Acetylcholinesterase (AChE) belongs to the family of carboxylesterase (EC 3.1.1.7.). It is serine protease and stabilizes level of acetylcholine (ACh), a neurotransmitter, by catalyzing the conversion of acetylcholine to choline and acetic acid [111]. AChE is mainly found at neuromuscular junctions and cholinergic brain synapses in the central nervous system, where its activity/ concentration terminates synaptic transmission when the enzyme is present in the active form. AChE is highly efficient catalyst with a very high catalytic activity; each molecule of AChE degrades approximately 25,000 molecules of ACh per second into choline and acetic acid. The produced choline is transported back into the nerve terminals in order to reuse it in synthesizing new ACh molecules.



Scheme 1.3. Cholinergic transformation reaction that occurs at nerve junction.

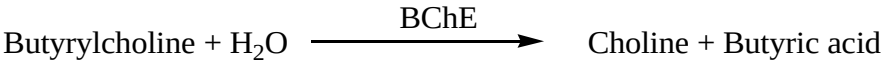
AChE belongs to the family of hydrolases whose active site is specified by a catalytic coordinated triad of three essential amino acids: histidine, serine and aspartic acid. The enzyme catalysis occurs when the triad's anionic binding site attracts the positively charged quaternary ammonium group of ACh. The serine hydroxyl group attacks and breaks down the ester after its deprotonation by a neighboring histidine group in the triad [112]. However, in the presence of an inhibitor such as an organophosphate, the nucleophilic serine hydroxyl group located at the active site is covalently bound to the phosphorus atom of the organophosphate. A similar reaction occurs with the carbonyl carbon of carbamates and this blocking of the triad serine inactivates the enzyme. The detection methods of organophosphate and carbamate pesticides are mostly based on the

principle of inhibition of cholinesterases by pesticides [113]. AChE has two active subsites, anionic and esteratic subsite. ACh mediates messages between the nerves which is responsible for muscle contraction. When ACh is released from the nerve into the synaptic cleft, it got recognized by ACh receptors present on the postsynaptic membrane which further transmits signal. Along with the ACh receptors, AChE is also present on the postsynaptic membrane which helps in the termination of the signal transmission by hydrolysing ACh. On hydrolysis, ACh split into choline and acetic-acid which are recycled by the body to again form acetylcholine to maintain the reserves of neurotransmitters so that they can be used by the body again during the time of need [114].

AChE biosensors work on inhibitory effects. When the analyte is not present in the solution, the substrate acetylthiocholine is converted into thiocholine and acetic acid. Thiocholine is oxidized by the applied voltage. In the presence of a suitable inhibitor, conversion of acetylthiocholine is decreased or even null. Basic principle of AChE biosensors is widely used for fast analysis of toxic compounds. The enzyme AChE is a biorecognition element sensitive to inhibition by organophosphates as well as carbamate pesticides, nerve agents, several natural toxins and some drugs. Hence, AChE is widely used as a potent recognition element for the construction of biosensors for pesticide detection. In the presence of inhibitor which forms covalent bond with serine present on the active site of AChE, leads to inactivation of the enzyme which in turn does not allow the muscles involved to relax, leading to paralytic conditions. The intensity of inhibition of AChE is proportional to the concentration of inhibitor compound and is also exploited as principle of detection method for concentration of OP compounds [115]. Furthermore, the anodic oxidation current is inversely proportional to the concentration of pesticides in samples and the exposed time as well. Inhibition can occur both reversibly and irreversibly. Irreversible inhibition (i.e. nerve agents, OP) is characterized by covalent bonding between the AChE enzyme and the inhibitor and thus requires either a new biosensor after the inhibitor measurement or a reactivation of the biosensor in use. Reversible inhibition (carbamates), on the other hand, is characterized by noncovalent interaction between inhibitor and AChE enzyme with the consequent restoration of the initial activity after the inhibitor measurement [116].

Biosensors based on AChE as well as butyrylcholinesterase were first reported during the 1980s [117]. Since then, there has been a continuous improvement of cholinesterase-based biosensors due to the gradual improvement of transducer devices and the availability of pure enzymes.

