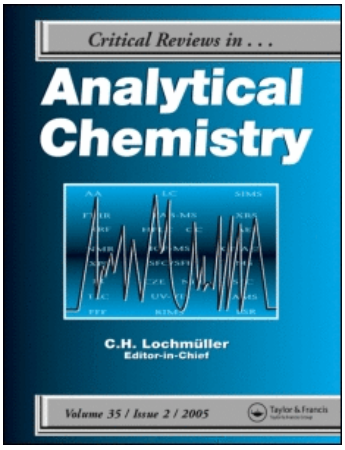

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Determination of Toxic Substances Based on Enzyme Inhibition. Part I. Electrochemical Biosensors for the Determination of Pesticides Using Batch Procedures

*Sílvia Solé, Arben Merkoçi, and Salvador Alegret**

Grup de Sensors and Biosensors, Departament de Química, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain

* Corresponding author; E-mail: salegret@gsb.uab.es

ABSTRACT: This article reviews the use of potentiometric and amperometric biosensors to determine pesticides using discrete analytical procedures. These measurements are based on the inhibition of an enzyme by the toxic substance under analysis. The present report reviews several such biosensors developed in the last decade and lists their analytical characteristics. The structure of the article is based on the enzyme immobilized in the respective biosensor. These enzymes are cholinesterase, tyrosinase, aldehyde dehydrogenase, acid phosphatase, acetolactate synthetase, and peroxidase.

KEY WORDS: pesticides, electrochemical detection, cholinesterase, tyrosinase, alkaline phosphatase, acid phosphatase, acetolactate synthetase.

I. INTRODUCTION

Most pesticides belong to the organophosphate and carbamate families. Their short lifetime in environment compared with that of organochlorate pesticides[1] (DDT, aldrin, lindane, etc.), combined with their great efficiency has fostered the abusive use of these agrochemicals. Similar chemicals have been synthesized for military use because they are very toxic to the nerve system.[2] These chemicals have been linked to bone marrow disease, carcinogenic processes, fertility disorders, cytogenic effects, neurological diseases, and respiratory and immunological dysfunction.[3]

The risk associated with these widely used chemicals calls for sensitive, fast, and reliable detection methods. Both gas and liquid chromatography have been described for this purpose. These techniques, coupled with mass spectrometry, are very sensitive methods that can discriminate among several pesticides belonging to the same family.[4] However, they are expensive, require laboratory facilities and specialized personnel, and show limitations when dealing with polar and thermolabile compounds because they require sophisticated extraction systems that are not amenable to field analytical procedures.[5]

Immunoassay techniques have been developed in the last 20 years to detect a number of pesticides, including fenitrothion, chlorpyrifos, and pirimiphos.[6] These methods use antibodies specific to the analyte under study. Their high specificity permits the determination of a single species. However, it is generally more desirable to have a method capable to determine the general toxicity of a given sample due to a family of products. In 1983 it was demonstrated that

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microorganisms might be used for the non-specific detection of pesticides.[7]

In any case, one of the methods that are more widely used as an alternative to the sophisticated equipment mentioned above is enzyme inhibition, particularly cholinesterases. Pesticides inhibit a large number of enzymes (cholinesterases, tyrosinases, peroxidases, glucose oxidases, etc.).[8] This approach furnishes a wide range of procedures for the quantification of pesticides. The choice of a given enzyme to detect its activity disruptor is based on the toxicity mechanism of the pesticide on living beings. Cholinesterases from insects would be the enzymes of choice for the detection of insectoacaricides such as organophosphate and carbamate pesticides.[9] On the other hand, dithiocarbamate fungicides act on living beings by inhibiting aldehyde dehydrogenases involved in amino acid biosynthesis especially valine, leucine, and isoleucine[10] while peroxidases and tyrosinases are a better option for the detection of herbicides that disrupt photosynthetic reactions.[11] The inhibition of these and other enzymes by several toxic substances has fostered novel analytical methodologies that are less expensive, simpler, and show lower detection limits when compared with the techniques mentioned above.

Biosensors bring the possibility of integrating the specific functional features of biological components with those of measurement instruments.[12,13,14] This is the case when enzymes are integrated to electrochemical transducers producing a biosensor device offering a selective recognition and a high sensitivity. Using this principle, several enzyme electrodes for the detection of inhibitors have been developed since the mid-1980s.[15] Later, devices for the detection of biocatalytic activators were also developed.[16] The amplifying nature of enzyme activity[17] together with the sensitivity of electrochemical transducers, especially amperometric devices, has spurred the development of biosensors sensitive to very low levels of toxic chemicals. The sensitivity of the device can be correlated to the toxicity of the substance under study.

Several procedures have been devised for the monitoring of the activity of an enzyme using electrochemical transducers. The assessment of this activity usually takes place by the direct measurement of electroactive products or co-substrates involved in the enzymatic reaction. It is possible to realize this monitoring indirectly also using synthetic mediators that favor the transfer of electrons between the electroactive species and the electrode. These procedures are used also in biosensors.

Enzyme immobilization on the transducers is an indispensable step in the development of biosensors. The simplest form of immobilization is to dissolve the enzyme in the buffer solution, depositing it on the electrode surface and covering it with a dialysis membrane.[18] Other immobilization techniques are based on the physical entrapment of the enzyme inside a synthetic gel layer (formed by the co-polymerization of acrylamide and bisacrylamide) or a chemical bond between the enzyme and a membrane or an organic or inorganic support or directly to the transducer (made of Pt, Au, C, etc.). The enzyme can be immobilized also by crosslinking or by co-crosslinking with an inert protein with gluteraldehyde and forming insoluble macromolecular aggregates.[19]

Evtugyn et al. (1998) published a review of the factors that determine the operational and analytical characteristics of biosensors based on enzyme inhibition both in solution and immobilized in a membrane.[20] Normally, immobilization offers a stable environment for an enzyme under extreme working conditions. This stability is lacking when the enzyme is free in the solution. Additionally, immobilization on a membrane allows that the biological material can be reused, lowering the cost of the analysis.

Enzymes in a living cell act in the midst of a highly organized structure. Inhibition operates differently when the enzymes are in their natural milieu or when they are immobilized or in a homogeneous phase. This fact, together with the type of inhibitor present, offers a wide range of pathways to the inhibition process.

These inhibitors may be grouped in two large sets: irreversible and reversible. In the case of irreversible inhibitors, the interaction takes the form of a covalent bond between the active center of the enzyme and the inhibitor. Reversible inhibition, on the other hand, is characterized by an equilibrium between the enzyme and the inhibitor defined by the equilibrium constant K_i. This constant is a measure of the affinity of the enzyme toward the inhibitor.

According to *Tran-Minh*,[15] the optimization of the electrochemical measurement resides essentially on the enzyme-inhibitor mechanism. The determination of inhibitors can follow one of two methodologies. In one, inhibition is produced by the simultaneous presence of the (reversible) inhibitor and the substrate (*kinetic measurement with mixed solutions*). In the other method, the enzyme is inhibited in the presence of the (irreversible) inhibitor without the substrate present (*steady-state measurement with separate solutions*).

Relative inhibition (RI) (s^{-1}) is measured in the kinetic measurement described below. In this case the estimated variable is the decreasing rate of the electrochemical signal (dI/dt) when pesticide is injected to the solution where the substrate is present. Relative inhibition is expressed as:

$$RI = \frac{dI / dt}{I_{ss}}$$

where I_{ss} is the initial analytical signal corresponding to the steady state when the substrate has been added and the inhibitor is absent.

In steady-state determination, the variable to measure is the inhibition percentage (I%). Two consecutive measurements of the enzyme activity in two separate experiments are needed. The first measurement estimates the intensity of the signal (I_{ss}) relative to the enzyme activity (the enzyme has not been in contact with the inhibitor) in the presence of the substrate. This is followed by the measurement of the intensity of the signal (I_i) when the same amount of substrate is present, but the enzyme has been exposed previously to the inhibitor (incubation) for a given time in a separated solution. This permits the calculation of the inhibition percentage as follows:

$$I\% = \frac{I_{ss} - I_i}{I_{ss}} x100$$

In addition to the presence of inhibitors, the response of the biosensor can be affected also by other interfering species that may be present in the sample. These interferents have an apparent effect on the sensitivity of the biosensor similar to the inhibitor determination.[15]

Despite a large number of advantages offered by the biosensors, it is important to bear in mind that the detection principle is based on inhibition, that is, on the loss of enzyme activity. This marks a limit to the number of measurements that can be made using a device containing a finite amount of immobilized material. That is why inhibition biosensors were used exclusively at first to measure reversible inhibitors.[20] The initial response of the biosensor could be recovered with several washing cycles with a buffer solution. Special reactivators for irreversible inhibitors were discovered later. This reactivation is specific for each inhibitor and each enzyme. Clinical antidotes such as 2-pyridine aldoxime methiodide (2-PAM) or 1,1'-trimethylenebis-4-(hydroxyiminomethyl)-pyridinium bromide (TMB-4) were used in

some instances for the reactivation of cholinesterases. Reactivation is a manoeuvre that lengthens the life of the biosensor but complicates the measurement protocol.

The present work intends to review the developments in the field of pesticide biosensors based on enzyme inhibition and electrochemical transducers applied as discrete or batch analytical procedures. The article covers the last decade where studies on different enzymes (cholinesterases, tyrosinases, phosphatases, etc.) immobilized on electrochemical transducers (potenciometric, amperometric, etc.) have produced various analytical advances.

II. CHOLINESTERASE INHIBITION

The hydrolysis of the choline esters is catalyzed by cholinesterases (ChE):

$$\text{Choline ester} + H_2O \xrightarrow{\text{ChE}} \text{Choline} + \text{carboxilic acid}$$

Biosensors based on the activity of acetylcholinesterases (AChE) or pseudocholinesterases have been designed. These devices combine biological systems with a wide range of transducers that can be potentiometic, spectrophotometric,[21] voltammetric,[22] piezoelectric,[23] etc.

Cholinesterase inhibition occurs because pesticides have a shape that resembles the shape of the substrate, thus blocking the active estearic center of the enzyme and inhibiting its activity. For example, an organophosphorus pesticide hinders the choline ester hydrolisis reaction because it blocks the active center of the enzyme. This inhibition is independent of the presence of the substrate.

$$\underset{\text{enzyme}}{\text{E-OH}} + \underset{\text{organophosphorus inhibitor}}{(RO_2)\text{-PO-X}} \xrightarrow{K_i} \underset{\text{phosphorilated enzyme}}{(RO_2)\text{-PO-OE}} + HX$$

where K_i is the inhibition constant.

On the other hand, carbamate compounds compete with the substrate for the active center of the enzyme. The blocking caused by these pesticides is prevented by a high substrate concentration. For this reason, organophosphorus pesticides produce a noncompetitive inhibition, while carbamates show a competitive one. In any case, these features may be tuned by adjusting the concentration of the substrate and the enzyme immobilization method.[8] For instance, an amperometric biosensor with immobilized AChE that was inhibited competitively by carbaryl when the substrate was present displayed a noncompetitive inhibition when the substrate was absent.[24]

Enzyme inhibition by pesticides was used for measurement purposes since the early 1970s using electrochemical methods.[25,26] Many research efforts have been applied to obtain enzyme-based biosensors with a higher stability,[21,27] a larger sensitivity, and lower detection limits. The scientific community has focused its efforts toward the application of these devices to real samples without a previous treatment.[28]

Three biosensor strategies can be found in the literature to quantify the decrease of cholinesterase activity. In all cases, the substrate defines the type of transducer used (see Figures 1 and 2).

