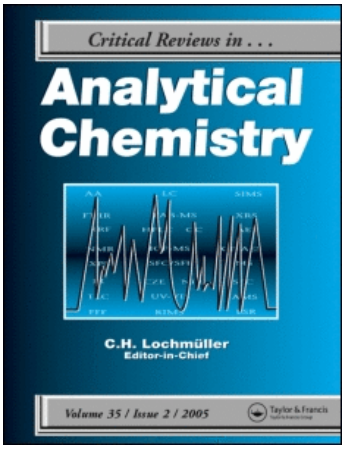

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Determination of Toxic Substances Based on Enzyme Inhibition. Part II. Electrochemical Biosensors for the Determination of Pesticides Using Flow Systems

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ABSTRACT: Enzyme inhibition coupled to flow injection systems set-ups are a powerful tool for the monitoring of environmental toxics. The present review examines the latest contributions to this field where electrochemical detection is used. The advantages and limitations of the FIA technique in the context of enzyme inhibition analytical procedures are discussed. The present review is organized in accordance to the enzyme (cholinesterases, tyrosinases, or alkaline phosphatase) used in the measurement.

KEY WORDS: pesticides, cholinesterase, tyrosinase, alkaline phosphatase, flow injection analysis, electrochemical detection.

I. INTRODUCTION

The development of biosensors to measure pesticides in discrete or batch systems using enzyme inhibition presents a number of advantages compared with classic manual procedures and in some cases to conventional instrumental methods (gas chromatography, liquid chromatography, etc.). These aspects have been reviewed thoroughly in the first part of this review.[1]

The practical application of discrete or batch systems based on inhibition biosensors is limited by the high number of steps needed for the measurement procedure. The biosensors used in these systems have shortcomings for the continuous batch measurement of pesticides and other inhibitors. They show stability problems that call for a special treatment of the sample and of the biosensor surface. It is advisable to automate the inhibition procedure if systems capable of monitoring inhibitors on-line are needed. These automated systems should comprise the treatment of the sample and of the biosensor surface.

Among automated systems, flow injection analysis (FIA) has become a very versatile and efficient technique for quantitative automated analyses since its introduction 20 years ago.[2] This responds to the straightforward integration of the operations involved, including sample injection, reagent transport at preset flow rates, easy handling of the flow system, and its ability to accommodate different detection systems. The combination of biosensors with flow injection (FI) techniques permits the automation and integration of the steps involved in inhibition analysis. Additionally, this technique allows for the control of the reagent addition steps, the measurement of enzyme activity, and also simplifies the optimization of the conditions of the reaction.

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There are two different strategies for the integration of the biological material in a flow system. The biological material is immobilized onto a transducer (forming a biosensor) or it is immobilized inside a reactor placed upstream from the transducer forming a bioreactor (see Figure 1). Both strategies show distinct advantages (Table 1) and the use of either one will depend on the analytical problem at hand.

An automated biosystem is produced when the biological material is integrated to a flow system. The automated analytical biosystems can be used for on line monitoring of toxic substances based on enzyme inhibition. They can be used also for sample screening prior to the use of sophisticated measurement systems.

In a continuous flow system not only the analytical process has to be automated, but also the regeneration of the biological material that has been inhibited[3,4] or at least the substitution of the inhibited enzyme.[5] In principle, the application of changeable parts (in biosensors or bioreactors) simplifies the operation of the flow system, enabling the continuous use of the system after it contacts an irreversible inhibitor.[6] However, the recovery of the activity of the inhibited material is one of the main obstacles for the automation of analyses using flow systems.

Although the application of FIA systems to inhibition measurements contains a number of operations (mixing of working solutions, incubation step, biosensor surface cleaning between measurements, etc.), the measuring time is reduced three to four times when compared with inhibition-based batch methods. These facts have provoked the rise in the number of reports on inhibition detection based on flow systems.

Just as in discrete systems,[1] the biosensor detection in flow systems can be electrochemical, optical, mass, etc. The present review focuses on the latest advances in the electrochemical detection of pesticides using enzyme inhibition detection in flow injection systems.

II. CHOLINESTERASES INHIBITION

Several systems combining cholinesterases and flow systems have been described. These systems use the enzyme either immobilized or in the solution. The reaction mechanisms are described in Part I of this review.[1] Systems reviewed here include mono-enzyme set ups with potentiometric and amperometric detection and bi-enzyme systems with amperometric sensing (ChE and ChO) both in the biosensor or bioreactor configuration (see Table 2).

Flow systems based on electrochemical detection using cholinesterase inhibition for the measurement of organophosphorus and carbamate pesticides reported during the last decade are presented below.

A. Potentiometric Detection

As stated in the first part of this review,[1] both the substrate and the number of enzymes to be used define the choice of the transducer type. If one enzyme is used (ChE) and the substrate is R-choline, the detection will be based on the potentiometric measurement of the pH change. The most representative systems using potentiometric detection are presented below. More information about the response characteristics and other experimental details are displayed in the Table 3.