Another enzyme that is related to AChE is butyrylcholinesterase (BChE). BChE (EC 3.1.1.8) is present in much lower concentrations than AChE and is usually thought to have a much more restricted neuronal distribution in the CNS. The number of BChE-rich neurons in the human cerebral cortex is approximately two orders of magnitude less that the number of AChE-rich neurons [118]. BChE catalyzes the hydrolysis of butyrylcholine (BCh) to yield butyric acid in addition to choline [119]. Artificial substrate butyrylthiocholine for BChE has been also used.



Scheme 1.4. Hydrolysis of BCh to form choline and butyric acid in presence of BChE.

The pH variation produced by the acid formation can be measured using electrochemical methods, such as potentiometry or using pH-sensitive spectrophotometric indicators or pH sensitive fluorescence indicators.

1.12 Organophosphorus Hydrolase (OPH) based biosensor

OPH is an organophosphotriester hydrolyzing enzyme; the enzyme has broad substrate specificity and is able to hydrolyze a number of organophosphorus pesticides such as paraoxon [120], parathion, coumaphos, diazinon, dursban etc. This enzyme hydrolyzes P-O, P-S and P-CN bonds via an S_N2-type mechanism [121] generating two protons, able to be electrochemically detected and an alcohol which in many cases is chromophoric and/or electroactive. The resulting hydrogen ion can be followed by potentiometry. Organophosphorus hydrolase can be integrated with an amperometric transducer to monitor the oxidation or reduction current of the hydrolysis products (equation). However, these biosensors show lower sensitivity values and higher detection limits than cholinesterase-based biosensors. Moreover, they can only detect some organophosphorus (OP) compounds [81].

The basic reaction catalyzed by OPH is as follows:

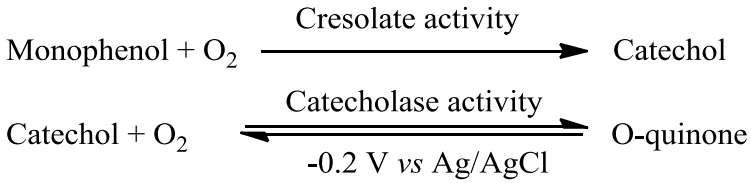
$$\text{aryldialkyl phosphate} + H_2O \xrightarrow{\text{OPH}} \text{dialkylphosphate} + \text{aryl alcohol}$$

Scheme 1.5. Working principle of organophosphorus hydrolase.

The enzyme was first discovered in soil microorganisms Pseudomonas diminuta and Flavobacterium spp. The use of OPH is extremely attractive for biosensing of OP compounds that act as substrates for the enzyme. The hydrolysis products can be monitored spectrophotometrically or electrochemically and thus the analyte can be directly determined as the rate of product formation is directly proportional to the concentration of the analyte. This method is the opposite of inhibition-based enzymatic detection as carried out with AChE and ALP. As such, it therefore has certain advantages over inhibition-based methods [122]. OPH methods do not require a continuous supply of consumable enzyme substrate as the analyte itself is the substrate. Unlike the AChE-based system which is inhibited by both organophosphate and carbamate pesticides and involves multiple step indirect measurement, the OPH-based system is simple, direct, and quick, measuring only the organophosphorus class of nerve agents and they are ideal for on-line monitoring and control of detoxification processes [123]. However, the commercial application of OPH-biosensor has been impeded by several factors, including high cost on enzyme production, purification and processing [124].

1.13 Tyrosinase based biosensor

Tyrosinase is a type of metalloprotein and contains a dinuclear copper centre. It has two binding sites, the substrate binding site which has an affinity for aromatic compounds and the oxygen site which has an affinity for coordinating agents that bind to the metal. Tyrosinase oxidizes monophenols in two consecutive steps: first, the enzyme catalyzes the o-hydroxylation of monophenol to corresponding o-diphenol (cresolate activity) which, in the second step, is oxidized to its corresponding o-quinone, whereby the enzyme is oxidized by molecular oxygen back to its native form (catecholase activity): [109]



Scheme 1.6. Working principle of tyrosinase.

The binuclear copper-active site of tyrosinase can exhibit various states in which the two copper atoms can be reduced or oxidized when substrate or water is bound to them. Three states of enzyme are expected to be involved according to the mechanism proposed to the oxidation of monophenols and o-diphenols into their corresponding o-quinones, Tyr_{oxy}, Tyr_{deoxy} and Tyr_{met}.