A. One Enzyme System

When using acetylcholine (ACh) or butyrilcholine (BCh) as a substrate, the reac-

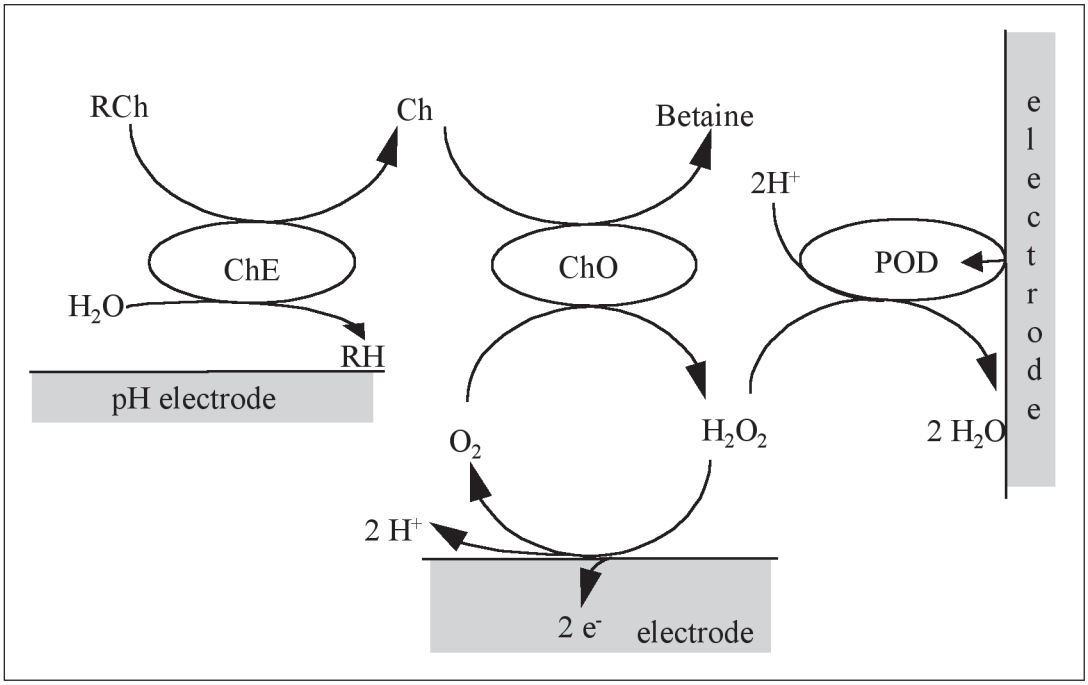

FIGURE 1. Measurement principle of the choline esterase (ChE) activity using R-choline (RCh) as substrate. According to the number of enzymes used in the recognition process, the transduction may be potentiometric or amperometric.

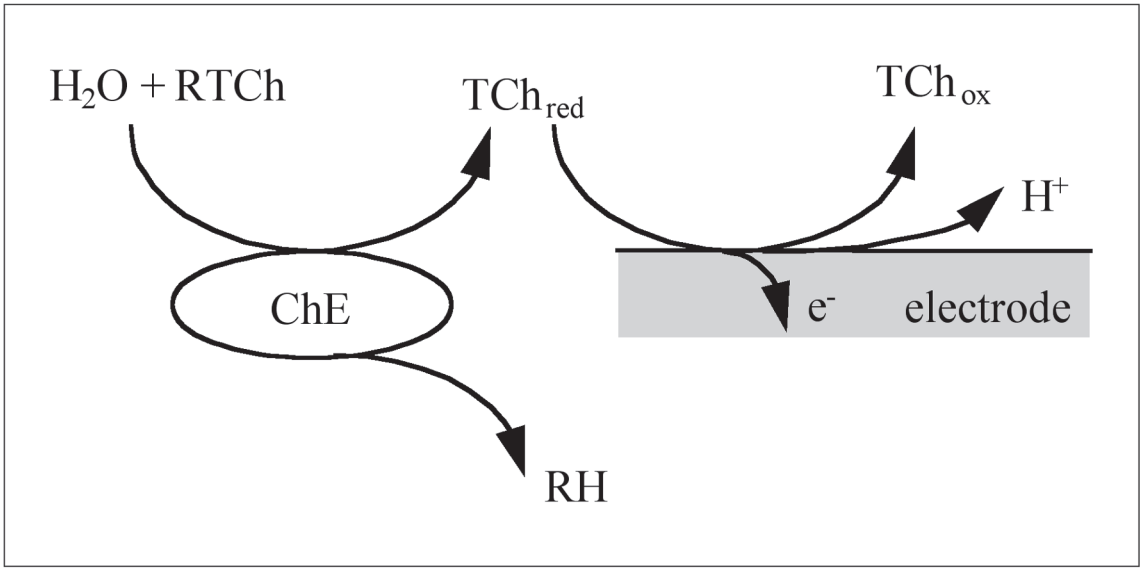

FIGURE 2. Measurement principle of the choline esterase (ChE) activity using R-thyocholine (RCh) as substrate. An amperometric transduction is employed usually.

tion products are choline (Ch) and the corresponding organic acid (see Figure 1). Since choline is not electrochemically active, the change of enzyme activity is detected by the pH variation due to the acid production at the surface of the biosensor. In this case the electrochemical method of choice is a potentiometric one.

There are no natural substrates producing electroactive species. When artificial substrates such as butyrylthiocholine (BTCh) or acetylthiocholine (ATCh) are used, the products of the reaction are thiocholine (TCh) and an organic acid (see Figure 2). Thiocholine can be oxidized anodically using platinum electrodes, but chemically modified carbon electrodes are a better option.

Another approach is the use of multienzyme systems.

B. Bi-Enzyme System

In this system cholinesterase (ChE) is coupled to a second enzyme as seen in Figure 1. This second enzyme has to transform one of the products of the hydrolysis reaction into another product that can be detected amperometrically.

When butyrylcholinesterase (BChE) or acetylcholinesterase (AChE) are used with choline oxidase (ChO), the enzyme system can be coupled to amperometric H_2O_2 or O_2 devices. Normally, the detection of O_2 is achieved with Clark electrodes and H_2O_2 with platinum, graphite, or screen-printed electrodes polarized to a fixed potential.

This technique permits the production of bi-enzyme electrodes similar to single-enzyme devices. Some authors state that they did not find significant differences between these two groups of electrodes.[29] This means that there are no significative interactions between the two enzymes used.

C. Tri-Enzyme System

Peroxidase (POD) may be added to the bi-enzyme system to build a trienzyme device. Potentiometric measurement of ChE activity based on mediatorless bioelectrocatalysis can be done at the electrode interface by three consecutive reactions (see Figure 1). The generation of H_2O_2 as a product of the second reaction provokes a potential change in the electrode. This change is due to the bioelectrocatalysis of peroxide, where POD is regenerated without the presence of a mediator. Direct electron transfer to POD takes place on the electrode causing the potential change. This potential shift is proportional to the H_2O_2 concentration and to the activity of the cholinesterase.

Multienzyme systems pose greater challenges than monoenzyme ones. However, they produce biosensors that are more specific and less prone to interferences.[30]

A. Potentiometric Detection

As stated above, the substrate sometimes determines the type of signal transduction to be used. For instance, when the substrate is a choline ester (RCh) the detection will be potentiometric. This detection is based on a pH change brought by the organic acid produced by the enzyme hydrolysis of the ester used as substrate.

A wide range of pH sensors has been used, including pH-ISFETs, glass electrodes, Pd/PdO electrodes,[31] and Ir/IrO electrodes[32] or polyurethane-based ion-selective electrode.[33] Enzymes were immobilized on these devices following several techniques (see Table 1).

1. Glass Electrode

By far the most widely used transducer in early biosensor designs was the pH-sensi-

tive glass electrode. Inhibition determination was not an exception in this respect. In 1990 *Tran-Minh,*[34] a pioneer in inhibition biosensor studies, used a glass electrode covered with a polyacrylamide gel containing the crosslinked enzyme (AChE).

A common theme in numerous eterior publications is the method followed for the immobilization of the enzyme and the production of the enzymatic membrane placed on the pH electrode. This is because the sensitivity of biosensors often depends on the immobilization method used. Kumaran et al.[35] immobilized the enzyme by co-crosslinking it with HSA and glutaraldehyde on a Nylon mesh. They also tried bonding the enzyme covalently to a polyamide membrane and its entrapping in a gel as described by Tran-Minh.[34] The different immobilization strategies showed approximately the same response characteristics (sensitivity and detection limit) when placed in contact with the toxic materials. Enzyme activity was preserved for more than 3 years if devices were kept dry. Additionally, these devices were not expensive and could be mass produced.

Three years later, Kumaran[36] progressed in his inhibition research by applying the polyamide membrane biosensor to the detection of pesticides in soil extracts. The biosensor produced a result in an hour with an acceptable precision even with samples containing pesticides in concentrations close to the detection limit of the biosensor.

Stein et al.[37] compared their results obtained with a device containing AChE directly co-crosslinked to the surface of the glass electrode and those produced by a biosensor where the enzyme was co-crosslinked to a cellulose nitrate membrane. They also compared the entrapment of the enzyme in a polyacrylamide gel on the same membrane. Their studies concluded that direct immobilization onto the electrode is not a good idea because it is difficult to manipulate and irreproducible. The use of membranes facilitates the substitution of the enzyme load, defines the thickness of the membrane controlling the diffusion process. Additionally, this technique involves procedures that are more reproducible, especially when the enzyme is trapped in a gel.

Further researches on the immobilization of enzymes on glass electrodes has appeared. In 1996, Budnikov et al.[38] designed potentiometric biosensors based on several enzyme membranes (*tracing paper, gelatine membrane,* and *cellulose trinitrate*). The authors concluded that the detection limit and the linear range for a given inhibitor depended on the nature of the inhibitor and on the enzyme support, that is, on the type of the membrane used. Of all these, tracing paper was deemed to be the best alternative because it is mechanically durable and the resulting membrane shows a high sensitivity. Fifteen to 20 measurements could be realized on samples with low pesticide content as long as the enzyme (BChE) was reactivated with TMB-4 after each analysis. After these measurements, or even before, if the concentration of the pesticide was high, the membrane was replaced with a fresh one.

Using these findings, G.A. Evtugyn used a biosensor based on a white tracing paper with immobilized BChE for screening the toxicity of a number of wastewater samples. The information gathered from the results, with or without sample pretreatment, was combined with the microbiological observation of a nutritional culture of *Paramecium caudataum,* to determine the source of the pollution and the identity of the inhibitors. This format represents a biosensor that is more robust and more appropriate for use in the field analysis.

2. pH Metal Oxide Electrode

These pH-sensitive electrodes are more robust than glass electrodes.

In 1990 Tran-Minh et al. simultaneously immobilized polyacrylamide gel containing

AChE on Pd/PdO or Ir/IrO electrodes, both sensitive to pH, on a glass electrode as described above. The resulting biosensors have good response characteristics and a high operational and storage stability. They also showed good detection limits especially for irreversible inhibitors (malathion and paraoxon).

Five years later, M. Trojanowicz[39] built a biosensor based on Ir/IrO electrode, using a cellulose triacetate membrane with the enzyme BChE. The biosensor was stable for several days.

Recently, Ghindilis et al. developed a potentiometric method to measure the inhibition of cholinesterase based on mediatorless bioelectrocatalysis. This implied the co-immobilization of three enzymes: butyrylcholinesterase, choline oxidase, and peroxidase on a graphite electrode (see Figure 1). As stated above, the rate of the electrode potential increase is proportional to the rate of the production of the peroxide that is in turn proportional to the activity of butyrylcholinesterase. This method lowers the detection limit to $2 \cdot 10^{-13}$ *M* for Trichlorfon and a better stability for 4 weeks. The regeneration of the electrode, following an inhibition that is not quite irreversible, is achieved by washing the device with the same buffer solution used in the analysis. However, this regeneration is limited to low inhibitor concentrations.[40,41]

Yang et al.[42] designed a potentiometric screen-printed electrode based on the same principle described by Diehl-Faxon and Ghindilis.

