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Organophosphorus Pesticides Determination by Electrochemical Biosensors

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1. Introduction

According to the definition given by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA, 2008), a pesticide is any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest (insects, mice and other animals, unwanted plants, fungi, microorganisms such as bacteria and viruses, and prions). Considering their chemical structure, the pesticides are organophosphorus, carbamates, organochlorines, and pyrethroid ones (U. S. EPA, 2009). Currently, more than 30 % of the registered pesticides in the world market (Hill, 2003) and about 45 % of those registered with U. S. Environmental Protection Agency (EPA) (Roger & Dagnac., 2006) are organophosphorus (OP).

The organophosphorus pesticides, because of their high toxicity, fast biodegradation, low bioaccumulation, and broad target spectrum are extensively used in the agricultural and veterinary practices for protection of field and fruit crops, and for parasites control in domestic animals. However, their intensive and indiscriminate application, as well as their high acute toxicity generated risks to man and his environment. The resulting public concern created a demand for the development of reliable, sensitive, simple and low-costing methods for their fast “*in field*” detection.

In this work are reviewed the principles of the emerging electrochemical biosensors based methods for organophosphorus pesticides determination during the last decades, as methods of choice for “*in situ*” and “*on line*” application. Two main analytical techniques are considered, involving respectively the direct enzyme transformation of the pesticide and its enzyme activity inhibition effect, both followed by the conversion of the signal produced by the interaction between the bioreceptor and the analyte, into electrical one. The advantages and the drawbacks of each of them are discussed. The recent trends in the development of electrochemical biosensors for OP pesticides quantification, including nanomaterials transducer modification and genetic engineering of the biological recognition element are revised. Special attention is paid to the electrochemical biosensors based methods application for OP pesticides residues detection in food and in the environment.

2. The electrochemical biosensors

The biosensors are analytical devices, products of the current progress in biotechnology and material science, in concert with the modern principles of transduction of the chemical information. They are considered, due to their selectivity and specificity, as a very promising variety of chemical sensors. The electrochemical biosensors in particular use a biological recognition element retained in direct spatial contact with an electrochemical transducer (Thévenot et al., 1999) to obtain an analytically useful signal by coupling biochemical and electrochemical interactions.

The biorecognition elements or bioreceptors, according to the biochemical event, are of two main types (Thévenot et al., 1999): biocatalytic and biocomplexing (bioaffinity based). The biocatalytic ones include enzymes, whole cells (bacteria, fungi, eukaryotic cells or yeasts) or cell organelles and particles (mitochondria, cell walls), and tissues (plant or animal tissues). The biocomplexing receptors are mainly antibodies, biomimetic materials, cell receptors, and nucleic acids.

The applied electrochemical transduction mode (Thévenot et al., 1999) is commonly potentiometric or amperometric one.

The potentiometric determinations are based on the measurement of the emf of a galvanic element, constituted by an indicator and a reference electrode. The potential of the indicator electrode depends on the analyte concentration, according to the Nernst equation, while the potential of the reference electrode remains constant. The exponential character of the relationship between the potential of the indicator electrode and the analyte concentration defines the wide dynamic concentration range of the determinations (3-4 decades), but also the low accuracy and precision of the method.

The amperometry involves the measurement, at a constant potential, of the current response of an indicator electrode, as a function of the concentration of the present electroactive specie. The amperometric detection presents several advantages: (i) controlling the process through the electrode potential; (ii) high sensitivity and precision of the determinations; (iii) linear calibration plot.

The retention of the biological component of the biosensor in contact with the transducer is performed by its immobilization. The biorecognition element being usually an enzyme, the term "enzyme immobilization" was defined at the First Enzyme Engineering Conference held at Hennicker, NH, USA, in 1971. It describes "enzymes physically confined at or located in a certain region or space with retention of their catalytic activity and which can be used repeatedly and continuously" (Powel, 1996). This method ensures several issues as the effective use of the enzyme and its stabilization, the localization of the interaction, the prevention of product contamination, etc. (D'Souza, 1982).

