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Automated resolution of dichlorvos and methylparaoxon pesticide mixtures employing a Flow Injection system with an inhibition electronic tongue

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ABSTRACT

An amperometric biosensor array has been developed to resolve pesticide mixtures of dichlorvos and methylparaoxon. The biosensor array has been used in a Flow Injection system, in order to operate automatically the inhibition procedure. The sensors used were three screen-printed amperometric biosensors that incorporated three different acetylcholinesterase enzymes: the wild type from *Electric eel* and two different genetically modified enzymes, B1 and B394 mutants, from *Drosophila melanogaster*. The inhibition response triplet was modelled using an Artificial Neural Network which was trained with mixture solutions that contain dichlorvos from 10^{-4} to 0.1 μM and methylparaoxon from 0.001 to 2.5 μM. This system can be considered an inhibition electronic tongue.

1. Introduction

The use of pesticides has strongly increased after the Second World War. Thereafter, high quantities of pesticides were introduced to protect seed and crops. A great number of pesticides are commonly and widely used to raise crop productivity (FAO, 1989; FAO/WHO, 2000) and attracted attention of legislators and scientists (FAO, 1989; USDA, 1992; Gascón et al., 1997; Turdean and Turdean, 2007). In this area, organophosphorous (OPs) compounds are widely used in agriculture due to their relatively low environmental persistence; however, their high acute toxicity places a serious risk on ecosystems equilibrium (Leon-Gonzalez and Townshend, 1990; Wilkins et al., 2000). The OPs persistence in the environment and their presence in water and food posses a very potential hazard to human health because they irreversibly inhibit the catalytic active sites of acetylcholinesterase (AChE), an essential enzyme of the central nervous system (CNS) of mammals; AChE catalyses the hydrolysis of acetylcholine which is a neurotransmitter in the CNS (Miroslaw et al., 2008). Due to toxicity of OPs, there is a considerable interest in the development of highly sensitive, low cost and reliable analytical methods for their detection.

On one hand, current analytical techniques such as gas chromatography (GC) and liquid chromatography (LC) may not be sensitive enough; on the other hand, GC and/or LC–MS (mass spectrometry detectors "MS") are excellent methods for identification and determination of pesticide mixtures. The last methods are time-consuming, very expensive and need skilled technicians. Nonetheless, GC and/or LC–MS instrumentation is economically inaccessible in many laboratories from developing countries; lately, many researches have focussed on the development of biosensors, which are well suited for the rapid, simple and selective analysis to quantify pesticides. Biosensors are a reliable and promising tool in this respect. Since organophosphorous compounds are inhibitors of acetylcholinesterase, amperometric biosensors based on enzymatic AChE inhibition can be developed, which have shown good results for analysis in waters and residues in food. The analytical determination allows getting a fast, sensitive and specific electric signal related to insecticide concentration. The enzymatic activity is measured through the anodic oxidation of the thiocholine (TCh) produced from hydrolysis of modified substrate acetylthiocholine (ATCh). The decrease in biosensor response is correlated with the amount of insecticide in samples and the time of incubation, as described in many publications (Hart et al., 1997; Andreescu et al., 2002; Marques et al., 2004; Vakurov et al., 2004; Nunes et al., 2004; Bucur et al., 2005, 2006; Law and Higson, 2005; Andreescu and Marty, 2006).

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However, the final measure is related to the total amount of inhibitors present, and thus, biosensor procedures do not permit to resolve pesticide mixtures. Moreover, the degree of inhibition of different pesticides to a given biosensor may be different, which makes quantification of an unknown sample having different chemical agents practically impossible. Recently, a new trend has appeared in the (bio)sensor field, which is the use of arrays of sensors to generate multidimensional data and their proper processing to obtain more detailed information (Holmberg et al., 2004; Gutiérrez et al., 2007); this approach has been termed electronic tongue (Vlasov et al., 1997).

Applications of chemometrics on multivariate sensor data has been used successfully in order to resolve different analytes mixtures (Richards et al., 2002), specially heavy metals (Mortensen et al., 2000) or other inorganic compounds (Gallardo et al., 2003). In those cases, Artificial Neural Networks (ANNs), as one of the available processing tools, is particularly suited for various tasks in data processing, such as memorising, associating, recognising patterns, or modelling non-lineal multivariate data. ANNs are non-parametric calibration methods specially created to process non-linear information: these tools have an ability to learn and extract X–Y relationships from a set of training samples (Gang et al., 2000). The fundamental processing element of an ANNs is the neuron. Neurons are arranged in layers that make up the global network architecture (Cartwright, 1993). Hence ANNs have been applied to liquid chromatography (UV or MS) and to voltammetric methods to quantify and resolve pesticides mixtures from different families, most of the time a combination of organophosphorous and carbamates or triazines (Ni et al., 2004, 2005). There are also recent works in the literature which have attempted the resolution of pesticide mixtures from the response of an inhibition biosensor array, either paraoxon/carbofuran (Bachmann and Schmid, 1999) or malaoxon/paraoxon (Bachmann et al., 2000); our own effort permitted to obtain a procedure to resolve dichlorvos/carbofuran mixtures, at lower concentrations and with shorter procedural times (Cortina et al., 2008). Few of these works have resolved a pesticide mixture from the same family, where the inhibition responses may be quite similar making its resolution more difficult. Thus, the target of the presented work has been to develop a very sensitive, simple, relatively inexpensive and trustworthy method of analysis to resolve dichlorvos and methylparaoxon pesticide mixtures, a case where both chemicals are organophosphates. To improve its reproducibility and speed of analysis, the procedure has been proposed employing disposable biosensors integrated with the Flow Injection Analysis (FIA) technique. Biosensors built are based on the use of three enzymes AChE, wild type from *Electric eel* and genetically modified B1 and B394 from *Drosophila melanogaster*. The enzymes are immobilised onto screen-printing electrodes by cross-linking in a water-soluble polymer with pendant azide-unit (PVA-AWP). The combined inhibition response of the amperometric biosensor array has been modelled by means of Artificial Neural Networks (ANNs) to build an electronic tongue based on inhibition biosensors. The method was applied to quantify mixtures of dichlorvos and methylparaoxon pesticides in real water samples without the need of eliminating interfering species.