Tyrosinase is inhibited by many different compounds such as carbamate and dithiocarbamate pesticides, atrazines, chlorophenols and thioureas and this characteristic has been used to develop biosensors for the enzymatic detection of many pesticides. In the enzymatic phenol sensors, tyrosinase is generally combined with an electrochemical transducer to sense the oxygen consumption of the overall enzyme reaction or the electroactive quinones produced in the enzymatic reaction. The latter provides advantages as the quinones are reduced at low potential and the substrate is electrochemically regenerated resulting in a considerable amplification of the signal, improving the sensitivity of an enzymatic assay. Regardless of these advantages, the electrochemical reduction of quinones remains incomplete as they are highly unstable in water and the intermediate radicals formed in both enzymatic and electrochemical reactions can easily polymerize to polyaromatic compounds that can inactivate the enzyme and foul the electrode surface [125].

Andreescu *et al.* [126] indicated that tyrosinase biosensors suffer from poor specificity since many substrates and inhibitors interfere with the enzyme's ability to detect the target compounds. The enzyme is further inherently unstable, reducing the lifetime and usability of the tyrosinase-based biosensors. However, tyrosinase detectors have some advantages over other types of enzymes. Tyrosinase can withstand high temperatures which ''facilitates sensor fabrication and substrate detection". Tyrosinase has a higher tolerance for organic solvents and biosensors have been constructed that operate with an

organic phase. A tyrosinase biosensor can also be operated faster than, for instance, an AChE sensor as it does not require pre-incubation [81,122].

1.14 Alkaline Phosphatase (ALP) based biosensor

Alkaline phosphatase (EC 3.1.3.1) catalyses the transfer of a phosphate group to water (hydrolysis) or alcohol (transphosphorylation). The basic reaction is as follows [81]:

$$\text{Phosphate monoester} + H_2O \xrightarrow{\text{ALP}} \text{alcohol} + \text{phosphate}$$

Scheme 1.7. Hydrolysis of phosphate group in presence of alkaline phosphatase.

This enzyme is characterized by a high pH (usually around pH 8-10) and broad substrate specificity. It uses a wide variety of phosphomonoesters. The enzyme is a dimeric metalloenzyme with two Zn ions and one Mg ion coordinated within the active site of each monomer. Enzyme activity can be reduced or inhibited by ethylenediamine tetra acetic acid (EDTA). ALP as sensing element has not been used as extensively as AChE for the detection of pesticides in the environment. In most cases, research was based on utilization of the enzyme within a biosensor and using different immobilization and transducer strategies. ALP is suitable for detection of organochlorine, organophosphate and carbamate pesticides although only a limited number of compounds were tested. No research has been performed to investigate the ability of this enzyme assay to detect pesticides in real samples and thus the effect of the environmental matrix is unknown. [122].

1.15 Acid Phosphatase (AP) based biosensor

Acid phosphatases are enzymes with a low pH that catalyze the reaction:

$$\text{Orthophosphoric monoester} + H_2O \xrightarrow{\text{AP}} \text{alcohol} + H_3PO_4$$

Scheme 1.8. Working principle of acid phosphatase.

This enzyme has been utilized to a limited extent for the detection of pesticides through the inhibition of the enzyme. Biocatalytic hydrolysis of glucose-6-phosphate in the presence of acid phosphatase is reversibly inhibited by organophosphorus and carbamate pesticides. Thus, amperometric detection of this inhibition requires a bienzymatic system with glucose oxidase (GOD) which utilizes the following reactions and final measurement of hydrogen peroxide:

$$\text{Glucose 6-phosphate} + H_2O \xrightarrow{\text{AP}} \text{glucose} + \text{inorganic phosphate}$$

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconolactone} + H_2O_2$$

Scheme 1.9. Catalytic reactions carried out by acid phosphatase and glucose oxidase.

The AP-GOD biosensor was tested with the organophosphate pesticides malathion, methyl parathion and paraoxon [109,122].