Until recently, one of the more widely used supports in the construction of microreactors was CPG5 (controlled-pore glass). Although it is an adequate support for the immobilization of the biological material, the hydrolysis at basic media may lead to the loss of the enzyme. Additionally, its commercial presentation does not have a uniform size and shape. This lack of uniformity obstructs the packing of the enzyme in the reactor, creating frequent problems for the adequate transport inside the flow system in the form of overpressures and bubbles.[7] Kumaran and Tran-Minh[8] (1992)

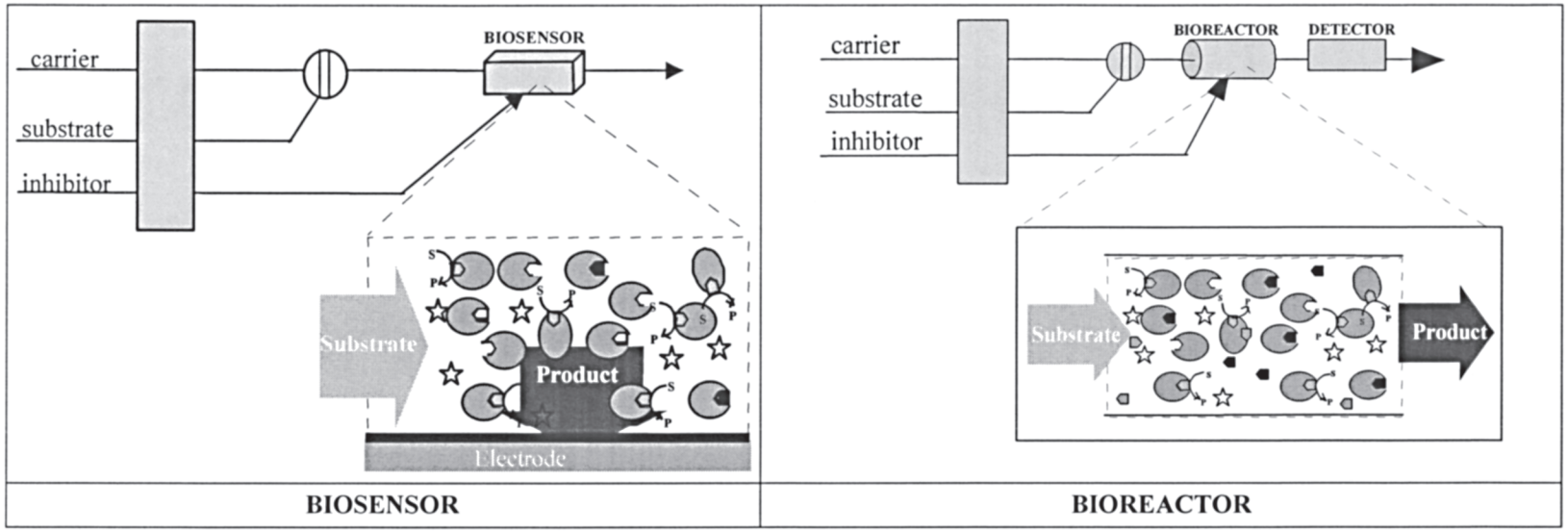


FIGURE 1. Biological material integration strategies used in FIA systems. (Enzyme); (Interferent); (Inhibitor); (Substrate)

TABLE 1
Some Advantages of Both Biosensor and Bioreactor Configurations in FIA System

BIOSENSOR	BIOREACTOR
More sensibility (as the detection and the reaction occur on the same transducer)	More quantity of biological material is permitted
Faster response time	A conversion till 100% is possible
Less dispersion of the sample	The recirculation and as result the preconcentration is permitted.
Shorter analysis time	Interferences can be avoided using a posterior treatment that follows enzyme reaction.
Robust	Measurement in equilibrium conditions.
Simple	Less influence of pH variation, ionic strength, temperature, presence of activated species or inhibitors in the sample matrix.
Compact	Better stability and shelf life time
Easy manipulation	
Less adsorption (as less contact surface)	

substituted CPGS with SBSR (single bead string reactor). This system (configuration B, Table 2) offers a better micromixture of the reagent and the sample, a lower dispersion of the sample, a low resistance to flow, and it is easy to build and inexpensive. It also facilitates the regeneration process. The reactivation was conducted in the presence of the reactivator alone (2-pyridine aldoxime methiodide (PAM)).

As shown in Section II.A of the first part of this review,[1] the small size, and the easy availability of solid-state potentiometric electrodes added to the easy measurement technique and its high selectivity render sensors highly desirable for field or on-line pesticide analysis. All of the solid-state AChE sensors mentioned previously (enzyme membranes on the surface of ISFET or hydrogen-ion-selective coated-wire electrodes) showed great promise, but some problems need to be overcome. The most problematic area in these sensors is the interface between the enzyme membrane and the solid sensitive phase of the electrode.[9,10] These sensors are not ideal; they are unstable and highly sensitive to interferences. In real systems, solid-state enzyme electrodes exhibit low signal drift but marked sensitivity to changes in ionic strength and buffer capacity.