2. Methods and material

2.1. Chemicals and reagents

Acetylcholinesterase (EC 3.1.1.7 type V-S from *E. eel*), acetylthiocholine iodide (ATChI), acetylthiocholine chloride (ATChCl), 5,5′-dithiobis (2-nitro-benzoic acid) (DTNB), bovine albumin (BSA), phosphate buffer (PBS) 0.1 M K_2HPO_4/KH_2PO_4 (pH/7.0) containing KCl 0.1 M were obtained from Sigma–Aldrich (St. Quentin-Fallavier, France), wild (B1) and genetically modified acetylcholinesterase (B394) form *D. melanogaster* were kindly supplied by PBS Protein Biosensor (Toulouse, France), Acetonitrile HPLC grade was supplied by Carlo Erba. The photocrosslinkable polymer poly(vinyl alcohol) with pendant-azide-units (PVA-AWP) was supplied by Toyo Gosei Kogyo (Tokyo, Japan). Methylparaoxon and dichlorvos were obtained from Chem service (West Chester, PA, USA). Other chemical reagents used were of analytical grade.

2.2. Screen-printing electrodes (SPEs)

The SPEs were provided by Gwent Technologies (UK). Each SPE has a three electrode configuration: the working electrode (WE) is a circle of 12.5 mm^2 of area, which surface is covered by Cobalt (II) phtalocyanine redox mediator specific for thiols, the auxiliar electrode (AE) and Ag/AgCl reference electrode (RE), both in a triangular shape with a geometric area of 10.5 mm^2 (Fig. 1). Each SPE is supported on a ceramic plate.

2.3. Biosensors production/enzyme immobilisation

Three different kinds of biosensors were produced using AChE from *E. eel*, and two different AChE's genetically modified from *D. melanogaster* (*B1* and *B394*). Each enzyme was immobilised onto the working electrode by entrapment in a polymeric matrix of PVA-AWP. In order to obtain the biosensors and to achieve the proper amount of immobilised enzyme (1 mU/electrode), 2 μL of enzymatic solution containing 30% enzyme solution in buffer (1.65 IU/mL) and 70% PVA-AWP were spread manually onto SPEs (WE surface). Afterwards, the electrodes were dried at room temperature and exposed for 3 h under neon lamp (15 W) at 4 °C, allowing the photo-polymerisation between azide groups. Finally, biosensors were dried at least for 72 h at 4 °C before being used.

2.4. Spectrophotometric measurements

Enzymatic activity was carried out with free enzyme applying Ellman's method which, is based on the fact that, thiocholine (TCh) produced from ATChI enzymatic hydrolysis, reacts immediately, quantitatively and irreversibly with DTNB producing 5-thio-2-nitrobenzoate (yellow product) that can be detected spectrometrically at 412 nm (Ellman et al., 1961). The reaction is represented as Scheme 1.

The enzymatic activity test was achieved as follows. In a spectrometric cell, were added: 700 μL of PBS, 90 μL H_2O (distilled), 100 μL DTNB 7.57 mM, 100 μL substrate (ATChI) 10 mM and 10 μL enzyme. DTNB was prepared in PBS solution. To avoid spontaneous hydrolysis of ATChI, it was prepared in 0.9% NaCl solution. The absorbance curve vs. time was obtained; the enzymatic activity expressed in U mL^{-1} was calculated.

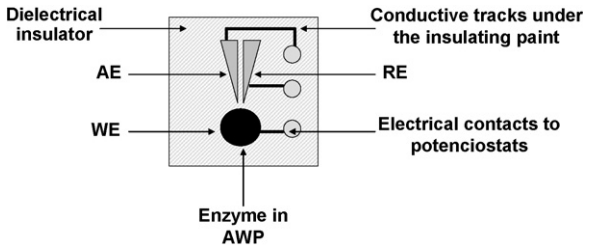

Fig. 1. Scheme of the screen-printing electrode (AE: auxiliary electrode; RE: reference electrode; WE: working electrode).