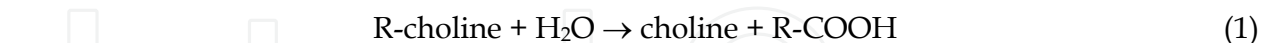
Biorecognition elements immobilization is achieved applying various techniques including (Thévenot et al., 1999): entrapment behind a membrane, entrapment within a polymeric matrix, entrapment within self-assembled monolayers, covalent bonding, bulk modification of entire electrode material (carbon paste or graphite epoxy-resin), etc.

The biosensors are self-contained (Thévenot et al., 1999), simple to handle and able to provide information in real time, without or with a minimum sample preparation (Andreescu & Marty., 2006). These performances, in concert with their sensitivity, selectivity and low cost, make them suitable for "*in field*" and "*on line*" analysis, and an excellent complement to the expensive and time-consuming classical analytical techniques.

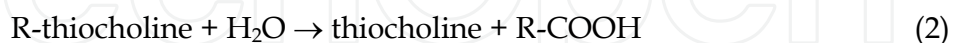
3. OP pesticides determination by electrochemical biosensors

3.1 Acylcholinesterases based sensors for OP pesticides determination

The acylcholinesterases (acetylcholinesterase EC 3.1.1.7 and butyrylcholinesterase EC 3.1.1.8) are enzymes, belonging to the class of the hydrolases. Acylcholinesterases based sensors exploit their ability to catalyze the following reactions:



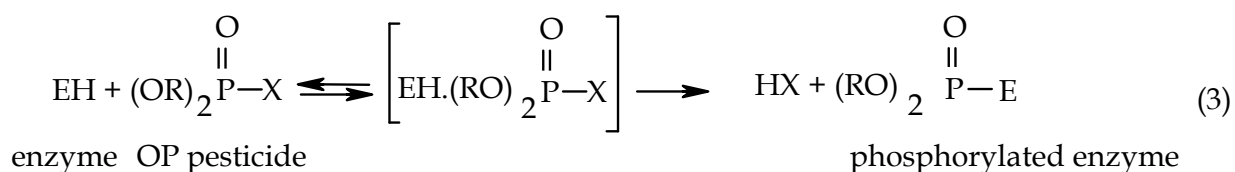
or



where R is usually an acetyl or butyryl moiety.

Acetylcholinesterase (AChE) demonstrates a high specificity toward acetylcholine, while butyrylcholinesterase (BuChE) is less specific and hydrolyses a number of choline esters, including acetylcholine. Apart from the natural substrates, acylcholinesterases catalyze the hydrolysis of the synthetic thiocholine esters, too (reaction 2).

OP pesticides determination using acylcholinesterases based sensors involves enzyme inhibition, according to the following mechanism (Aldridge, 1950):



The resulting reduction in reagents consumption and products release is correspondingly detected applying electrochemical techniques and is correlated to the OP pesticides concentration.

These inhibition based acylcholinesterases sensor for OP quantification have been extensively reviewed (Andreescu & Marty, 2006; Anzai, 2006; Jaffrezic-Renault, 2001; Noguer et al., 1999; Prieto-Simón et al., 2006; Rodriguez-Mozaz et al., 2004; Solé et al., 2003a; Solé et al., 2003b; Tran-Minh, 1985; Turdean et al., 2002). Attention has been paid to two main types of them, according to the transduction mode: the potentiometric and the amperometric ones.

The potentiometric sensors detect the pH shift resulting from the decrease, in the presence of OP pesticides, of the acid release during the enzyme catalyzed hydrolysis of the choline esters (reaction 1). The detection is performed in a single step, using a range of pH-sensitive transducers, varying from the traditional pH glass electrodes (Tran-Minh, 1985) to the ion-selective field effect transistors (ISFET) (Yuqing Miao et al., 2010). The equipment is simple and includes commercially available devices. Drawback of the method, apart of the non-linearity of the biosensor response and the related error of the determination is the increased response time. It varies from 2 to 10 min (Nikol'skaya & Evtyugin, 1992) in dependence of the time needed to reach the equilibrium at the interface between the biosensor and the solution, the measurements being performed under no current flow conditions. In addition, the sensitivity of the analyses of OP pesticides is lower in comparison to that performed using amperometric biosensors (Solé et al., 2003).

The amperometric acylcholinesterases based sensors of first generation take advantage of the following reactions sequence, illustrated in Fig. 1:



or



where ChE is acylcholinesterase and ChO is choline oxidase.