Several methods have been developed to overcome these problems.[11] One approach involves amperometric detection, which is discussed in the next section.[12]

It is in this context that Ivnitskii and Rishpon[13] developed a new solid-state AChE sensor based on the AChE-acetylthiocholine-hexacyanoferrate (III) reaction (see Figure 2). The response of the sensor was induced by two phenomena: the Donnan effect[14] due to the enzyme layer and the redox potential. Unlike pH-sensitive sensors, this type of sensor was operated at high ionic strength and a high buffer capacity, raising the sensitivity of the biosensor.

B. Amperometric Detection

Although the use of amperometric detectors in flow systems pose a complication because of the passivation of the surface of the electrode, the devices reported show a higher sensitivity to pesticides (see Table 4). Amperometric detection is associated with a single enzyme (ChE) and an electroactive substrate such as R-thiocholine or 4-aminophenyl acetate (PAPA). When the substrate is a R-choline, two enzymes (ChE and ChO) are needed for the measurement because this substrate is not electroactive.[1]

TABLE 2
Proposed FIA Systems for Automatic Enzyme Inhibition Determination

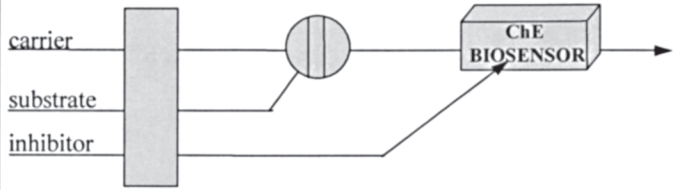
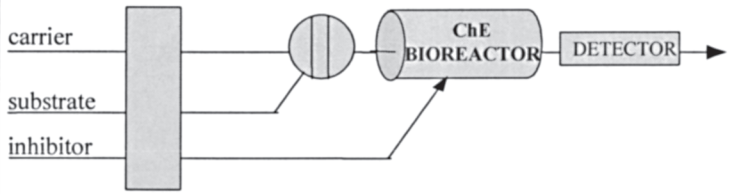
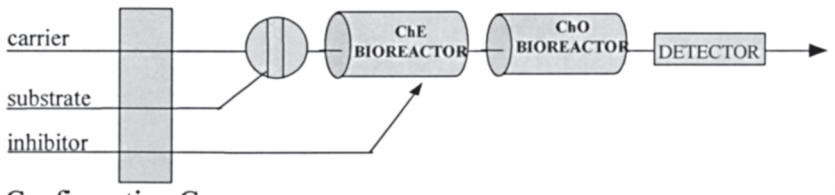
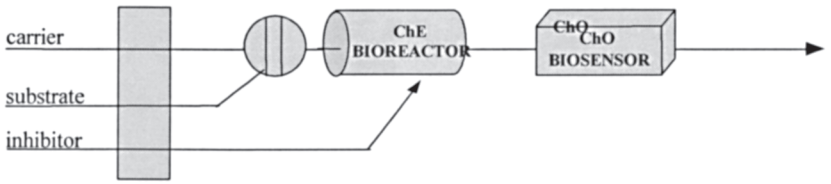
FLOW SYSTEM	DETECTION	REFERENCE
 Configuration A	*Potenciometric* *Amperomtric*	[13] [6, 17, 21, 23, 24, 28-30]
 Configuration B	*Potenciometric* *Amperometric*	[8] [19]
 Configuration C	*Amperometric*	[5]
 Configuration D	*Amperometric*	[16]

TABLE 3
Response Characteristics of FIA Systems Based in Cholinesterase Inhibition and Potentiometric Transduction

Transducer	Enzyme/Support/ immobilization procedure	Configuration	Substrate (concentration) / $V_{Injection}$	Inhibitor/ Detection limit / $V_{injection}$ / Incubation time	Sample	Regeneration (time)	Carrier/ Flow rate	[Reference] Year
pH electrode	AChE / glass beads /covalent	SBSR (single bead string reactor)	ACh^+ (0.5 mM) / 250 µl	Paraoxon / 25 ppb Carbofuran / 6 ppb Dichlorovos / 50 ppb Malathion / 0.5 ppb (....) Vinj = 6.75 ml	Sea Water	2-PAM (20 µM) (15 min)	HEPES 2.5 mM + $MgCl_2$ 20 mM + NaCl 100mM + gelatin 0.01 % / 0.45 $ml \cdot min^{-1}$	[8] 1992
Glassy carbon disc electrode	AChE / polyethylenimine membrane / crosslinking + covalent	Biosensor	ATCh (0.04 mM) / 50 µl	Dichlorvos 1.5 µM (1-2 min) Dichlorvos 0.2 µM (10 min) Paraoxon 10^{-8} M (10 min) Vinj = 50 µl	---	2-PAM (0.5 mM) (35 min)	Citrate buffer 100 mM, NaCl 50 mM, $MgCl_2$ 0.01 M, $K_3[Fe(CN)_6]$ 0.1 mM pH 7.5 0.25 ml/min	[13] 1994