3. pH-Sensitive Field Effect Transistors

The first immobilisation of cholinesterases on ISFETs aimed at the detection of pesticides at a ppm level was reported in 1990 by Yuan et al.[43] They immobilized BChE by co-crosslinking it with BSA and glutaraldehyde. This layer was protected with a Nylon mesh to prevent the detachment of the enzyme to the solution.

Shortly afterward, Vlasov et al. modified the usual co-crosslinking immobilization technique substituting BSA for gelatine,[44] producing single-use membranes. The detection limits were lower than those where the inhibition occurred in a homogeneous phase because the matrix forming the membrane protect the enzyme and screen partially the inhibitors.

The reproducibility of the crosslinking immobilization technique depends on the characteristics of the support membrane (thickness, enzyme concentration, adhesion, lifetime, etc.). Because of this limitation, other methods were assayed where the enzyme was bonded covalently to the ISFET surface with triethoxysilylpropylamine and glutaraldehyde.[45] Although the resulting devices have short response times, they are sensitive to dynamic changes in the system (agitation, temperature, etc.), making the results very irreproducible and not amenable for real samples.

Two years later, in 1993, A.M.N. Hendji[46] used a differential system featuring two identical pH-FETs. On the first ISFET, AChE or BChE was immobilized following the co-crosslinking technique with BSA and glutaraldehyde (ENFET). A second blank ISFET, without the enzyme, was used as a reference (REFET). The use of 2-PAM for 30 min produced a regeneration of 60%. When a longer reactivation time was assayed, the membrane characteristics were modified.

Volotovsky et al.[47] reported a multiparametric inhibition biosensor based on a membrane built with a gel immobilizing three enzymes (urease, peroxidase, butyrylcholinesterase) on a field-effect transducer. This biosensor was applied to the environmental monitoring of toxic materials such as heavy metals, cyanides, organophosphorus and carbamate pesticides, each of which inhibited an enzyme. The authors concluded that the immobilized enzymes act independently, and that their

number can be enlarged to monitor a wider variety of toxic materials in the same analysis.

In a recent report, a BChE-FET sensor was used by Wan et al.[48] to detect trichlorfon as a common pesticide. They used two different procedure to immobilize enzymes: entrapment in a photocross-linkable membrane and cross-linking in a BSA-glutaraldehyde membrane. Meanwhile Starodub et al.[49] used between other enzymes AChE and BChE immoblized over ISFET gates for the determination of chlorpyrifos. They used also PAM to reactivate the enzyme.

4. Features of the Potentiometric Biosensors Used in the Determination of Pesticides

It may be concluded that although the covalent immobilization of enzymes is ideal because it ensures a good stability and reproducibility, it has not been a viable option for potentiometric inhibition biosensors. This is because the reactions needed to produce the covalent bonding of the enzyme to the glass or the silicon dioxide of the ISFET alter the properties of these transducers. Additionally, potentiometric biosensors with covalent bonding are easily affected by dynamic changes (stirring, temperature changes, etc.), making them irreproducible. Furthermore, covalent immobilization can alter or block the active center of the enzyme, diminishing the enzyme available in the membrane.

Of all the support materials used for the immobilization of enzymes, membranes are one of the best options. Tracing paper membranes in particular offer the lowest detection limits and the highest stability, permitting their use in real samples.[8]

Looking at the information presented in Table 1 one can see that enzyme trapping using a gel or crosslinking the enzyme on a Nylon mesh produces potentiometric biosensors that can be stored for about 3 years.[35] These immobilization techniques retain physically the enzyme and maintain its activity. The resulted high-porous membrane increased diffusion, minimizing kinetic limitations. Additionally, membranes raise the mechanical, thermal, and chemical stability, producing longer storage times.[19]

Incubation times are 15 to 30 min and typical detection limits go from 10^{-9} to 10^{-12} *M,* depending on the pesticide used.

From an analytical point of view, it appears that the introduction of ISFETs has not had a significant impact on the quality of the data produced. On the other hand, ISFETs have brought a technological advance because they are used to produce inhibition biosensors potentially compatible with microelectronic fabrication techniques.

Moreover, the use of enzyme systems coupled to POD on graphite, capable of bioelectrocatalytic reduction of H_2O_2 by direct (mediadorless) electron transfer (see Figure 1) are an attractive procedure for the use of mass fabrication procedures.[42]

B. Amperometric Detection

The amperometric detection of the enzymatic inhibition of the choline esters hydrolysis can take place following three different paths:[50] The most usual is the determination of choline formation by monitoring hydrogen peroxide generated in the second reaction catalyzed by the choline oxidase (ChO) (see Figure 1). The second option is to measure the concentration of the oxygen consumed in the same reaction using a Clark electrode. The third amperometric method implies the use of thiocholine esters as artificial substrates for ChE and the direct anodic measurement of the thiocholine using a biosensor with or without mediator (see Figure 2).

The most widely used transducer for these kind of measurements is the platinum electrode, although carbon paste electrodes and carbon-based screen-printed electrodes have been used also.

TABLE 1
Response Characteristics of Potentiometric Biosensors Based on Cholinesterases (ChE) Inhibition

Transducer	Enzyme	Enzyme Support / Immobilization Method	Substrate (concentration)	Inhibitor/ Detection Limit / / Incubation Time	Storage Time	Regeneration (time)	Application	Reference [Year]
Glass electrode	AChE	Polyacrylamide gel / Entrapment	AChCl (5 mM)	Malathion / 10^{-10} M / 1 hora Paraoxon / 10^{-9} M / 1 hora	---	2-PAM 1mM	---	[34] 1990
Glass electrode	BChE	Nylon mesh / HSA co-crosslinking	BChCl (1.5 mM)	Carbofuran / 4 ppm / 30 min Carbaryl / 10 ppm / 30 min Paraoxon / 0.2 ppm / 30 min	3 years	---	---	[35] 1992
	BChE	Polyamide membrane / Covalent	BChCl (1.5 mM)	Carbofuran / 1 ppm / 30 min Carbaryl / 4 ppm / 30 min Paraoxon / 0.2 ppm / 30 min	3 years	Membrane change	----	
	BChE	Polyacrylamide gel / Entrapment	BChCl (1.5 mM)	Carbofuran / 2 ppm / 30 min Carbaryl / 4 ppm / 30 min Paraoxon / 0.3 ppm / 30 min	3 years	---	---	
Glass electrode	AChE	/ BSA co-crosslinking	AChCl (300 mg·mL^{-1})	Diclorvos // 30 min	50 days	---	---	[37] 1993
	AChE	Cellulose acetate or nitrate / BSA co-crosslinking	AChCl (800 mg·L^{-1})	Diclorvos // 30 min	2-3 days	Membrane change	---	
	AChE	Cellulose acetate / Entrapment in polyacrylamide gel	AChCl (800 mg·L^{-1})	Diclorvos // 30 min	> 50 days	Membrane change	---	
Glass electrode	BChE	Polyamide membrane / Covalent	BChCl ($1.2 \cdot 10^{-3}$ M)	Fenitrothion/21 ppm / 10 min Diazinon / 35 ppb / 10 min Parathion ethyl/3.0ppm/10min Mevinphos / 1.4 ppm / 10 min Heptonophos/ 650 ppb / 10 min	3 years	Membrane change	Extracted from soil	[36] 1995

Transducer	Enzyme	Enzyme Support / Immobilization Method	Substrate (concentration)	Inhibitor/ Detection Limit / / Incubation Time	Storage Time	Regeneration (time)	Application	Reference [Year]
Glass electrode	BChE	Tracing paper / Crosslinking	BChI ($2 \cdot 10^{-3}$M)	Zolone // Diazinon // DDVP //	> 8 months	TMB-4	---	[38] 1996
	BChE	Gelatine membrane / Crosslinking	BChI ($2 \cdot 10^{-3}$M)	Zolone // Diazinon // DDVP //	> 15 months	(15 –20 measurements)	---	
	BChE	Cellulose trinitrate/ Crosslinking	BChI ($2 \cdot 10^{-3}$M)	Zolone // Diazinon // DDVP //	> 6 months	Membrane Change	---	
Glass electrode	BChE	White tracing paper / Crosslinking	BChI	Reversible pesticides Irreversible pesticides	3 months	Buffer washing TMB-4 1 % (10-15 min)	Waste water samples	[8] 1997
Pd/PdO i Ir/IrO	AChE	Polyacrylamide gel / Entrapment & crosslinking		Paraoxon / 0.3 ppb / Malathion //	---	---	---	[34] 1990
Ir/IrO	BChE	Cellulose triacetate / Entrapment	BChCl (8.3 mM)	Paraoxon / /	---	---	---	[39] 1995
Composite	BChE / ChO / HRP	Glutaraldehyde / Covalent (HRP), crosslinking (ChO), crosslinking (ChE)	BCh^+ (10 mM)	Trichlorfon/$2 \cdot 10^{-13}$ M/ 15min	4 weeks.	Buffer washing	---	[40] 1996
	ChO / HRP and BChE (sol)	Glutaraldehyde / Covalent (HRP), crosslinking (ChO)	BCh^+ (10 mM)	Trichlorfon/$2 \cdot 10^{-9}$ M/ 15 min				

TABLE 1 (continued)

Transducer	Enzyme	Enzyme Support / Immobilization Method	Substrate (concentration)	Inhibitor/ Detection Limit / / Incubation Time	Storage Time	Regeneration (time)	Application	Reference [Year]
Black carbon TCB-and TCB-355	BChE / ChO / HRP	/ Adsorption (HRP), crosslinking (ChO), crosslinking (ChE)	BChCl (1mM)	Trichlorfon / 5nM / 10 min	1 month	-	---	[41] 1996
Composite	HRP / ChO / ChE	Glutaraldehyde / Covalent (HRP), crosslinking (ChO, ChE)	BChCl (1mM)	---		(Membrane change)	---	
Graphite (screen printed)	AChE / OCh / HRP		$ATCh^+$	Trichlorfon / pM / < 15 min	2 months	Single-use	---	[42] 1998
pH-ISFET	BChE	Nylon mesh / BSA co-crosslinking	BCh^+ (10^{-2} M)	Feniltrothion / ppm-ppb / Malathion / ppm /		---	---	[43] 1990
pH-ISFET	BChE	/ Gelatine co-crosslinking	BChI (10^{-3} M)	Tacrin / 10^{-9} M / 15 min DFP / $5 \cdot 10^{-5}$ M / 15 min DDVP / 10^{-6} M / 15 min		Single-use	---	[44] 1991
pH-ISFET	AChE AChE	/ BSA co-crosslinking / Covalent	AChCl (441 $mg \cdot L^{-1}$)	Diclorvos / 0.3 $mg \cdot mL^{-1}$ /	23 days	2-PAM (4 h) + buffer solution (2 h)	Potable water	[45] 1991
pH-ISFET	AChE	/ BSA co-crosslinking	ACh^+ (20 nM)	DFP / 10^{-12} M / 20 min Paraoxon methyl /10^{-7} M/ 20 min Trichlorfon/ 10^{-7} M / 20 min	3 days	2-PAM (1/2 h)	-----	[46] 1993
	BChE	/ BSA co-crosslinking	BCh^+ (20 nM)	DFP / 10^{-12} M / 20 min Paraoxon methyl / 10^{-6} M / 20 min Trichlorfon / 10^{-6} M / 20 min				

Transducer	Enzyme	Enzyme Support / Immobilization Method	Substrate (concentration)	Inhibitor/ Detection Limit / / Incubation Time	Storage Time	Regeneration (time)	Application	Reference [Year]
pH-ISFET	BChE, POD, UrE	Gel / BSA co-crosslinking	BCh^+	Carbofuran / 5 μM / 10 min	---	---	---	[4 7] 1998
FET	BChE	/Photocross-linkable membrane	BChCl (18mM)	Trichlorfon / 10^{-6} M / 10 min	9 months	2-PAM (60 min)	--	1999 [48]
	BChE	/BSA-glutaraldehyde crosslinking	BChCl (18mM)	Trichlorfon / 10^{-6} M / 10 min	35 days	2-PAM (60 min)	--	
Polyurethane-based-ISE	BChE	/HPU (hydrophilic polyurethane)	BCh^+ (10^{-2}-10^{-4}M)	Paraoxon/1h	4 weeks	-	-	(1999) [33]

Described below are the main biosensors used for the detection of pesticides reported in recent papers. Table 2 shows the technical details of these biosensors.