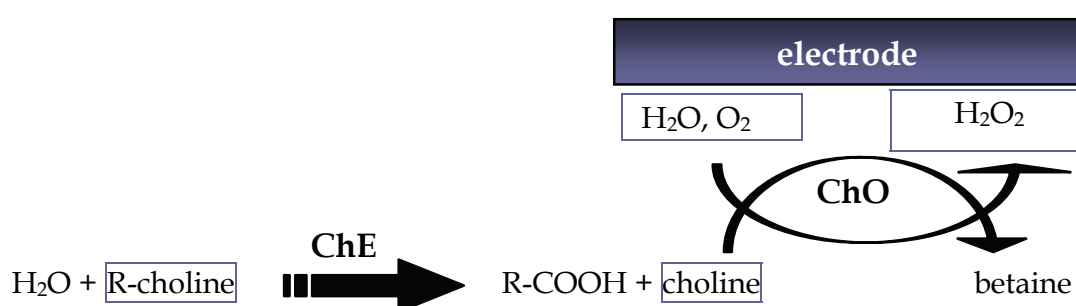
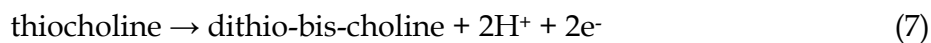


Fig. 1. Inhibition based amperometric biosensor of first generation

R-choline hydrolysis catalyzed by the acylcholinesterases (reaction 1) does not involve electroactive species. Thus, the process has been coupled with the choline oxidase catalyzed betaine oxidation (reaction 4). The current of the oxidation of the produced H_2O_2 (reaction 5) or the current of the reduction of the consumed O_2 (reaction 6), is registered as a sensor response.

The drawbacks of the sensors of first generation are: (i) the sophisticated design, as two enzymes have to be integrated; (ii) the need of optimization of the experimental conditions and the kinetics of the process to ensure the linearity of the biosensor response as a function of the analyte concentration; (iii) the possible interferences at the potential of H_2O_2 oxidation (+0.6 V vs. SCE); (iiii) the fluctuations in the oxygen concentration.

The formation of the analytical signal of the amperometric acylcholinesterases based sensors of second generation results from the combination of the following biochemical and electrochemical reactions:



The enzymatic hydrolysis of the thiocholine esters (reaction 2) generates products able to be easily oxidized. The current of thiocholine oxidation is recorded as a sensor response (reaction 7). Fig. 2 illustrates the occurring processes.

The advantages of the amperometric biosensors of second generation are: (i) the simple detection principle implicating direct thiocholine oxidation and the use of a single enzyme; (ii) the simple biosensor construction, the system being monoenzymatic one.

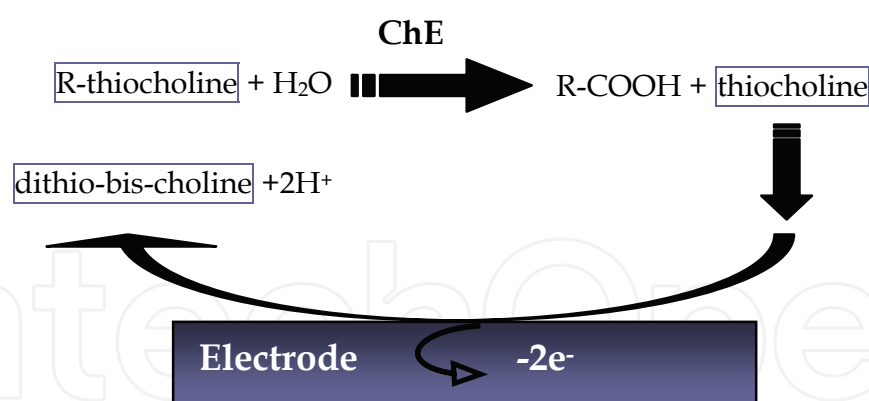


Fig. 2. Inhibition based amperometric biosensor of second generation

The main problems come from: (i) the spontaneous hydrolysis of the thiocholine esters, leading to overestimation of the anodic current response; (ii) the passivation of the platinum anodes by the sulfur-containing compounds; (iii) the high potential of thiocholine oxidation (+0.8 V vs. SCE) at conventional metal and graphite transducers (Martorell et al., 1994; Marty et al., 1992; Marty et al., 1993; Marty et al., 1995; Sužnjević et al., 1985) as a cause of possible interferences.