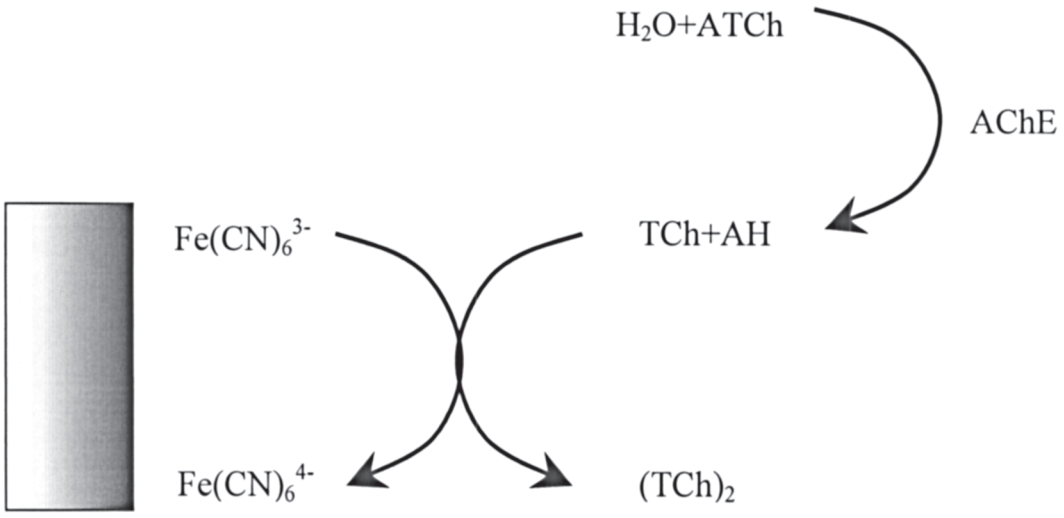


FIGURE 2. Mechanism of the reaction that occurs at the surface of the AChE biosensor.

Kindervater (1990)[5] used a bi-enzyme system featuring acetylcholinesterase (AChE) and choline oxidase (ChO) (configuration C, Table 2). The substrate is hydrolyzed to choline in the first reactor with AChE immobilized on magnetic particles. Choline is then oxidised to betaine in a second reactor where ChE is immobilised on CPGS. The hydrogen peroxide produced by the second reaction is detected amperometrically on the surface of a working electrode polarized at 700 mV. The flow system is designed in the manner that the sample is wasted out at the exit of the first reactor where it inhibits the AChE. In this fashion the pesticide is prevented from contacting the enzyme ChO. The residual enzyme activity of AChE is detected by injecting substrate. The main advantage of this system is that the magnetic field is turned off and the magnetic particles with the inhibited AChE are released once the analysis is done and the system is then ready to receive a new injection of magnetic particles with fresh AChE immobilized on it. Additionally, the magnetic bioreactor offers low resistance to the flow.

Using a chromatography column with hydroxyethylmethacrylate gel and a mobile phase containing acetylcholine, Salamoun and Remien[15] separated different inhibitors [tetramethylammonium (TMA), galantamine (Gal) and physostigmine (Phy)]. The AChE/ChO enzyme system is immobilized in a second microcolumn placed after the chromatography column. A fixed potential of 450 mV (Ag/AgCl) applied to a platinum electrode detects the presence of hydrogen peroxide. This system raises the selectivity since only AChE-inhibiting compounds are previously separated in the chromatography system. The miniaturization of the system reduces costs and requires less enzyme.

A new report by Botrè et al.[16] aims at reducing the cost of the system. They use two enzymes, AChE bioreactor and the amperometric detection based on a ChO biosensor (configuration D, Table 2). The novelty is the use of immobilized tissue from a plant rich in AChE (grapefruit shell, *Albedum pomi citreum*). The design of the system with separate enzymes permits the recirculation of the sample through the bioreactor, raising the contact time between the inhibitor and the enzyme. This compensates for the lower enzyme activity that results from using whole tissue instead of purified enzyme. Also, the quitine used to immobilize the AChE is inexpensive and biocompatible.