If it is not otherwise stated, in the following E_{app} (applied potential) is referred to Ag/AgCl).

1. Platinum Electrodes

Platinum electrodes are widely used in amperometric biosensors. Platinum is an inert metal with high conductivity and as result with high current density. It presents elevated sensitivity when transducing the catalytic activity of the enzyme.

Bernabei[51] covered a platinum transducer (E_{app} = 650 mV) with a Nylon membrane containing immobilized enzymes using a crosslinking technique. The report of this work compares the response obtained with AChE and ChO enzymes co-immobilized in the membrane and with the ChO immobilized and AChE free in the solution. The authors conclude that, although the response time is shorter when both enzymes are co-immobilized, the system with AChE in the solution is better because less enzyme is used. Additionally, this biosensor with only ChO immobilized is not affected greatly by the pesticides, and the lifetime of the device is lengthened.

In later reports an immunoaffinity membrane was used[52] with the choline oxidase bonded covalently to the preactivated membrane. This biosensor was applied to waste water analysis in rivers and in the sea. The method correlated well to the chromatographic reference method (GLC). The biosensor was used in surface waters in several parts of Italy[53] demonstrating enough sensitivity in real samples analysis and enough robustness to be applied to samples from different sources and different composition.

Marty et al. developed a novel membrane. On a platinum electrode (E_{app}= 700 mV) they placed a cellulose nitrate membrane where a bi-enzyme system (AChE and ChO) had been immobilized using photocrosslinking with PVA-SbQ.[54] The resulting biosensors were very stable for a period of 1 year. In a second report[55] they used the same polymer to trap a single enzyme (AChE) while using ATCh as the substrate (E_{app}=410 mV). A monoenzyme system solves all problems derived from consecutive enzyme reactions, that is, the often conflicting requisite of a different optimal pH and a different stability for each of the enzymes. Given the difficulty of regenerating the activity of the enzyme with nucleophilic agents, the authors recommended the recalibration of the sensor after each inhibition/reactivation cycle. They also recommended changing the membrane altogether.

The same authors validated the biosensor in organic media some years later (1995).[6] They compared the biosensor to a solid-phase on-line extraction system followed by liquid chromatography incorporating a diode array as the detector (LC-DAD). The same samples were dried and frozen and then hydrated and injected to a flow system using the biosensor as the detector. The system, working in an aqueous medium, requires 2 to 3 h to complete the analysis. This time is sufficiently long for the degradation of the pesticides into their oxo-metabolytes. The biosensor shows a high sensitivity to these products, delivering better results than the method of reference.

In 1998, Curulli et al.[56] developed a new choline oxidase biosensor based on the electropolymerization of nonconducting polymers at the surface of the electrode. This layer presents better diffusional properties, screens out interferents, and prevents fouling. The biosensor was used in the analysis of organophosphorus pesticides using BChE in solution.

Okazaki et al.[57] co-immobilized AChE and ChO by co-crosslinking with BSA onto a Pt electrode. They used it in determination of

TABLE 2
Response Characteristics of Amperometric Biosensors Based on Cholinesterases (chE) Inhibition

Transducer / Modifier / Working potential	Enzyme	Enzyme support / Immobilization method	Substrate (concentration)	Inhibitor / Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Platinum electrode // 0.65 V (Ag/AgCl)	AChE / ChO	Nylon mesh / crosslinking	BChCl (0.05 mM)	---	---	Membrane change /	---	[51] 1991
	ChO AChE (sol)	Nylon mesh / crosslinking	BChCl (0.05 mM)	Paraoxon / 2 ppb / 30 min		--		
Platinum electrode // 0.65 V (Ag/AgCl)	ChO BChE (sol)	Immunoafflinity membrane / Covalent	ACh^+ (0.3 mM)	Parathion methyl// Paraoxon methyl// Parathion // Paraoxon//	---		Waste water in river and in the sea	[52] 1992
Platinum electrode // 0.65 V (Ag/AgCl)	BChE / ChO	Immunoafflinity membrane / Covalent	BCh^+ (0.3 mM)	Paraoxon / 0.5 ppb / 30 min	---	---	Surface waters (Italy)	[53] 1993
	ChO BChE (sol)	Immunoafflinity membrane / Covalent	BCh^+ (0.3 mM)	---	---	Membrane change		
Platinum electrode // 0.7 V (Ag/AgCl)	AChE / ChO	Cellulose nitrate/ Photo-crosslinking (PVA-SbQ)	ACh^+ (5 mM)	Paraoxon / 10 nM / 30 min	1 year (dry)	Water + phosphate buffer + obidoxime / (20 minuts)	----	[54] 1992
Platinum electrode // 0.41 V (Ag/AgCl)	AChE	/ Photo-crosslinking (PVA-SbQ)	$ATCh^+$ ($5 \cdot 10^{-3}$ M)	Parathion / 10^{-4} M / 30 min Phonophos / 10^{-6} M / 30 min Monocrophos/ 10^{-7} M /30min Paraoxon / 10^{-10} M / 30 min Aldicarb / 10^{-4} M / 30 min Carbofuran / 10^{-8} M / 30 min	1 year	2-PAM obidoxime	---	[55] 1993

TABLE 2 (continued)

Transducer / Modifier / Working potential	Enzyme	Enzyme support / Immobilization method	Substrate (concentration)	Inhibitor / Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Platinum electrode // 0.41 V (Ag/AgCl)	AChE	/ Photo-crosslinking (PVA-SbQ)	---	Phenamiphos // Carbaryl // Fenitrothion // Parathion Ethyl // Paraoxon / 10^{-10} M /	---	2-PAM (1 mM) (30 minuts)	Aigua	[6] 1995
Platinum electrode //	ChO BChE (sol)	/ Electropolymerized film	---	Paraoxon / < 2 ppb /	---	---	---	[56] 1998
Platinum electrode // 0.5 V (Ag/AgCl)	AChE, ChO	/ BSA co-crosslinking	AChCl 1 mM	Dichlorvos /	--	2-PAM	--	[57] 2000
Clark electrode //	AChE / ChO	O_2 permeable affinity membrane/ Covalent	AChCl 10^{-2} M	Paraoxon / 1 μM /	3 weeks	2-PAM (1 mM) (10 min)	Non-aqueous media	[58] 1997
	ChO AChE (sol)	O_2 permeable affinity membrane/ Covalent	AChCl 10^{-2} M	Paraoxon / 1 nM /	4 weeks			
Clark electrode //	AChE /ChO	/ kappa-carrageenan gel discs	BChCl 26.6 μM (mixed organic media)	Paraoxon / 4.5 $\mu g \cdot L^{-1}$ / 20 min Malathion / 4.8 $\mu g \cdot L^{-1}$ / 20 min Parathion ethyl/ 0.5$\mu g \cdot L^{-1}$ /20min Aldicarb / 4.8 $\mu g \cdot L^{-1}$ / 20 min Carbaryl / 0.5 $\mu g \cdot L^{-1}$ / 20 min	15 days	---	---	[30] 1999

Transducer / Modifier / Working potential	Enzyme	Enzyme support / Immobilization method	Substrate (concentration)	Inhibitor / Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Graphite / CoPC / 0.4V (Ag/AgCl)	BChE	Polyamide mesh / crosslinking	BTChI 0.55 mM	Hostaquick / 0.2 mg·mL^{-1} / 3 min Seedox / 80 mg·mL^{-1} / 3 min	3 months	---	---	[59] 1991
Graphite / CoPC / 0.3 V (Ag/AgCl)	AChE (sol)	---	ATCh^{+} 0.5 mM	Paraoxon / 11μg·L^{-1} / 3 min Carbaryl / 56 μg·L^{-1} / 3 min Heptenophos/76 μg·L^{-1} /3min Malathion/1600μg·L^{-1} / 3 min		---	---	[60] 1992
	AChE	Nylon mesh / crosslinking	ATCh^{+} 0.5 mM	Paraoxon / 12 μg·L^{-1} / 3 min Carbaryl / 19 μg·L^{-1} / 3 min. Heptenophos/ 1800 μg·L^{-1} / 3 min Malathion/ 840 μg·L^{-1} / 3 min	1 month	---	---	
	AChE	Immunoaffinity membrane / crosslinking	ATCh^{+} 0.5 mM	---	---	---	---	
	BChE (sol)	---	ATCh^{+} 0.5 mM	Paraoxon / 1.7 μg·L^{-1} / 3 min Carbaryl / 340 μg·L^{-1} / 3 min Heptenophos/10 μg·L^{-1} /3min Malathion/ 4700 μg·l^{-1} / 3min	---	---	---	
	BChE	Nylon mesh / crosslinking	ATCh^{+} 0.5 mM	Paraoxon / 1.5 μg·L^{-1} / 3 min Carbaryl / 250 μg·L^{-1} / 3 min Heptenophos /8.4 μg·L^{-1} / 3min Malathion / 920 μg·L^{-1} / 3min	1 month	5-10 uses one membrane		

TABLE 2 (continued)

Transducer / Modifier / Working potential	Enzyme	Enzyme support / Immobilization method	Substrate (concentration)	Inhibitor / Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Graphite // CoPC (acetylcellulose) / 0.25 V (Ag/AgCl)	BChE	Immunoaffinity membrane / crosslinking	BTChI 0.5 mM	Paraoxon / 0.3 nM / 10 min Dichlorvos / 1.2 nM / 10 min Carbaryl / 11 nM / 10 minuts	---	-----	---	[61] 1992
Glassy carbon // 0.25 V (SCE)	AChE	Nylon mesh / BSA co-crosslinking	PAPA 1.2 mM	Carbaryl / 4 nM / 3 minuts Paraoxon / 13 nM / 3 minuts	40 days	2-PAM (24.8mM) (4h)	---	[62] 1994
Rotating graphite electrode // 0.8 V (SCE)	AChE	/ Covalent (Carbodiimide)	ATChI $2 \cdot 10^{-4}$ M	Trichlorfon /10^{-9}M / Galantani/ Hidrobromicum/10^{-10} M Oxofosfol / 10^{-10} M/	40 days	---	---	[63] 1994
Composite // 0.7 V (Ag/AgCl)	AChE	/ Entrapment (Biocomposite)	ATChCl 10^{-4} M	Carbofuran / 2.2 $\mu g \cdot L^{-1}$ / Others / 20-30 µg/L /		Polishing process	---	[64] 1994
Composite / TCNQ / 0.3 V (Ag/AgCl)	AChE o BChE	Silica particles/ Entrapment (Biocomposite)	ACh^+ o BCh^+ 10^{-4} nM	Carbofuran / 22.1 $\mu g \cdot L^{-1}$/5min Paraoxon / 2.8$\mu g \cdot L^{-1}$ / 5 min Chlorfenviphos / 3.6 $\mu g \cdot L^{-1}$ /5min	Depending on the use	Polishing process	---	[65] 1997
Glassy carbon rotating electrode // 0.25 V (SCE)	AChE	Gold-covered nylon mesh / crosslinking	PAPA 1.2 mM	Paraoxon / $5 \cdot 10^{-8}$ M / Carbaryl / $5 \cdot 10^{-8}$ M /	90 days	2-PAM (4 h) + buffer solution (2 h)	---	[66] 1996
Reticulated vitreous carbon (RCV) //0.25 V (Ag/AgCl) Meldola Blue (sol)	AChE AChE	/ Adsorption Agarose gel / entrapment	ATChI 0.4 mM ATChI 0.4 mM	Paraoxon / 0.5 nM / ---	1 month	---	---	[67] 1998