1.16 Glutathione S-transferase based biosensor

Glutathione S-transferases (GSTs) constitute a super family of ubiquitous, multifunctional enzymes which play a key role in cellular detoxification, protecting macromolecules from attack by reactive electrophiles. Based on sequence homology and immunological crossreactivity, human cytosolic GSTs have been grouped into seven families, designated GST Alpha, Mu, Pi, Sigma, Omega, Theta and Zeta [127]. The primary function of the enzyme, particularly in higher organisms, is generally considered to be the detoxication of both endogenous and xenobiotic alkylating agents such as epoxides, unsaturated aldehydes and ketones, alkyl and aryl halides and others [106].

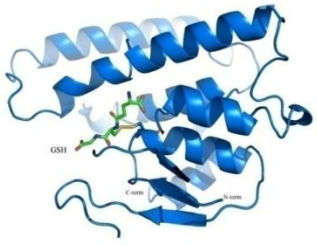


Figure 1.12. 3-D image of bacterial glutathione S-transferase enzyme.

Mode of action in a GST-catalyzed conjugation reaction, one molecule of reduced GSH and one molecule of a second substrate are combined to form a thioester. The reaction proceeds via substrate binding, the activation of the thiol group of GSH and subsequent nucleophilic attack by the anionic GSH on the bound hydrophobic compound. This conjugation neutralizes the electrophilic sites of the lipophilic substrate and protects the cellular components, especially the nucleophilic oxygen and nitrogen of DNA from electrophilic attack of nucleophiles. Conjugation also renders the product more water soluble and therefore more readily excretable from the cell. GSTs have a high affinity towards GSH and because this tripeptide is present at high intracellular concentrations the GSH binding site of GST may always be occupied. The 'active site residue' in the N-terminal domain interacts with and activates the sulphydryl group of glutathione [128]. This conjugation reaction functions as a detoxification mechanism of endogenous compounds and exogenous compounds such as xenobiotics (X) as it is represented in the following equation [129]:

$$GSH + X \xrightarrow{GST} GSX$$

Scheme 1.10. Conjugation reaction of GSH with xenobiotics catalyzed by GST.

Different substrates are in use for *in vitro* study of the GST catalyzed conjugation with GSH, the most commonly used one among them is 1-chloro-2,4-dinitrobenzene (CDNB) [130]. Conjugation of GSH with CDNB produces a yellow colored complex that absorbs at 335 nm [131].

1.16.1 Key features of GST enzyme

- GST helps in maintaining the intracellular redox homeostasis by GSH mediated detoxification of free radicals, acting to safeguard the cells.
- Since GST acts as the catalyst of any reaction in which glutathione (GSH) acts as a nucleophile, this implies that any compound bearing a sufficiently electrophilic atom, such as many organochlorine compounds, may be subjected to such attack [132]. Thus, GST can be used in ecological risk assessment of OC contaminated environment.

- Members of the GST family (E.C. 2.5.1.18) are important candidates as they may regulate an individual's ability to metabolize environmental carcinogens for commonly occurring cancers [133]. Growing evidence suggests that GST enzymes determine cytotoxicity of a variety of chemotherapeutic drugs [134].

- Since GST can provide hydrophobic environment, therefore, it is possible for hydrophobic compounds (most of the pesticides) to bind with the enzyme which in turn will help in assessment of toxic hydrophobic metabolites.

Thus, GSTs are of interest in recent time to pharmacologists and toxicologists which are proposing new ventures for development of GST based biosensors.

1.16.2 Literature review on biosensors based on GST

Andreou *et al.* [135] has developed a fiber-optic biosensor based on immobilized glutathione S-transferase for the determination of atrazine which is a chlorinated triazine herbicide used to control certain weeds in corn, sugarcane, soybean, barley, grape and citrus fruit plantations and as a general weed control agent on industrial and non-agricultural land. The sensing material comprised multiple layers, including sol–gel-immobilized bromocresol green and a poly (vinylidene fluoride) membrane coated with glutathione S-transferase. Glutathione S-transferase catalyzed the nucleophilic attack of glutathione on atrazine, liberating HCl and resulting in a localized pH change. Detection was performed by monitoring the absorbance change of the bromocresol green due to the change in pH. The sensor displayed a linear range of 2.52–125 μM with a limit of detection of 0.84 μM. Choi *et al.* [136] developed optical biosensors to detect the captan which is based on the inhibition of glutathione S-transferase (GST) by captan. The amounts of captan were determined by measuring the absorbance change of products amounts, which caused by the inhibition of immobilized enzyme by inhibitors (captan). The sensing scheme is based on enzyme reaction that GST converts the substrates, 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH), into yellow products, S-(2,4-dinitrophenyl) glutathione. In the absence of inhibitors, the substrates are completely converted into yellow products, while in the presence of inhibitors, the amounts of yellow product are reduced. They related this absorbance change due to the different amounts of yellow products to the amounts of captan in the proposed sensor system. The