TABLE 4
Response Characteristics of FIA Systems Based in Cholinesterase Inhibition and Amperometric Transduction

Transducer/ Mediator/$E_{applied}$	Enzyme/Support / Immobilization System	Configuration	Substrate (Concentration) / $V_{Injection}$	Inhibitor/ Detection limit (M) $V_{injection}$ / Incubation time	Application	Regeneration (time)	Carrier / Flow-rate	[Reference] Year
LC amperometric detection (Commercial) / 0.7 V	AChE / magnetic particles/covalent ChO / CPGs	Bioreactor + Bioreactor	ACh^+ /	Carbofuran 0.5 µg/L (*stopped flow* 30 s)	--	Switching off the magnet	Phosphate buffer 0.1 M pH 7.5/ 1.0 $ml \cdot min^{-1}$	[5] 1990
Platinum electrode // 0.45 V (Ag/AgCl)	AChE / glass microfibre filter ChO / beads	Microreactor	ACh^+ (0.1 mM)	TMA / 1 nM Gal / 1p M 2 µl / 60 $µl \cdot min^{-1}$	--	Buffer solutiona	Phosphate buffer 0.1 M pH 7.4, EDTA 0.1 mM, ACh^+ 10^{-4} M / 60 $µl \cdot min^{-1}$	[15] 1992
H_2O_2 sensing amperometric electrode (commercial)//	AChE (tissue) // crosslinking ChO / nylon membrane	Bioreactor +Biosensor	ACh^+ (0.5 mM)	Aldicarb / 100 ppb Malathion / 1 ppb Paraoxon / 10 ppb (sample recycling time : 40 min)	--	2-PAM 10^{-4} M (20 ml)	Phosphate buffer 0.1 µM pH 7.5 / 0.5 $ml \cdot min^{-1}$	[16] 1994
Glassy carbon // 0.25 V (Ag/AgCl)	AChE / nylon grids	Biosensor	PAPA (1.2 mM) / 150 µl	Carbaryl / $4 \cdot 10^{-7}$ M Paraoxon / $4 \cdot 10^{-7}$ M (150 µl) / t< 5 min	Lagoon water, fruits	2-PAM (0.248 $mg \cdot ml^{-1}$) (4h) + buffer solution (2h)	Phosphate buffer 0.08 M pH 8 / 0.55-0.34 $ml \cdot min^{-1}$	[17] 1995
Pt thick film electrode // 0.6 V	AChE / magnetic particles	Magnetic bioreactor	$ATCh^+$ (6 mM) / 22 µl	Carbofuran/ 3 $µg \cdot l^{-1}$ Paraoxon-etil / 3 $µg \cdot l^{-1}$ Paraoxon-methyl / 10 $µg \cdot l^{-1}$ Malaoxon / 20 $µg \cdot l^{-1}$ 22 µl / 1min flow+ 9 min *stopped flow*	Drinking and waste water	Switching off the magnet	Phosphate buffer 0.01 M pH 8 / 1 $ml \cdot min^{-1}$	[19] 1995

Transducer/ Mediator/$E_{applied}$	**Enzyme/ Support / Immobilisation System**	**Configuration**	**Substrate (Concentration) / $V_{Injection}$**	**Inhibitor/ Detection límit (M) $V_{injecrion}$ / Incubation time**	**Aplication**	**Regeneration (time)**	**Carrier / Flow-rate**	**[Reference] Year**
Rotating disc glassy carbon electrode // 0.25 V (calomel electrode)	AChE / Gold-coated nylon meshes /	Biosensor	PAPA () / 150 µL	Paraoxon / $5 \cdot 10^{-8}$M Carbaryl / $5 \cdot 10^{-8}$ M	--	0.0248 mg·ml^{-1} 2-PAM (4h) + phosphate buffer (2h)	Phosphate buffer 0.08 M pH 8 / 0.33 ml·min^{-1}	[20] 1996
Screen-printed/ CoPC / 0 V (Ag/AgCl)	AChE (1U/electrode) // Covalent	Biosensor	$ATCh^+$ (1 mM) / 20 µL	Dichlorvos / $6 \cdot 10^{-9}$ M Paraoxon / $7 \cdot 10^{-11}$ M	--	no (single use)	Phosphate buffer 0.1 M pH 7.0 /	[21] 1997
Screen-printed/ CoPC / 0 V (Ag/AgCl)	AChE (0.05U/electrode) // Covalent	Biosensor	$ATCh^+$ (1 mM) /20µL	Paraoxon / $4 \cdot 10^{-11}$ M / 20 min				
Reticulated vitreous carbon electrode (RCV) / 0.25 V (Ag/AgCl)	AChE / Superporous agarosa gel/	Biosensor	$ATCh^+$ (0.4 mM) /	Paraoxon 0.5 nM 100 µl / 1ml·min^{-1}	--	----	Phosphate buffer 0.1 M pH 7, Meldola Blue 5 µM / 1ml·min^{-1}	[23] 1998
Platinum wire / 0.41 V (Hg/Hg_2Cl_2)	AChE / / foto-crosslinking	Biosensor	$ATCh^+$ ($2 \cdot 10^{-4}$ M) /	Paraoxon / $4 \cdot 10^{-9}$ M / 10 min	--	2-PAM 1mM (7 min)	Phosphate buffer 50 mM, NaCl 30 mM, KCl 1.3 mM, $MgCl_2$ 2 mM pH 8 / 1.5 ml·min^{-1}	[24] 1998

TABLE 4 (continued)