Transducer / Modifier / Working potential	Enzyme	Enzyme support / Immobilization method	Substrate (concentration)	Inhibitor / Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Graphite (*Screen-printed)*/ TCNQ/ 0.1 V (Ag/AgCl)	BChE	/crosslinking	$BTCh^+$ 1.23 mM $BTCh^+$ 2.46 mM	PMSF / --- DIFP / ---	---	Single-use	---	[70] 1991
Graphite (*Screen-printed)*/ Ru/ 0.7 V (SCE)	ChO and AChE (sol)	/ Adsorption /Electropolymerization	AChCl 0.5 mM AChCl 0.5 mM	Posdrin / >1 nM / 10 min Dichlorvos/ >1 nM / 10 min ---	--- ---	Single-use Single-use	--- ---	[69, 71] 1995
Graphite *(Screen-Printed)*/ Ru/ 0.7 V (SCE)	ChO and AChE (sol)	/ Adsorption	AChCl 0.5 mM (mixed organic media)	Carbofuran / 2 $\mu g \cdot L^{-1}$ /10 min	---	Single-use	Fruits and vegetables in organic solvents	[72] 1997
Graphite *(Screen-printed)*/ CoPC/ 0.1 V (SCE)	AChE (sol)	---	ATChI	Paraoxon / $5 \cdot 10^{-8}$ M / 10 min Dichlorvos /	---	Single-use	---	[73] 1994
	AChE	/ crosslinking	ATChI	Paraoxon/ $3.24 \cdot 10^{-7}$M/ 10min Dichlorvos/$1.7 \cdot 10^{-6}$M / 10min				
Graphite (*Screen-Printed)*/ CoPC/ 0.1 V (Ag/AgCl)	AChE	/ printing ink (crosslinking)	$ATCh^+$ 0.5 mM	Paraoxon / 10^{-8} M / 30 min Dichlorvos (vapona, dibrom)//		Single-use	---	[74] 1997
	BChE	/ Printing ink (crosslinking)	$BTCh^+$ 0.5 mM	Malathion//		Single-use	---	

TABLE 2 (continued)

Transducer / Modifier / Working potential	Enzyme	Enzyme support / Immobilization method	Substrate (concentration)	Inhibitor / Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Graphite (*Screen-Printed*) / CoPC (acetylcellulose)/ 0.35 V (Ag/AgI)	AChE (ee)	/BSA co-crosslinking	1 mM AChI	Aldicarb / 190 $\mu g \cdot L^{-1}$ / 10 min Carbaryl / 0.92 $\mu g \cdot L^{-1}$ / 10 min Carbofuran/0.080$\mu g \cdot L^{-1}$/10min Methomyl/0.19 $\mu g \cdot L^{-1}$ /10 min Proporxur/0.68 $\mu g \cdot L^{-1}$ / 10 min	---	no	Mostres vegetals	[75] 1997
	BChE (hr)	/BSA co-crosslinking	BChI 1mM	Aldicarb /3200 $\mu g \cdot L^{-1}$ / 10 min Carbaryl / 1300$\mu g \cdot L^{-1}$ / 10 min Carbofuran / 150 $\mu g \cdot L^{-1}$ /10min Methomyl/ 0.88$\mu g \cdot L^{-1}$ /10 min Proporxur />$10^{-4}\mu g \cdot L^{-1}$ /10 min				
Graphite (*Screen-Printed*) / CoPC /0.1 V (Ag/AgCl)	AChE + stabilisers (GafQuatf + lactitol)	/ Printing ink	AThI 0.25mM	Vapona//	---	Single- use	---	[76] 1998
Grafit (*Screen-Printed*) / CoPC (acetylcellulose)/ 0.35 V (Ag/AgI)	AChE BChE	/Entrecreuament amb BSA	AThI 1 mM	Aldicarb/0.125 $mg \cdot L^{-1}$ /10min Carbaryl/ $5 \cdot 10^{-4}$ $mg \cdot L^{-1}$/10min Carbofuran/10^{-4} $mg \cdot L^{-1}$/10min Methomyl / 10^{-4} $mg \cdot L^{-1}$/10min Propoxur/$5 \cdot 10^{-3}$ $mg \cdot L^{-1}$/10min	---	Single- use	Potatoes, carrot, sweat, pimento juice or extracts	[77] 1998 [78] 1999]
Stainless steel filament// 0.67 V (SCE)	AChE / ChO	Lipid bilayer activated with biotin / Covalent	AChCl 19.6 mM	Trichlorfon / 3.9 nM / 7 min Eserina / 0.368 nM / 7 min	3 h	--- (Membrana change)	---	[29] 1997

dichlorvos as a model organophosphate and regenerated the enzyme activity using PAM.

2. Clark Electrodes

The main advantage of Clark electrodes over more classic potentiometric or amperometric systems is the presence of a gas-diffusing membrane forming a barrier for interferents.

Additionally, its use in nonaqueous media has several advantages: electrolytes do not have to be added to the working solution to raise its conductivity, and it shows a reduced bacteriological contamination that entails an oxygen consumption that interferes with the detection of enzyme activity with a Clark electrode.[30]

Detection systems based on these amperometric gas electrodes are bienzymatic comprising AChE and an excess of ChO to quantify inhibition from oxygen depletion occurring during the second biocatalytic reaction (see Figure 1). Taking advantage this depletion, Fennouh et al.[58] designed a device sensitive to anticholinesterases based on a Clark electrode covered with an hydrophobic polypropylene film as a protection against soluble pollutants. A second affinity membrane (Ultrabind) also permeable to oxygen is applied containing covalently bound AChE and ChO. Inhibition is compared when both enzymes are immobilized and when only ChO is immobilized and AChE is dissolved. The biosensor with only ChO immobilized allows inhibition in an organic medium where the pesticide is more soluble. A better inhibition of the free AChE is noticed in this case and with mixed nonaqueous media.

Campanella built a biosensor trapping the enzyme between two kappa-carrageenan gel discs. These discs were placed between the gas-permeable membrane of the Clark electrode and a dialysis membrane. The biosensor allowed pesticide analysis in mixed media consisting of water and an organic solvent.

3. Graphite Electrodes

The use of graphite electrodes is normally linked to monoenzyme systems (AChE or BChE) with substrates (ATCh or BTCh) that release electroactive products (see Table 2).

Skládal (1991)[59] used a carbon graphite electrode modified with cobalt phtalocyanide (COPC) as the amperometric transducer. BChE was immobilized to a polyamide mesh using crosslinking. This modified mesh was used to cover the electrode forming a biosensor. COPC acts as a mediator for the electron transfer. This lowers the working potential (E_{app} = 400 mV), reducing the interferents that may be present in the sample. Additionally, the membrane works in an optimal pH range, better than the range used when working with enzymes immobilized in polyacrylamide gels. An additional advantage is that it shows a linear response to pesticide concentration.

This author reports better detection limits for an incubation time of 3 min, when a Nylon mesh is used to immobilize AChE o BChE.[60] as seen in Table 2. They also tried immobilizing the enzymes on an immunoaffinity membrane. However, the inhibition percentages were lower and response times longer.

Using this mediator, Skládal built a modified graphite electrode with COPC and acetylcellulose using the same immunoaffinity membrane.[61] These modifications produced a lower working potential (250 mV) and a detection limit 1000 times lower when the response was processed mathematically.[60]

la Rosa et al.[62] immobilized AChE cocrosslinking it with BSA on a Nylon mesh that was fixed to an electrode made of glassy carbon. In this case they used chronoamperometry as the electrochemical technique and 4-aminophenyl acetate as the substrate (PAPA) synthesized from the catalytic hydrogenation of 4-nitrophenyl acetate. This substrate uses lower working potentials (250 mV) without a mediator. This biosensor also shows lower detection limits with a lower incubation time (3 min).

In 1994, Stoytcheva et al.[63] developed a rotating graphite disc with AChE immobilized covalently using carbodiimide. The advantage was that the covalent bond ensures a better stability and a quick response because there is no membrane. The graphite permits the electrochemical oxidation of its surface. This modification facilitates the bonding of proteins. This method optimizes the proximity of the active centres of the enzymes to the electrochemical surface.

In our laboratories Martorell et al.[64] trapped the enzyme in a rigid polymer matrix made with an epoxy resin and graphite. The biosensor acts as a reservoir of the biological material, lengthening the lifetime of the device. However, the main advantage of this biosensor is in the regeneration step. A simple polishing produces a new and reproducible surface containing fresh enzyme. However, the elevated working potential (700 mV) favors the action of interferents that may be present in the sample as they could be oxidized at this potential. Tetracyanoquinodimethane (TCNQ), a mediator, is added to the biocomposite to lower the working potential.[65] The reproducibility and the stability of the device are bettered if AChE is immobilized on previously silanized particles. This prevents enzyme leakage to the solution. Another important advantage is that the sensor is robust and can be shaped into different configurations.

Pariente in 1996[66] described a new protocol for the immobilization of enzymes to a gold-covered Nylon mesh acting as a support. This configuration keeps the pores open increasing the sensitivity. Because there is not mass-transfer limitations, this method produces biosensors with larger quantities of protein. The enzyme is physically separated so the electrode may be reused.

Recently, Khayyami's[67] group of used a new material consisting of a composite made of reticulated vitreous carbon (RCV) or a variation where the RCV is modified with a superporous agarose gel. These composites, especially the second one, are capable of immobilizing a larger quantity of enzyme. These carbonaceous materials have high conductivity, high mechanical rigidity, high surface area, low pressure drop to fluid flow, and low background noise.

4. Screen-Printed Electrodes

Screen printing technology is very useful for the mass fabrication of biosensors. A conducting track is printed on a flat support (polyester, plastic, ceramic, glass). Pastes of various compositions are printed on this track. This constitutes a quick and simple method for the fabrication of single-use planar devices. A single-use biosensor has multiple advantages because it prevents contamination across samples, prevents the denaturalization of the enzymes with use, produces devices with a constant sensitivity and a high reproducibility. A large variety of inks are currently under development and some are commercially available.

Pastes doped with noble metals have been used for the conducting tracks. However, their high curing temperatures make them incompatible with the simultaneous application of enzyme layers.[68] Graphite pastes are more compatible in this respect, because they can be cured at room temperature and show good detection limits for hydrogen peroxide when doped with catalytic particles.[69]

Kulys in 1991[70] described a second generation of planar bioanalytical instruments to measure pesticides featuring an enzyme (BChE) and a mediator (TCNQ). They were produced with screen-printing techniques and detected the substrate (BTCh) at 100 mV.

Cagnini et al.[69,71] obtained lower detection limits when they doped the graphite paste with various noble metals (Pd, Pt, Ru), especially with 0.5% ruthenium (E_{app}= 700 mV). Two immobilization methods were assayed: adsorption and electropolymerization. The first method yielded devices with a better response: as it had been demonstrated by Bernabei et

al.,[51] when a bi-enzyme (AChE/ChO) system is used, better detection limits are achieved if AChE is in the solution.

These devices were used years later for the analysis of real samples of fruits and vegetables.[72] Since the pretreatment of the sample involves the extraction of pesticides in organic solvents, these devices were applied in mixed media made of a buffer solution and non-aqueous solvents (acetonitrile). The results show that this biosensor is useful for the screening of large number of samples, proceeding to a more detailed analysis on those samples tested positive by the biosensor.

In 1994 Hart et al.[22] studied the electrochemical mechanism of a COPC-modified screen-printed device using cyclic voltammetry. The voltammograms showed that, with dissolved AChE, it is possible to analyse pesticides at lower potentials (100 mV), hence lowering interferences. In a later report the same authors immobilized AChE crosslinking it on the COPC-modified planar device. This biosensor was applied to the analysis of several pesticides.[73] Results showed that pesticides containing sulfurs such as parathion and malathion need previous derivations (oxidation) before inhibiting the AChE. Analysis time is lowered if the enzyme is immobilized on the surface.