method was found to be useful for detecting captan at up to 2 ppm and sufficient enzyme activity was maintained for 30 days. An electrochemical biosensor based on GST enzyme was proposed by Oliveira *et al.* [129] which was constructed by immobilization of GST onto a glassy carbon electrode (GCE) via an aminosilane–glutaraldehyde covalent attachment. The principle of this enzymatic biosensor consisted on the GST inhibition process promoted by molinate, a pre-emergent thiocarbamate herbicide, in the presence of the substrates GSH and 1-chloro-2,4-dinitrobenzene (CDNB) which leads to a decrease in current peak measurement by differential pulse voltammetry (DPV).

So, the thesis work was aimed at development of a biosensor for different classes of pesticides using direct use of the enzyme GST and also through physical entrapment of GST in a suitable immobilization matrix, optimization of parameters for stability, longevity and real samples analysis, so as to contribute some efforts towards food safety and quality control.

1.17 Scopes, aim and objectives of the present investigation

It is known from the available literature that not much effort have been devoted in last two decades towards the development of better efficient GST biosensors for pesticide quantification. Therefore, the field is still an open area of research, to achieve further improvement of the GST based biosensors in terms of their stability (both operational and storage), reproducibility, reusability, specificity and selectivity. The ease and cost of fabrication also important factors those need attention from the perspective of applicability to routine analysis. Moreover, most of the pesticide biosensors developed till date are capable of operating in either phosphate buffer or in a solution of 5% acetonitrile in phosphate buffer, or in 10% ethanol in selected cases.

So, our primary goal in the present investigation is to develop an easy and cost effective method for GST biosensor fabrication with focus on achieving high stability, reproducibility and reusability. Also we aim to evolve some efficient mechanism for analyzing pesticide residue in organic extract using the developed biosensor.

1.17.1 Aim and Objectives

Aim: To develop a versatile biosensing protocol that can be applied to a wide varieties, if not all, of pesticide classes and that can work efficiently in fully or moderately concentrated organic solvents.

Under the above background the objectives of the present investigations are as follows:

- To select GST enzyme for development of new biosensing protocol.
- To study electrochemical behavoiur of GSH-CDNB reaction in different solvents like methanol, ethanol etc.
- To focus on the development of Cyclic Voltammetry (CV) and Chronoamperometry (CA) technique for biosensing of pesticides.
- Kinetics of inhibition of the said reaction by different classes of pesticides will be studied.
- To select a suitable matrix for GST immobilization.
- To perform preliminary electrochemical study of the feasibility of enzyme immobilization in the selected matrix.
- To optimize the conditions for stable immobilization, conditions for maximum signal output and reproducibility.
- To fabricate the sensor probe under the optimized conditions and application to test sample (pesticide) analysis.
- To work out suitable method or optimized conditions for pesticide analysis in real samples (organic extract) using the prepared biosensor.

1.18 Plan of work

- Selection of a suitable enzyme for biosensing of different classes of pesticides. Since GST is capable of detoxifying different classes of compound, so it may possible that GST can provide a biosensor with broad spectrum applicability.
- Study the effect of different organic solvent on the stability of the enzyme.
- Optimum parameter such as pH, enzyme amount, concentration of supporting electrolyte, saturated substrate concentration etc. will be studied for stability of the sensor as well as for reproducibility and maximum signal output.
- Inhibition characteristic of different pesticides towards the enzyme and hence towards the sensor response will be studied.
- The sensor will be applied to sample analysis in methanol solution.
- Validation of the new method will be checked through gas chromatography-mass spectrometry (GC-MS).
- Selection of a suitable martix for enzyme immobilization to enhance the stability and reusability of the enzyme.
- Characterization of the fabricated biosensor by using cyclic voltammetry, chronoamperometry, electrochemical impedance spectroscopy and scanning electron microscopy.

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