The non-enzymatic thiocholine hydrolysis could be reduced by dilution in a NaCl (0.09 %, w/v) solution and storage in ice (Andreescu & Marty, 2006).

Potential lowering could be achieved applying an alternative route to obtain response-generating electroactive species, comprising the following stages: (i) acylthiocholine enzymatic hydrolysis (reaction 2); (ii) chemical reduction of the produced thiocholine (reaction 8) using an appropriate electron mediator; (iii) electrochemical mediator regeneration (reaction 9):



(M_{ox} and M_{red} are the oxidized and the reduced forms of the mediator M).

The redox potential of the mediator determines the potential of the working electrode. The current of its oxidation is recorded as a sensor response.

This detection principle was exploited using acylcholinesterases sensors, including electrodes chemically modified with mediators such as phthalocyanines (Harlbert & Baldwin, 1985; Hart & Hartley, 1994; Skladal, 1991), Prussian blue (Ricci et al., 2004), tetracyanoquinodimethane (Kulys & D'Costa, 1991; Martorell et al., 1997), ferrocene (Evtugyn et al., 1996), etc. The redox reaction (8) could be performed in a homogeneous phase, too (Neufeld et al., 2000; Ovalle et al., 2009).

Fig. 3 illustrates the measurements carried out using an acetylcholinesterase based amperometric sensor of second generation applying two different strategies. The first one (Fig. 3A), consists in the registration of the amperometric biosensor response to OP pesticide additions in the presence of acetylthiocholine with a constant concentration. The second one (Fig. 3B) involves biosensor current response recording for increasing acetylthiocholine

concentrations before and after the incubation of the enzyme with the pesticide. The latter method is suggested for the determination of slow acting inhibitors (Tran-Minh, 1985).

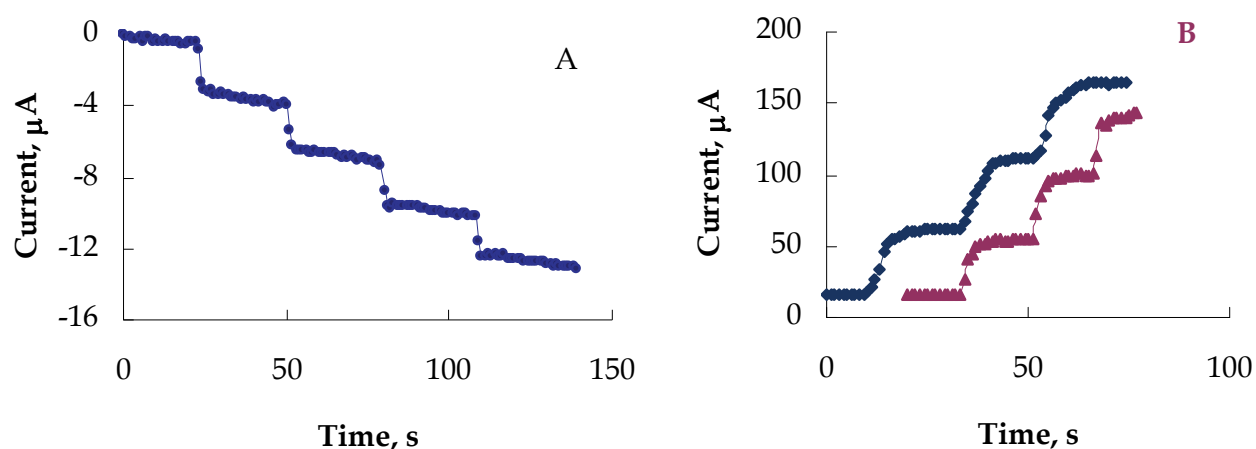
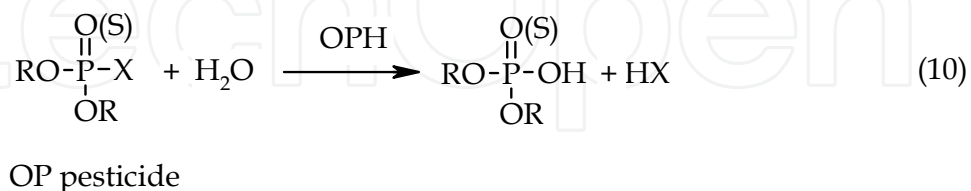