Transducer/ Mediator/$E_{applied}$	Enzyme/ Support / Immobilisation System	Configuration	Substrate (Concentration) / $V_{Injection}$	Inhibitor/ Detection límit (M) $V_{injecrion}$ / Incubation time	Aplication	Regeneration (time)	Carrier / Flow-rate	[Reference] Year
Epoxy carbon-paste electrode / / 0.61 V	BChE /nylon /crosslinking	Biosensor	$BTCh^+$ ($1.2\ 10^{-4}$ M) / 45 µl	Diazinon / $4{\cdot}10^{-9}$ M / 10 min	--	TMB-4 0.1 % (10 min)	Phosphate buffer 0.002 M, Na_2SO_4 0.1 M	[6] 1999
Epoxy carbon-paste electrode / / 0.61 V	BChE / cellulose nitrate / crosslinking	Biosensor	$BTCh^+$ ($1.2\ 10^{-4}$ M) / 45 µl	Diazinon / $1.5{\cdot}10^{-9}$ M / 10 min	--	TMB-4 0.1 % (10 min)	Phosphate buffer 0.002 M, Na_2SO_4 0.1 M	
Epoxy carbon-paste electrode / / 0.61 V (Ag/AgCl)	BChE / White tracing paper / crosslinking	Biosensor	$BTCh^+$ ($1.2\ 10^{-4}$ M) / 45 µl	---	--	---	Phosphate buffer 0.002 M, Na_2SO_4 0.1 M /	
Screen-Printed // 0.3 V (Ag/AgCl)	AChE/ nylon membrane	Biosensor	$ATCh^+$ (1 mM) / 5 µl	DDVF // 30 µl/ 3min	---	2-PAM (0.1 mM) 1 µl/min	Phosphate buffer 0.1 M + KCl 0.1 M a pH 7.5 + $K_3[Fe(CN)_6]_3$ / 100 µl/min	[29] 2000
Screen-printed / / 0.76 V (Ag/AgCl)	AChE / magnetic particles/covalent	Biosensor	$ATCh^+$ /	Paraoxon / $6.7{\cdot}10^{-12}$ M	--	Switching off the magnet	PBS pH 7.0 /	[30] 2000

This permits the future application of this system to the detection of ACh, and AChE inhibitors (micotoxins, for example) in biological samples.

La Rosa et al. (1995)[17] used a flow system to automate their analyses based on enzyme inhibition. The automation, presented previously in 1994,[18] permits the analysis of real samples of lake water and vegetable samples such as kiwis, apples, and grapes. The sensitivity achieved was comparable with standard methods such as LC. These authors used an amperometric flow cell that contained a Nylon membrane with immobilized AChE (configuration A, Table 2). The main advantage of the described method, as stated in the first part of this review,[1] is the use of the substrate 4-aminophenyl acetate (PAPA) that allows for lower working potentials (250 mV vs. Ag/AgCl) without a mediator. In this fashion the time of analysis is reduced (as lower preincubation times produced the same sensitivity) and interferences are reduced.

Günther and Bilitewski[19] (1995) simplified the work of Kindervater[5] following now configuration B, Table 2 using a single enzyme (AChE) immobilised to magnetic particles activated with amino groups. The results obtained facilitated the application of the flow system to the screening of tap and residual waters. The correlation was good without false negatives. The same tests were applied to colourimetric detection and a good correlation was noticed between the two detection methods.

Pariente et al.[20] included gold-coated nylon meshes with the enzyme AChE immobilized in a flow system and using PAPA as the substrate. The modified meshes did not limit transport, so lower flow rates were used raising the sensitivity without prolonging the recovery time of the baseline and thus raising the frequency of analysis.

In addition to systems with bioreactors, the literature shows reports of the direct use of ChE biosensors (configuration A, Table 2) with substrates that produce electroactive products (RTCh) that had been used previously in discrete systems.[1] These reports are reviewed highlighting important aspects of their application to flow systems.

Rippeth et al.[21] used their AChE biosensor based on a screen printed electrode modified with cobalt phtalocyanine (COPC) as the transducing system. When compared with results from discrete set ups,[22] the conclusion is that flow systems yield a higher sensitivity as the dilution is lower.

Khayyami et al.[23] also carried out studies of the behavior in flow conditions of a biosensor featuring AChE immobilized in reticulated graphite (RVC). The optimization of the system produced good detection limits, especially for the electrode modified with agarose gel because it immobilizes more enzyme. However, this modification entails longer times for the analysis of the samples because the polymer increases the sample dispersion and consequently widens the FIA peaks.

Jeanty and Marty (1998)[24] applied a biosensor described in 1995[25] to pesticide analysis using flow injection. AChE was immobilized to a photocurable polymer on the surface of a platinum electrode. The inhibition test lasts 1 h and includes a regeneration step with 2-PAM (2-pyridinealdoxime methiodide). This is a lower time than that reported for discrete systems. The stability of the immobilization was demonstrated as the biosensors were used for 3 weeks and six daily cycles.