Later, other enzyme immobilization improvements were described by Hart et al.[74] It implied having the enzyme as an ink that contained all the reagents needed for immobilization (BSA, glutaraldehyde, hydroxyethyl cellulose (HEC)). These inks are viscous enough to be printed on a thin graphite layer previously modified with COPC.

Skládal[75] made screen-printed electrodes using the composites employed in previous work.[59] The graphite ink was modified with the mediator COPC. This permitted that the working potential be lowered from 700 to 350 mV reducing interferences. Additionally, he assayed several isoenzymes of AChE and BChE. Better results were obtained with AChE from electric eel. BChE is not useful for the analysis of carbamates. These biosensors permitted the analysis of vegetable juices without the need of pretreatment or the use of extraction steps, reducing the time needed for an analysis to 20 min.

Another important contribution to this field was described by Hart[76] in 1998. Three enzyme layers were screen-printed to increase the stability of these enzymes. Each layer contained a different stabilizer (hydroxyethyl cellulose (HEC), dimethyl-laminoethyl methacrylate (GAFQUAT 755N) 2% + lactitol 10 %, polyethyleneimine (PEI) 25 % + HEC 3%). Lactitol provided an additional stability during the curing process. This protection is due to the interaction of its hydroxyl groups with the water present at the surface of the enzyme. Because of it, it was possible to measure the activity of the enzyme long after its inhibition had occurred.

Finally, Nunes[77] applied screen-printed devices modified with co-crosslinked AChE and COPC to the detection of carbamate pesticide in vegetable samples. The analysis was realized directly on vegetable juices or extracts. Juices yielded the best results. The screen-printed format allowed drop analysis that is very attractive for field studies. The same authors[78] improved the performance of the previously developed biosensor by lowering its enzymatic charge and consequently lowering the substrate with the purpose of increasing sensitivity and stability.

5. Other Electrodes

Rehák et al. designed inhibition measurements with a stainless steel filament modified with a lipid bilayer. The phospholipids were functionalized with biotin groups. This modification permits the bonding with the bi-enzyme system formed by AChE and ChO functionalized with the avidin group. This new design produced biosensors that were more stable, displayed a better interaction between the two enzymes, and showed

an increased sensitivity than the one achieved by Mionetto,[79] who used the same bi-enzyme system. The resulting biosensors are built easily, require less quantity of enzyme (μmols) and can be improved by introducing a mediator (TCNQ, for instance) in the bilayer.

6. Characteristic of Amperometric Biosensors Sensitive to Pesticides

Inhibition amperometric biosensors are usually based on a bi-enzyme system (ChE/ChO). Immobilization is achieved usually using adsorption reinforced by crosslinking. An alternative consists in the immobilisation of both enzymes using photo-crosslinking. This procedure yields a higher stability and a lifetime of 1 year.[54] A good integration between the two enzymes is needed to produce good detection limits and a high sensitivity with a reduced response time. This integration is facilitated by lipid membrane systems because they present a less rigid trapping system. A variation on the bi-enzyme approach is to leave the AChE in the solution. This improves the response of the ChO biosensor. The pesticide does not contact biosensor during a previous incubation cycle. It also obviates the regeneration stage because the inhibited enzyme is discarded (ChE), lengthening the lifetime of the device.

However, the tendency is to use a single enzyme system with a substrate (thiocholine ester) that produces an electroactive product (thiocholine). This system is simpler to handle and the number of variables influencing the sensitivity of the device are reduced. Although graphite and platinum are good conductors, they require a high working potential.[60] The detection of thiocoline occurs at elevated voltages (700 to 800 mV) that favor the presense of interferents and the fouling of the surface of the electrode. This is why the electrode is modified with an electrocatalyst that lowers the working potential, especially in graphite-based devices. Cobalt phtalocyanide (COPC) is a widely used catalyst. 4-aminophenyl acetate (PAPA) has been used also as a substrate of AChE allowing the detection of the hydrolysis product at 250 mV.

Clark electrodes, on the other hand, use a gas-permeable membrane that diminishes the influence of interferents, because there are few gases dissolved at room temperature in the more common samples.

Finally, electrodes based on rigid composite technologies seem a novel alternative. These composites provide physical and chemical advantages in addition to their low cost. The matrix of the biocomposite can contain the enzyme and other substances that improve the electrochemical response of the transducer and reduce the working potential (mediators for instance), acting as a reservoir for these materials. Additionally, these materials are compatible with mass production techniques.

As stated above, one of the main limitations of inhibition biosensors is the regeneration stage, which is seldom realized in full. The reactivation of the biosensor surface after it has been subjected to pesticide incubations is usually achieved chemically using reactivating products (2-PAM, etc.). However, the current trend in amperometric detection using biosensors is the application of single-use, inexpensive devices. Screen printing techniques produce surfaces with reproducible bioelectroactive characteristics.

C. Electrochemical Biosensors for the Detection of Cholinesterase Inhibitors

Pesticide analysis using electrochemical biosensors is an economical procedure that renders low detection limits.

Even though potentiometric designs are also fast and precise, they show higher detection limits (see Table 1). Also, the sensi-

tivity to H^+ ions is limited by the buffer solution that consumes a portion of the protons released by the enzyme reaction. This diminishes the magnitude of the potential change.

As it can be seen when comparing Table 1 and Table 2, amperometric methods are generally more sensitive (showing detection limits that are similar to those of potentiometric methods but with lower incubation times) and in many cases produce a signal proportional to the concentration of the analyte. In spite of the few examples found in literature, amperometric designs are suitable for the analysis of pesticides in partially aqueous media.

Even though they have great potential as analytical tools, there are few applications reported for inhibition biosensors in real samples.[6,8,28,45,52,53,77] However, in Germany there is a standardized protocol for a cholinesterase inhibition test[80] based on biosensors. This limited application is due to the main shortcomings of biosensors in general: stability and precision.

III. TYROSINASE INHIBITION

Tyrosinase is a protein with a bifunctional copper. It presents two binding sites one of which has affinity for aromatic compounds (the substrate binding site), and the other has an affinity for coordinating agents that bind to the metal (oxygen site).

A great number of inhibitors have been identified: carbamate and dithiocarbamate pesticides, atrazines, chlorophenols, aromatic carboxylic acids, pyridinones, thioureas, and many compounds with copper chelating properties. However, they do not show inhibition in the presence of metals.

Moreover, the copper site catalyzes two reactions: the hydroxilation of monophenols to *o*-diphenols (*cresolase activity)* and the oxidation of *o*-diphenols to *o*-quinones *(catecholase activity)*.[81]

As shown in Figure 3 in this system, the species produced electrochemically in the detection of *o*-quinone (catechol) is the substrate,[82] and this supposes a signal amplification.

The main advantage of tyrosinase over other enzymes is its remarkable tolerance to high temperatures. This facilitates biosensor fabrication and substrate detection.[83]

Besombes[84] showed for the first time that a tyrosinase electrode can be used to monitor chlorophenols and carbamate pesticides in aqueous media. The electrode was based on the adsorption of a mixture of enzyme and an amphyllic pyrrole monomer on the surface of the electrode. When a potential of 750 mV is applied, electropolymerization takes place on the surface of the electrodes. The main advantage of this biosensor is that it is insensitive to many ionic species present in water (Na^+, Ca^{2+}, heavy metals, etc.). Moreover, this system can be applied to real samples as an alarm for the presence of contamination sources. It also works with a reversible inhibition, facilitating the regeneration process.

According to Zaks and Klibanov,[85] the substitution of the aqueous medium for an organic medium has an inverting effect on the inhibitor selectivity for the enzyme. This is the same as stating that a poor inhibitor in an aqueous medium can be powerful in an organic solvent. This has also widened the range of analytes amenable to inhibition analysis. It has also facilitated the solubility, of many pesticides with low water solubility such as ethyldithiocarbamates, dichlorophenoxiacetic, thiourea, benzoic acid, and dimethyl mercury. These were analyzed for the first time by Wang, who followed the inhibition with an enzyme electrode in organic phase.[86] Tyrosinase is immobilized on the surface of a glassy carbon electrode using the Eastman AQ-55 polymer that traps it. The speed of the response and the reversibility of the inhibition reaction also allow the use of this biosensor in flow injection (FIA) systems for the detection of diethyldithiocarbamates at submillimolar levels.

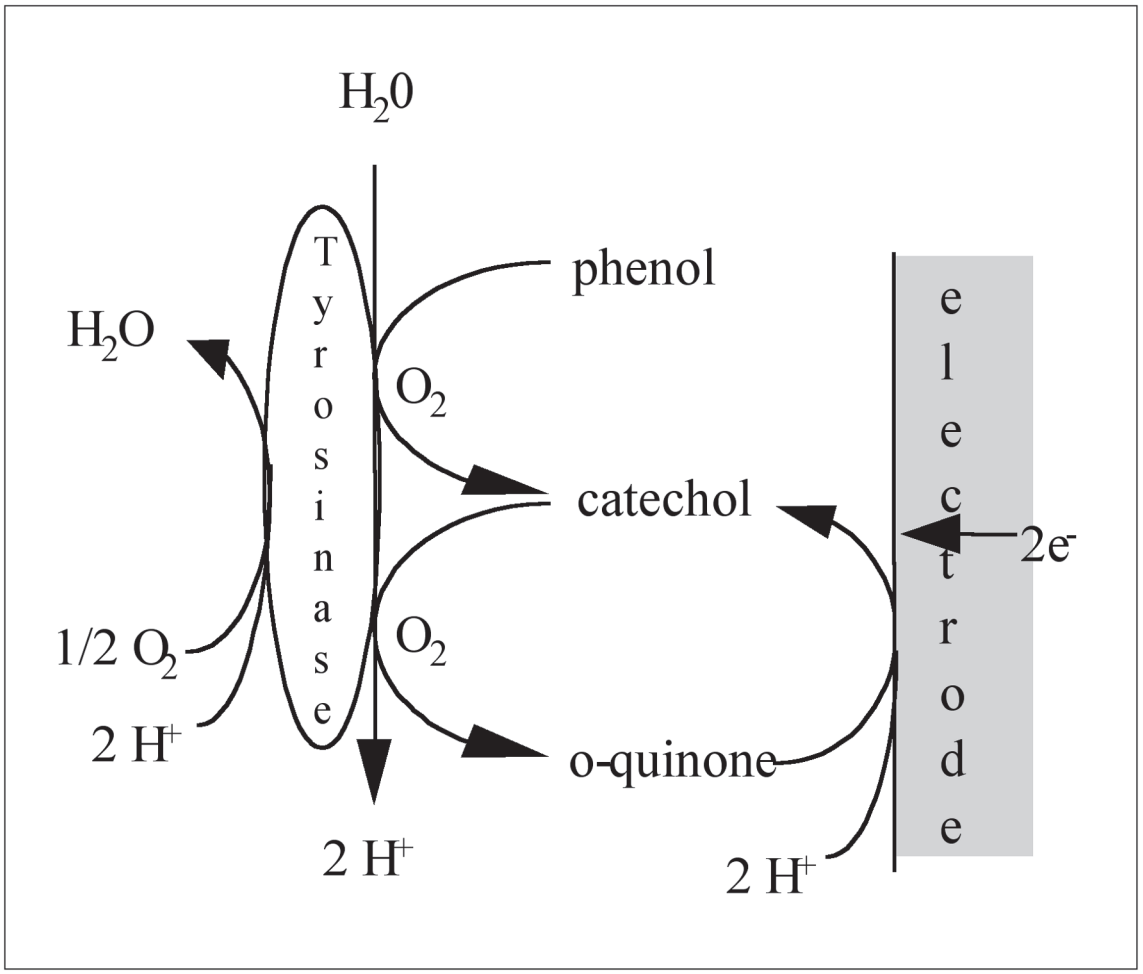

FIGURE 3. Measuring principle of the activity of tyrosinase.