Fig. 3. Amperometric response of an acetylcholinesterase based sensor to: (A) increasing chlorofos concentrations (1.0 to 4.0 mmol L⁻¹, with an increment of 1.0 mmol L⁻¹) and a constant acetylthiocholine concentration (0.2 mmol L⁻¹); (B) increasing acetylthiocholine concentrations (0.2 mmol L⁻¹, 0.4 mmol L⁻¹ and 0.6 mmol L⁻¹) before and after 30 min enzyme incubation with chlorofos (4.0 mmol L⁻¹). Applied potential +0.80 V vs. Ag, AgCl, pH 7, 26°C ± 0.5°C, and electrode rotation speed of 1000 rpm. Membrane fraction of rat brain was used as a biorecognition element and source of AChE.

The great advantage of the inhibition based acylcholinesterases sensors for OP pesticides quantification is their sensitivity. The major drawbacks are due to the fact that the determination is not direct, as well as to the lack of selectivity and the need of enzyme reactivation/regeneration.

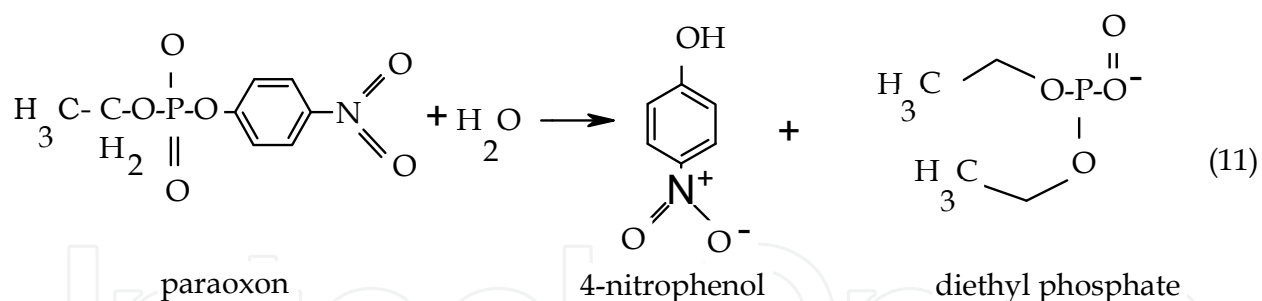
3.2 Organophosphorus hydrolase based sensors for OP pesticides determination

Organophosphorus hydrolase (OPH, EC 3.1.8.1) is a bacterial enzyme, catalyzing the hydrolysis of a range of OP pesticides (paraoxon, parathion, coumaphos, diazinon, dursban, methyl parathion, etc.), according to the following reaction:

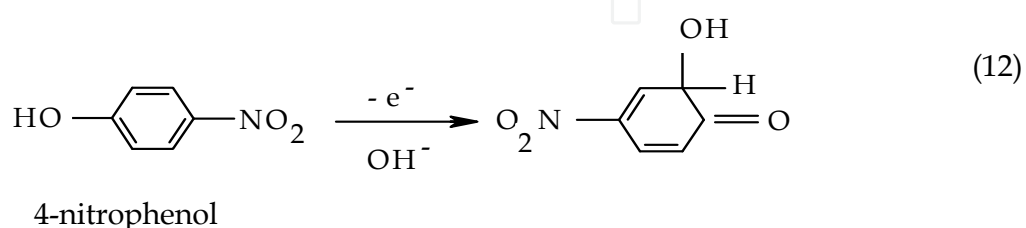


The hydrolysis involves a pH change, as well as electroactive species generation, thus allowing the development of potentiometric and amperometric sensors for OP pesticides quantification (Anzai, 2006; Chough et al., 2002; Lei et al., 2007; Mulchandani et al., 2001a; Mulchandani et al., 2001b; Prieto-Simón et al., 2006; Rodriguez-Mozaz et al., 2004; Wang et al., 2003).