Recently, Evtugyn[6] applied his cholinesterase biosensors to flow systems to detect reversible and irreversible inhibitors (diazinon). He compared the three membranes (white tracing paper, Nylon, and cellulose nitrate) used as the support for BChE. These results were compared to those produced with BChE free in the solution. The detection limits for Nylon and cellulose nitrate were similar to those of the free enzyme. Results with tracing paper were not reproducible, contrary to what was observed

in discrete systems.[26,27] This inconsistency is due to the resistance posed by paper to the mass transport of the reagents and its lack of permeability.

A novel detection strategy has been produced by Makower et al.[28] who built an enzyme affinity system to detect organophosphorus compounds. The strategy is based on a preincubation of the analyte with an excess of a bienzyme conjugate consisting of BChE as the receptor and HRP used to generate and amplify the analytical signal. The solution containing BChE-HRP-analyte passes through an affinity column containing immobilized paraoxon. The excess conjugate bonds irreversibly to the paraoxon. The peroxidase produces the analytical signal of the eluent and is proportional to the organophosphorus compound in the sample. The application of the FIA system facilitates the procedure and lower detection limits of 1 p*M* diisopropylfluorophosphate are achieved.

Neufeld et al.[29] presented a disposable screen-printed electrode, covered by an enzymatic membrane and located in a micro flow injection system for the identification and quantification of highly toxic organophosphorus compounds. The use of $Fe(CN)_6^{3-}$ in the working solution permits the detection at 300 mV, due to the fact that the TCh product of the enzyme reaction reduces the $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$ and the consecutive reoxidation by the electrode generates a measurable electric signal.

The main limitation of the previously described systems is the regeneration of the biological material because it lengthens the time needed to complete one analysis. To improve the regeneration step, a biosensor with a renewable surface based on magnetic particles integrated to a flow system is designed in our laboratory.[30] The main advantage of this system is that once an analysis has been done, the biological phase is liberated as the magnetic field is released. After a brief washing step, the system is ready to receive a new injection of magnetic particles. Although Günther[19] had described a similar system previously, our main innovation was that the magnetic particles are immobilized on the transducer, constituting a new biosensor at each injection step of magnetic particles. This brings the biological material and the transducer closer together, potentially raising the sensitivity and reducing the response time because the preincubation and the detection take place in the same flow cell. Additionally, our system[30] is more compact and robust than other systems reported previously and featuring magnetic bioreactors.[5,19]

C. Conductimetric Detection

Rodrigues et al. (1997)[31] described a conductimetric flow injection system to measure paraoxon. It is based on the measurement of acetate produced by an enzyme reaction of acetylcholine and acetylcholinesterase. The enzyme is immobilized on controlled pore particles (CPGS) that fill a polyethylene tub forming a reactor (configuration B, Table 2).

Acetate is mixed in a second channel where sulfuric acid transforms it to acetic acid. The acetic acid passes through a gas-diffusing membrane and picked up in another channel that takes it to the measuring cell where a conductivity change is sensed.

This system has the advantages inherent to flow biosystems. Additionally, it does not require long incubation periods, the reactivation of the enzyme with TMB-4 [1,1'-trimethylene-*bis*(4-formylpyridinium bromide)] is fast and complete. The gas-diffusing membrane raises the selectivity because there are few volatile substances at room temperature that may pass through it.

III. INHIBITION OF OTHER ENZYMES

Several studies that feature the design of electrodes with tyrosinase to measure pesti-

cides in batch systems have been reported.[1] However, there are few reports of these inhibition systems that use FIA.

Atrazine inhibits the Hill reaction of photosynthesis by hydrogen bonding to electron transport enzymes within the chloroplast. Tyrosinase is plentiful in the chloroplast and McArdle and Persaud[32] took advantage of this fact and designed one of the few FIA systems based on tyrosinase inhibition. They built a biosensor immobilizing the tyrosinase by crosslinking on a poly(pyrrole)-coated gold surface. The modified gold disk was placed in a flow cell. The resulting system analyses atrazines by detecting their inhibiting effect on tyrosinases (see Table 5).

Yamamoto et al.[33] developed a flow system to measure organophosphorus pesticides using a bioreactor and a biosensor consecutively. The bioreactor has alkaline phosphatase (ALP) and the biosensor has alcohol oxidase (AOD). The detector is an oxygen electrode. The response to DDVP is linear and one analysis cycle takes 10 min (Table 5).

Recently, Sacks et al.[34] designed an amperometric sensor for parathion by using parathion hydrolase. The enzyme parathion hydrolase catalyzes the hydrolysis of parathion to *p*-nitrophenol (PNP) that is detected amperometrically using a microflow injection system based on a screen-printed electrode.