Recently, Everett[87] developed an enzyme system where tyrosinase was immobilized by crosslinking to the surface of a glassy carbon disk. The substrate in this case was 1,2-naftoquinone-4-sulfonate (NQS) (see Figure 4). In the presence of a pesticide, inhibition lowers the speed of the enzyme recycling of NQS. This sensor is particularly sensitive to pesticides that are more water soluble than dichlorvos, as is the case of diethylcarbamate and its hydrolysis product (3-chloroaniline) that can only be measured in organic media. Some researchers are working in mixtures of water and miscible solvents to achieve a better sensitivity of these hydrophobic analytes.[28,58,88]

Wang et al.[83] used a screen-printed electrode with the enzyme immobilization step integrated with the printing process. In this case the tyrosinase was mixed with the graphite and the polymer and then printed on a ceramic alumina plate. The thermal resistance of the tyrosinase allows a high temperature to be used as required by the curing of the composite (110ºC). As there is no membrane involved, shorter response times can be achieved (1 min). Additionally, preincubation is not needed as is the case of AChE biosensors.[89] An adequate homogenization of the enzyme with the paste ensures a high reproducibility between sensors.

Pita et al.[90] studied inhibition applying the reversed micelles systems to the work-

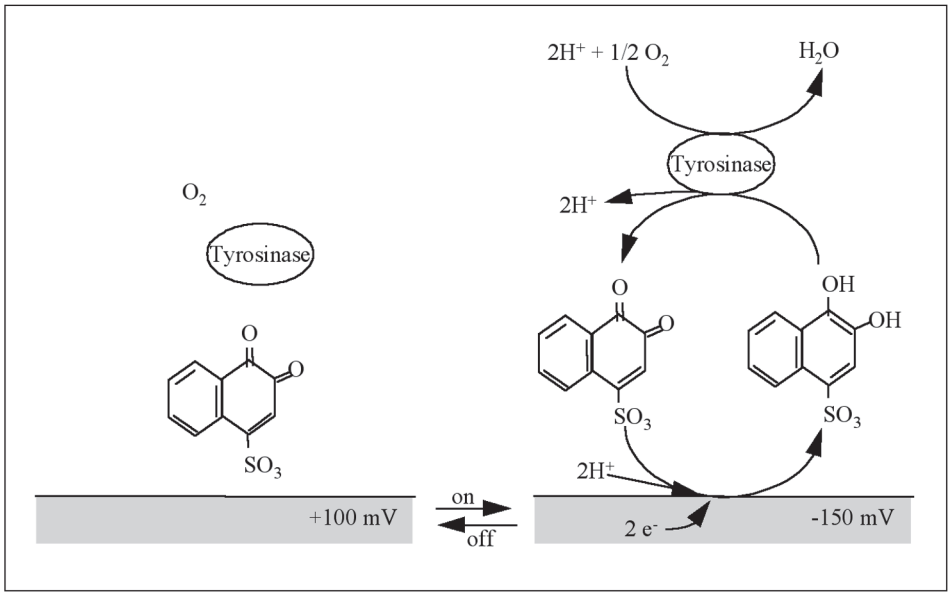

FIGURE 4. Measuring mechanism of tyrosinase activity when NQS is used as substrate. The reaction takes place when a reducing potential of -150 mV is applied and than is ended when a +100 mV potential is applied. The current produced is monitored by chronoamperometry.

ing medium. The reverse micelles system involves ethyl acetate as the organic solvent, dioctyl sulphosuccinate as the emulsifying agent, and a phosphate buffer as the dispersed phase. The use of this medium facilitates the immobilization of the enzyme that can be adsorbed directly to the surface of a graphite disk without losing its activity. Additionally, the analysis of products that do not dissolve well in water can be realized controlling the optimal water content and ensuring the hydration of the enzyme. The proposed system can discriminate between dimethyl- and diethyldithiocarbamate from other compounds of the carbamate family, with the exception of pirimicarb. The biosensor was applied successfully to the determination of ziram in apples sprayed with this pesticide.

Details about the response characteristics of amperometric biosensors based on tyrosinase inhibition are presented in Table 3.I.

IV. INHIBITION OF OTHER ENZYMES

Dithiocarbamate fungicides inhibit aldehyde dehydrogenase (AlDH) irreversibly.[55] It may be thought that these pesticides could be measured by the inhibition of this enzyme monitoring the reduced cofactor. However, oxidation potentials for NADH are very high, and many interferents would be present. However, the authors have achieved this oxidation with the enzyme diaphorase in the presence of the mediator hexacyanoferrate(III) (see Figure 5) permitting the reduction of the working potential (E_{app} = 81 mV).

Two alternatives were under study: the coimmobilisation of both enzymes in a photopolymer matrix of PVA-sbQ or the immobilization of the diaphorase in the polymer matrix while AlDH in solution. Results are shown in Table 4.

This method yields a procedure that is more sensitive than chromatography and is able to measure dithiocarbamate fungicides that have a low solubility in organic sol-

TABLE 3
Response Characteristics of Amperometric Biosensors Based on Tyrosinase Inhibition

Transducer/ Working potential	Immobilization method	Substrate (concentration)	Inhibitor/ Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Rotating graphite disc electrode / -0.2 V (Ag/AgCl)	Electropolymerized film	Dopamine (6 μM)	Chloroisopropylfenil carbamate / 2 μM /	7 days	Buffer solution	---	[84] 1995
Glassy carbon/ -0.2 V (Ag/AgCl)	Entrapment (Eastman AQ-55D)	Phenol (0.2 mM) (organic media)	Diethyldithiocarbamate //	---	Phenol (1mM) t=5 min	----	[86] 1993
Glassy carbon/ -0.15 V (Ag/AgCl)	crosslinking	NQS	Diazinon / 5 μM / Dichlorovos / 75 nM /	---	PBS	---	[87] 1998
Graphite (*Screen-printing*) / -0.2 V (Ag/AgCl)	Biocomposite	Catechol ($5 \cdot 10^{-5}$M)	Diethyldithiocarbamate// 0 min	60 days	Single-use	River Water (Rio Grande)	[83] 1996
Glassy carbon/ -0.2 V (Ag/AgCl)	Adsorption	Phenol (0.4 mM) (reversed miscellar media)	Ziram / 0.074 μM / 0 min Diram / 2 μM / 0 min Zinc diethyldithiocarbamate / 2 μM / 0 min	---	Miscellar media + ethylacetate (10 min)	apples	[90] 1997

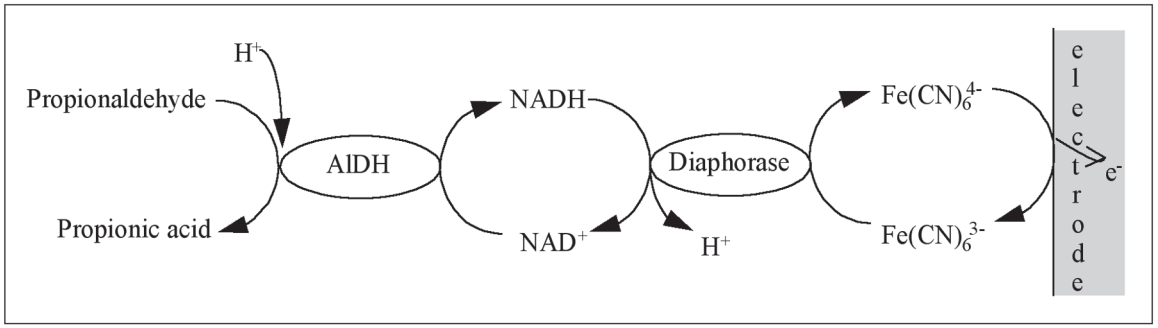

FIGURE 5. Measuring principle of aldehyde dehydrogenase activity using a mediator and a second enzyme, diaphorase.

vents. It is thought that the low stability can be bettered if diaphorase is substituted by NADH oxidase to reoxidate the NADH. BSA and glutaraldehyde were introduced in the gel to prevent leaking.[91]

Later studies,[92] showed that the inhibition of aldehyde dehydrogenase by dithiocarbamate fungicides was shown to be competitive with respect to NAD^+ and non competitive with respect to propionaldehyde. However, it is an irreversible inhibition, and the membrane has to be changed after it has been in contact with the pesticide. Additionally, diaphorase leaks from the membrane because of its low molecular weight.

A new bi-enzyme system has been described to solve the limitation posed by the irreversible inhibition of organophosphorus compounds. This system uses acid (AP) or alkaline (AlP) phosphatase and glucose oxidase (GOD) (see Figure 6). Organophosphorus compounds inhibit reversibly the catalytic action of the phosphatase. This discards the need of a regeneration steps and lengthens the lifetime of the resulting biosensors. Alkaline and acid phosphatase are used because their substrates have a structure that resembles organophosphorus insecticides, and these products can be powerful inhibitors of both alkaline and acid phosphatase.

This bi-enzyme system has been applied to the construction of inhibition amperometric biosensors applied to the detection of insecticides. The corresponding results and technical characteristics appear on Table 5.

Su et al.[93] (1995) used a three-electrode array (auxiliary, reference, and working electrodes) built with screen-printing. Glucose oxidase (GOD) adsorbed at the surface of the working electrode and the alkaline phosphatase (AlP) is added to the electrolytic cell at a concentration of 8 IU/ml. Applying a potential of 700 mV on the working electrode permits the detection of H_2O_2 produced by the enzyme reaction as shown in Figure 6.

Mazzei et al.[94] (1996) presented two different systems. In the first one, acid phosphatase (AP) and glucose oxidase (GOD) were coimmobilized to the surface of a H_2O_2 electrode. A layer of potato tissue was placed on the same platinum electrode in a second design. The tissue is rich in AP, and it contains GOD that had been adsorbed previously**.** Although this system does not have regeneration problems, GOD can also be inhibited by some organic compounds that can interfere with the transduction of the response.

Gouda[95] quantified oxygen decrease at the surface of a Clark electrode with a Teflon® membrane containing a layer of potato tissue rich in acid phosphatase. A second membrane with immobilized GOD was placed on this first membrane using cocrosslinking with gelatine. This new report represents a simple and economical way of measuring pesticides. Additionally, the detection limit achieved by Mazzei[94] was improved.

Sulfonylurides and imidazolinones, used as herbicides, are potent inhibitors of

TABLE 4
Response Characteristics of Biosensors Based on Aldehyde Deshydrogenase (AlDH) Inhibition

Transducer/ Working potential	Enzymatic system	Immobilization method	Substrate (concentration)	Inhibitor/ Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Platinum electrode / 0.081 V	AlDH/diaphorase	/Photo-crosslinking	Propionaldehyde (10^{-3} M)	Maneb / 0.05 ppm / 30 min	---	---	---	[55] 1993
	Diaphorase and AlDH (sol)	/Photo-crosslinking	Propionaldehyde (10^{-3} M)	Maneb / 0.1 ppm / 30 min	---	---	---	
Platinum electrode / 0.081 V (Ag/AgCl)	AlDH /diaphorase	/ BSA photo-crosslinking	Propionaldehyde (0.1 M)	Maneb / 0.188 μM /10 min Zineb / 0.91μM /10 min	2 months	---	---	[91] 1993
Platinum electrode / 0.1 V ($Pt//Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$)	Diaphorase and AlDH (sol)	/Photo-crosslinking	Propionaldehyde (50 μM)	Maneb / > 10 ppb / 10 min Zineb / > 10 ppb / 10 min Mancozeb /> 10 ppb / 10 min	2 months	---	Food matrices	[92] 1997
	AlDH /diaphorase	/Foto-crosslinking	Propionaldehyde (50 μM)			Membrane change		