For instance, OPH catalyzed hydrolysis of parathion, methyl parathion, paraoxon, fenitrothion, etc. yields 4-nitrophenol (reaction 11):



The current of 4-nitrophenol oxidation, proportional to the OP pesticide concentration, is recorded as a biosensor response (reaction 12):



The OPH based sensors for OP pesticides determination allow their selective quantification, applying a simple measurement procedure. However the reported detection limit (Mulchandani et al., 2006) is higher than that reached using acylcholinesterases based sensors. Another disadvantage represents the complex, long-lasting, and expensive procedure for OPH extraction and purification, performed in specialized microbiological laboratories (to note that this enzyme is not commercially available) (Prieto-Simón et al., 2006).

4. Electrochemical biosensors for OP pesticides determination in food and in the environment

The World Health Organization (WHO), the Food and Agricultural Organization of the United Nations (FAO), the Codex Alimentarius Commission, the EU Commission, and the U. S. Environmental Protection Agency (EPA) are among the principal organizations enacting the allowable pesticide residues levels in food, drinking water and environmental samples. The European Council Directive 98/83/EC on the quality of water intended for human consumption (Council Directive 98/83/CE) for instance, sets the limit value of the individual pesticides in drinking water at $0.1 \mu\text{g L}^{-1}$ and that of the total pesticides at $0.5 \mu\text{g L}^{-1}$. According to the U. S. EPA Office of Ground Water and Drinking Water (OGWDW), the health advisory levels for some OP pesticides in drinking water are: diazinon $3 \mu\text{g L}^{-1}$, parathion-methyl $2 \mu\text{g L}^{-1}$, disulfoton $1 \mu\text{g L}^{-1}$, fenamiphos $2 \mu\text{g L}^{-1}$, etc., the following 22 OP pesticides being on the U. S. National Pesticide Survey List: diazinon, dichlorfos, dicrotophos, dimethoate, diphenamiphos, sulfone, disulfoton, disulfoton sulfone, disulfoton sulfoxide, fenamiphos sulfone, fenamiphos sulfoxide, fenitrothion, methyl paraoxon, mevinphos, monocrotophos, omethoate, parathion ethyl, phosphamidon, stirophos, terbufos, tetrachlorvinphos, and merphos. Currently, EPA is reassessing pesticide residue limits in food to ensure that they met the safety standard established by the Food Quality Protection Act of 1996 (FQPA, 1996).

The electrochemical biosensors for OP pesticides determination, despite of their analytical potential, have found until now a limited application for real samples analysis. Some relevant data are presented in Table 1.

Pesticide	LOD	Sample	Reference
Diazinon	35 ppb	Spiked soil	Kumaran & Morita, 1995
Trichlorfon	0.1 $\mu\text{mol L}^{-1}$	Water	Wan et al., 2000
Parathion	10 ng mL^{-1}	Spiked river water	Sacks et al., 2000
Paraoxon	0.5 $\mu\text{g L}^{-1}$	Spiked river water	Bachmann et al., 2000
Paraoxon	1 nmol L^{-1}	Potato	Simonian et al., 2001
Paraoxon	0.6-0.8 $\mu\text{mol L}^{-1}$	Spiked river water	Jeanty et al., 2002
Paraoxon	2 $\mu\text{g kg}^{-1}$	Orange juices	Schulze et al., 2002
Paraoxon	2 $\mu\text{g kg}^{-1}$	Peach pap	Schulze et al., 2002
Paraoxon	2 $\mu\text{g kg}^{-1}$	Baby food	Schulze et al., 2002
Paraoxon-ethyl	10 nmol L^{-1}	Soil samples	Dzyadevych et al., 2003
Paraoxon-methyl	0.5 $\mu\text{mol L}^{-1}$	Soil samples	Dzyadevych et al., 2003
Trichlorfon	0.3 $\mu\text{mol L}^{-1}$	Soil samples	Dzyadevych et al., 2003
Paraoxon	0.1 $\mu\text{g L}^{-1}$	Grapes	Boni et al., 2004
Pirimiphos-methyl-oxon	10 nmol L^{-1}	Wheat and apple	Crew et al., 2004
Pirimiphos-methyl	38 ng mL^{-1}	Durum wheat	Del Carlo et al., 2005
Paraoxon	1.20 $\mu\text{g L}^{-1}$	Milk	Zhang et al., 2005
Paraoxon	1 $\mu\text{g L}^{-1}$	Milk	Yang et al., 2005
Paraoxon	0.5 nmol L^{-1}	Water	Joshi et al., 2005
Parathion	9-10.3 $\mu\text{g L}^{-1}$	Spiked water	Pedrosa et al., 2008
Chlorpyriphos-oxon	2 $\mu\text{g L}^{-1}$	Waters	Hildebrandt et al., 2008
Chlorpyriphos-oxon	2 $\mu\text{g L}^{-1}$	Beverages	Hildebrandt et al., 2008
Dimethoate	5.6x10 ⁻⁴ ng mL^{-1}	Chinese cabbage	Ning Gan et al., 2010