IV. FINAL REMARKS

The use of biosensor-based automated analytical systems was linked originally to the on-line monitoring of biotechnological processes.[35] At present, the development of on line monitoring techniques is a priority for government agencies dealing with environmental monitoring, evaluation, and remediation. In this context, work has been done to integrate flow injection analysis (FIA) with biological material (see Table 6) to analyze organophosphorus and carbamate pesticides continuously.

The most widely used FIA systems for this monitoring are monoenzyme systems with amperometric detection following a biosensor configuration. The use of a single enzyme simplifies the automation, reduces the cost, and eliminates problems associated to parallel reactions posed by different set of optimal working conditions when several enzymes are involved. However, single enzyme systems show limitations in regard to interferences. On the other hand, both amperometric detection and the biosensor configuration aim at raising the sensitivity to paraoxon in the order of 10^{-11} *M*.

However, bioreactor configuration also produce good detection limits by lengthening the time of analysis as their design, separated from the transducer, permits the recirculation of the sample.

Despite the advantages of inhibition-based FIA systems to detect pesticides, there are a number of limitations that obstruct their application. One problem is the low stability of the biological components. A second obstacle is the difficulties posed by the regeneration step as most regeneration solutions have a limited action.

The use of renewable screen-printed biosensors as detectors in flow systems is a step forward. However, the automated substitution of the device is still a limitation. A second alternative, based on the biological modified surface, is the use of magnetic particles that act as the support for the biological material. These particles facilitate the disposal and the renovation of the biological material and appear to be the most simple, robust, and reproducible solution to produce inhibition analysis using flow systems.

The described AChE systems are very sensitive, with model solutions containing one single toxic. However, when there are other pollutants in the sample, they interact with the active center of the enzyme unpredictably, masking or augmenting the response to the toxic of interest.

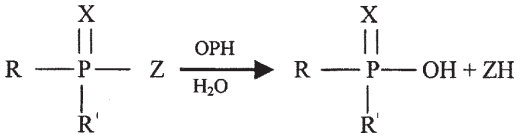
TABLE 5
Response Characteristics of FIA Systems Based on Different Enzymes and Transduction Modes

Transducer	Enzyme/Support / Immobilization method	Configuration	Substrate (concentration) / $V_{Injection}$	Inhibitor/ Detection limit (M) $V_{injection}$ / Incubation time	Aplication	Regeneration (time)	Carrier / Flow-rate	[Reference] Year
Gold plate ceramic chip	Tyrosinase/ gold plated surfaces / covalent-crosslinking	Biosensor	Catechol violet ($6.5 \cdot 10^{-6}$ M) / 120 µl	Atrazine/$<5 \cdot 10^{-6}$M 10 min	Artificial sea water	De-ionized water / substrate	Phosphate buffer pH 7.0 / 0.5 ml·min^{-1}	[32] 1993
Oxygen electrode (commercial)	AlP AOD	Bioreactor + Biosensor		DDVP/ <0.5 mM/10 min	--			[33] 1998

TABLE 6
Characteristics of Flow Analytical Systems

Characteristics	Biosensors in FIA
Manipulation	The knowledge of FIA technique is required
Maintenance	Simple compared to sophisticated analytical instrumentation
Sterility	By using a sterile membrane
Flexibility	High; construction based on different modules
Reliability	Demonstrated for *on-line* applications
Continuous information in the signal	High; the determination of peaks form permits various diagnostics
Dynamic range	5-6 orders using gradient technique
Recalibration	Easy and possible automation including a standard sample control.
Multicomponent analysis	Easy due to the gradient technique and the possibility to integrate multisensors.
Reproducibility	Elevated, due to the reproducible injection and immobilisation of reagents onto the biosensor surface.

Another alternative reported recently for pesticides determination is based on the direct measurement of organophosphorus compounds using its hydrolysis with organophosphorus hydrolase (OPH), as described in the previous review.[1] E. I. Rainina[36] immobilized *E. coli* with high OPH content in a reactor or in a microcolumn in the same fashion as previoulsy reported in a batch system.[37] He obtained detection limits in the ppm level using a pH electrode as the detector of the hydrogen ions liberated by the following reaction:



where X is oxygen or sulfur, R is an alkoxy group, R' is an alkoxy or a phenyl group and Z is the group phenoxy, thiol, cyanide, or fluoride.

The use of cells instead of enzymes is advantageous. It brings higher stability for a wider range of pH and ionic strength, but the sensitivity of this biosensor is limited by the sensitivity of the potentiometric measuring method.

The same authors[38] combined the organophosphorus hydrolase (OPH) with AChE biosensor to discriminate neurotoxins from a real sample and therefore measure the concentration of each of the two toxic families. They designed a flow system where the sample can be incubated previously with the reactor containing immobilized OPH. This

enzyme completely hydrolyzes organophosphorus compounds and eliminates their inhibiting effect on AChE. Only carbamates inhibit the AChE in the reactor. The residual activity of AChE is measured by injecting substrate that will be hydrolyzed to choline that goes to a third reactor with immobilized choline oxidase that consumes O_2 that can be measured with a commercial Clark electrode.

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