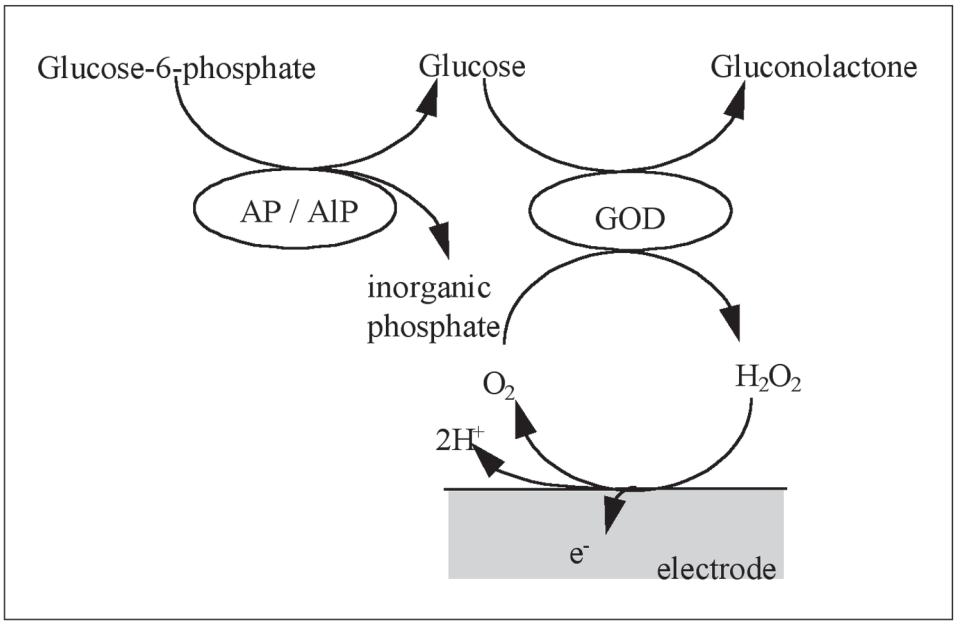

FIGURE 6. Measuring principle of the acid phosphatase catalytic activity.

acetolactate synthase (ALS), a key enzyme for the biosynthesis of the branched chain of the amino acids valine, leucine, and isoleucine. ALS inhibition involves a structural change of the enzyme. This system has used as a biosensor (see Table 6). Marty et al.[55] did semiquantitative studies to determine if this change can take place inside the polymer matrix. Results show that the enzyme can be inhibited despite pesticide diffusion problems in the matrix. Detection limits using ALS on a carbon dioxide electrode (see Figure 7) show an improvement over HPLC methods.

An enzyme sensor was developed to detect sulfonylurides. It was based on the inhibition of oxigenase activity from ALS. Seki et al.[96] trapped this enzyme in a photocurable matrix that was in turn immobilized on the surface of a Clark electrode (see Figure 7). These herbicides could only be quantified using conventional and cumbersome analytical methods (gas chromatography coupled to mass spectrometry, HPLC, etc.). The biosensor provides a quick, sensitive, and inexpensive detection method for these substances.

Finally, Wang used peroxidase inhibition by ethyldithiocarbamates pesticides in acetonitrile.[86]

V. FINAL REMARKS

The studies presented to date show the simplicity and the ease of the application of electrochemical biosensors to pesticide analysis. Although they are an alternative to chromatographic methods, biosensors have shortcomings: their robustness, both physical and chemical, is limited by the stability of the biological component due to inhibition.

Even though electrochemical biosensors are an option for pesticide analysis, a number of problems have to be solved before they can have a significant commercial impact. Some of these problems are related to the irreversible character of enzyme reactions. As mentioned above, regeneration techniques are still limited either because complete activity of the enzyme cannot be recovered or because it entails a thorough manipulation of the sensor or a regeneration period that is too long. Because of this, single-

TABLE 5
Response Characteristics of Biosensors Based on Alkaline Phosphatase (AlP) and Acid Phosphatase (AP) Inhibition

Transducer/ Working potential	Enzymatic system	Enzyme Support / Immobilization method	Substrate (concentration)	Inhibitor/ Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
Graphite *(screen printed)*/ 0.7 V (Ag/AgCl)	GOD AlP (sol)	/Adsorption	Glucose-6-phosphate (25 mM)	Dichlorvos / 15 ppb / min	---	no (reversible)	---	[93] 1995
Platinum electrode/ 0.65 V (Ag/AgCl)	AP / GOD	Nylon mesh/ Covalent	Glucose-6-phosphate (0.5 mM)	Malathion/ 3 $\mu g \cdot L^{-1}$/ 20 min Parathion Methyl /0.5$\mu g \cdot L^{-1}$/ 20 min Paraoxon/ 5$\mu g \cdot L^{-1}$/ 20 min	2 week	no (reversible)	---	[94] 1996
	AP / GOD	Tissue (potato rich in AP) / GOD adsorption	Glucose-6-phosphate (0.5 mM)	Malathion/ 1.5 $\mu g \cdot L^{-1}$/ 20 min Parathion Methyl /0.5$\mu g \cdot L^{-1}$/ 20 min Paraoxon/ 1.5$\mu g \cdot L^{-1}$/ 20 min	2 week	no (reversible)		
Clark electrode/ -0.65 V (Ag/AgCl)	AP / GOD	Tissue (potato rich in AP) / GOD (gelatine co-crosslinking))	Glucose-6-phosphate (38.7 mM)	Paraoxon / 1 μM / 10 min	8 days	no (reversible)	---	[95] 1997

TABLE 6
Response Characteristics of Biosensors Based on Acetolactate Syntethase (ALS) Inhibition

Transducer	Enzyme support / Immobilization method	Substrate (concentration)	Inhibitor/ Detection limit / Incubation time	Storage time	Regeneration (time)	Application	[Reference] Year
CO_2 electrode	/Entrapment and photo-crosslinking	Pyruvate (50 mM)	Tiphensulfuron methyl/ >19 ppb/	2 weeks	---	---	[55] 1993
Clark electrode	/ Entrapment and photo-crosslinking	Pyruvate (2.2 mM)	Sulphometuron methyl/10^{-6}M/ 30min Chlorosulphuro methyl /10^{-6}M/ 30 min Tiphensulfuro methyl /10^{-6}M/ 30 min Imazaquin /10^{-6}M/ 30 min Imidazolinone /10^{-6}M/ 30 min	---	---	---	[96] 1996

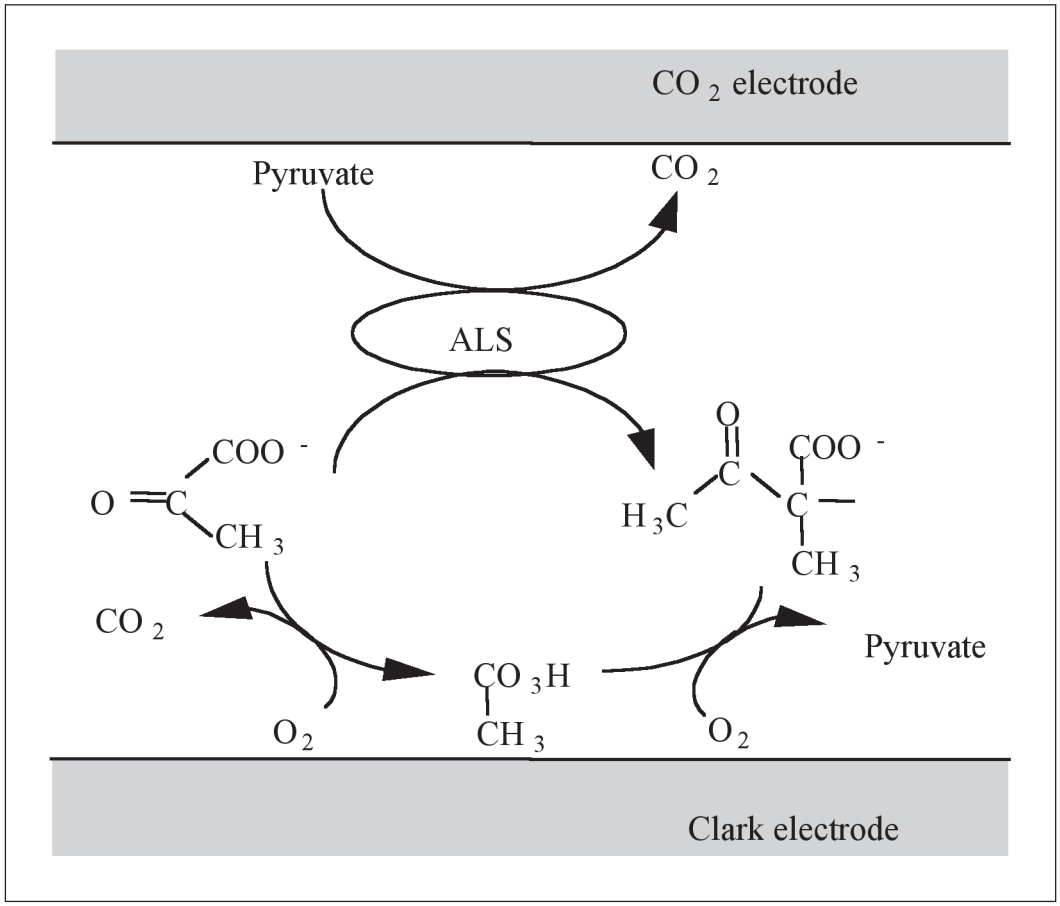

FIGURE 7. Measurement of acetolactate syntethase (ALS) activity. The transduction can be via a CO_2 electrode or a Clark electrode according to the proposed mechanism

use devices seem to be the most appropriate format for these devices.

A common feature of enzyme inhibition applied to complex environmental samples is the global selectivity of the measurement. Should a narrower selectivity be required, these samples ought to be analyzed by an array of several biosensors. Because of this, Danzer and G. Schwedt[97] immobilized three enzymes on a pH-sensitive glass electrode using a cellulose membrane. These enzymes were acetylcholinesterase, acid phosphatase and alkaline phosphatase. They showed that the combination of the three sensors yielded more information about pesticides and heavy metals on the sample.

Experimental designs and biosensor optimization by the simplex factorial method were treated in a first publication. The data obtained were analyzed on a second paper[98] using the method of multidimensional variance and discriminant analysis (MVDA) or multivariance techniques such as cluster or factor analysis. The results obtained with the three biosensors show that the application of chemometric methods yields additional information (discerning antagonic and additive interactions or the synergic influences among the variables) from a mixture of pollutants present in a real sample.

As stated previously, the sensitivity to pesticides depends on the source of the enzyme.[59,99,100] Until now the more readily available enzyme was the one used. At present this limitation has been overcome by cloning the gene that codifies AChE from differ-

ent species and also its *in vitro* expression in eukaryotic cells.[2] This also permits to mutate the enzymes, modifying its primary structure. This changes the catalytic properties of the enzyme, making it feasible to raise the sensitivity for a group of inhibitors.

Recently, a new enzyme system has been studied. It comprises the immobilization of organophosphorus hydrolase (OPH), a biological catalyst that hydrolyzes efficiently organophosphorus compounds (parathion, coumaphos) and biological agents of military importance (soman, sarin, VX, etc.). Catalytic hydrolysis of each molecule of these products releases 2 H^+. Mulchandani et al.[101] has built and characterized a potentiometric biosensor using *E. coli* cells that express OPH on their surface. Immobilization takes place by adsorbing the cells to a poycarbonate membrane that is fixed to the surface of a pH electrode. A second approach simplified the process by immobilizing the enzyme directly using co-crosslinking at the electrode surface.[102] This allowed a more direct, quick, and selective detection of organophosphorus nerve agents without needing the regeneration of the biological material.

These biosensors offer a system that is fast, simple, and selective for the monitoring of organophosphorus compounds. Furthermore, they can be used in the monitoring and on-line control of detoxifying processes. However, the detection limits are in the micromolar range and they are insufficient to be applied to environmental samples. This limitation made these authors build a screen-printed device where they trapped OPH.[103] The polarization of this electrode to a fixed potential of 850 mV allows the detection of the hydrolisis product (*p*-nitrofenol). In this case the detection limit reaches the nanomolar range. However, the system reported cannot quantify the toxicologic index (expressed as the amount of inhibitor that causes an inhibition percentage equivalent to that of a known amount of pesticide (paraoxon o parathion) chosen as a reference).

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