Table 1. OP pesticides determination in real samples, applying electrochemical biosensors

5. Recent trends in the development of electrochemical biosensors for OP pesticides determination

Two main strategies to improve electrochemical biosensors performances emerged during the recent years: nanomaterials transducer modification and genetic engineering of the biological recognition element.

The nanotechnological approach in electrochemical biosensors development (Balasubramanian & Burghard, 2006; Eftekhari, 2008; Gorton, 2005; Guo & Wang, 2007; Kerman et al., 2008; Kumar, 2007; Luo et al., 2006; Merkoçi & Alegret, 2005; Merkoçi, 2009; Pumera et al., 2007), takes advantage of the electrocatalytical properties of the nanostructures, their action as electron transfer mediators or electrical wires, large surface to volume ratio, structural robustness, and biocompatibility. Therefore, it yielded the following chief issues: electrode potential lowering, enhancement of the electron transfer rate with no electrode surface fouling, sensitivity increase, stability improvement, and interface functionalization.

Various nanomaterials used as acetylcholinesterase immobilization matrices in electrochemical biosensors for organophosphorus pesticides determination, along with biosensors performance characteristics such as sensitivity, linear dynamic range, and detection limit are evaluated and summarized in the review work of Periasamy et al. (Periasamy et al., 2009). As demonstrated, the nanomaterials transducer modification confers long storage stability of the biosensors, and enables OP pesticides detection in the nanomole – picomole range.

The alternative route leading to biosensors sensitivity, selectivity and stability increase involves the incorporation in the biosensing platform of biorecognition elements with tailor designed properties. These performances are achieved through appropriate site-directed mutagenesis ensuring increased biorecognition element affinity for the target analyte favoring the accessibility of the active site, enhanced electron transfer, and oriented or more stable immobilization (Campàs et al., 2009; Lambrianou et al., 2008).

Genetically modified enzymes are extensively used in inhibition based biosensors for OP pesticides determination (Bachmann & Schmid, 1999; Bucur et al., 2005; Marques et al., 2005; Nunes et al., 2001; Valdés-Ramírez et al., 2008), allowing attaining LOD as low as 10^{-17} M (Sotiropoulou et al., 2005).

6. Conclusion

In the recent decades, new methods for OP pesticides determination were developed, taking advantage of the unique analytical performances of the electrochemical biosensors. They made possible the reliable, fast, sensitive, simple and low-costing, “on line”, “on site”, and in real time pesticides quantification. This review gives a survey on the state of the art of organophosphorus compounds analysis using enzyme-based electrochemical sensors, pointing out on their advantages, drawbacks, real samples application and characteristics improvement.

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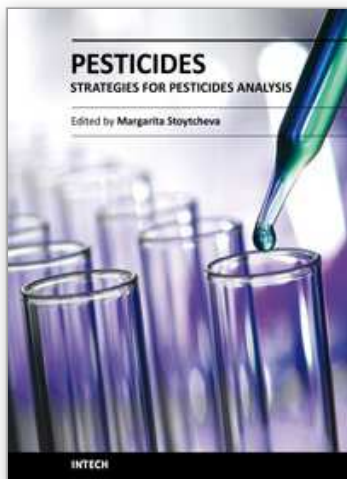
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This book provides recent information on various analytical procedures and techniques, representing strategies for reliability, specificity, selectivity and sensitivity improvements in pesticides analysis. The volume covers three main topics: current trends in sample preparation, selective and sensitive chromatographic detection and determination of pesticide residues in food and environmental samples, and the application of biological (immunoassays-and biosensors-based) methods in pesticides analysis as an alternative to the chromatographic methods for "in situ" and "on line" pesticides quantification. Intended as electronic edition, providing immediate "open access" to its content, the book is easy to follow and will be of interest to professionals involved in pesticides analysis.

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