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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.

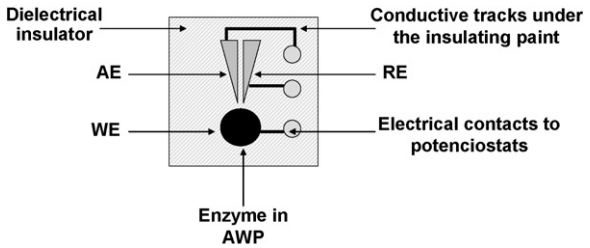
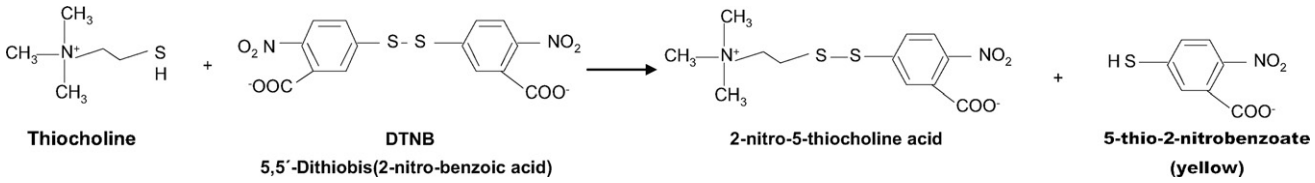


Fig. 1. Scheme of the screen-printing electrode (AE: auxiliary electrode; RE: reference electrode; WE: working electrode).



Scheme 1.

2.5. FIA manifold

The FIA manifold scheme is shown in Fig. 2. The device was constructed with the following components: An ISM932 peristaltic pump (Ismatec, Switzerland), three 12 VDC 30 PSI pinch valves (Cole Palmer, USA), (V1–V3 in the figure) driven by a solenoid valve controller (Uniscam Instruments, UK), a knotted reactor made from a PTFE tube of 30 cm × 3 mm (L × i.d.), three P120 potentiostats (Uniscam Instruments, UK) with wall-jet flow injection cells in which the incoming flow is perpendicular to the WE (P1–P3 in figure). These potentiostats are well adapted to the use of disposable screen-printed amperometric biosensors. Finally, a PC with SAFEGUARD 1.1 software (Uniscam Instruments, UK) was used to control the pinch valves, the peristaltic pump and the potentiostats and also to record the analytical signals from each potentiostat.

2.6. Detection procedure

Due to the fact that the FIA peak height is proportional to the activity of the enzyme immobilised onto the electrodes, the pesticides analysis was carried out in three steps. In the first step, the initial response of each biosensor was measured (I_1), and then standard pesticides or sample solutions were passed through the system for 15 min. After that, the system was washed with PBS and water; finally the residual activity of each biosensor was measured (I_2). The inhibition percentage ($I\%$) was determined according to the following expression $I\% = ((I_1 - I_2)/I_1) \times 100$; the ($I\%$) in each case was correlated with the pesticide concentration. The activity of each AChE immobilised onto the SPEs was determined electrochemically monitoring the thiocholine produced by enzymatic hydrolysis of ATChCl. Since Cobalt (II) phtalocyanine was employed as redox mediator, the applied potential was 200 mV vs. Ag/AgCl pseudo-reference electrode. The electrochemical signal is related with the height on the FIA peaks.

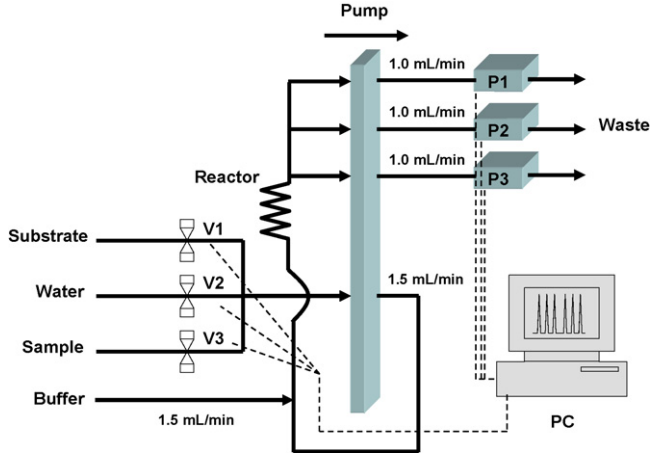


Fig. 2. Scheme of the Flow Injection Analysis system (FIA manifold) (V1–V3: pinch valves; P1–P3: potentiostats) at the system.

The operational step for one analysis cycle is described as follows: (1) injection of substrate (acetylthiocholine chloride), 38 s; (2) injection of water for 200 s, in order to wash the system for the substrate and obtain separated peaks; (3) repetition of the two first steps four times. Thus, the initial response was measured four times for each biosensor to verify stability; (4) injection of sample solution for 15 min to allow the pesticide inhibition; (5) repetition of the first two steps four times in order to detect the residual activity of the enzyme on the biosensor; (6) in order to eliminate all pesticide residues, after the measurement of each pesticide mixture, the FIA system was washed with acetonitrile–water 1:1, for 20 min.

The pesticide samples and the mixtures were prepared fresh before their usage. The highest concentrations were dissolved in acetonitrile and kept at 4 °C and the lower ones by appropriate dilution in PBS solution.

2.7. Artificial Neural Network model

To model the combined response of the two pesticides studied with the three-biosensor array, ANNs were used; the calculations were made by developing the corresponding program in MATLAB (MATLAB 6.1, Mathworks, USA) and by using the Neural Network Toolbox (Neural Network Toolbox 4.0.2, Mathworks, USA). In all cases, the ANNs used were feedforward networks and were trained by employing backpropagation algorithms, viz. Bayesian Regularization (Demuth and Beale, 1992).

In order to model the mixed response to dichlorvos and methylparaoxon pesticides, a total amount of 17 training solutions were manually prepared accordingly to a full factorial design with four levels and two factors (4^2), plus a centre point. The concentration ranges of these solutions were obtained from a previous work in a batch system (Valdés-Ramírez et al., 2008). Moreover, an additional set of 8 solutions was prepared, the concentrations of which were generated randomly, but inside the training space. These solutions, called test set, did not participate in the training process and they served to evaluate the model's predictive ability.

3. Results and discussion

3.1. Inhibition constants

The inhibition constants, k_i, were calculated by performing enzyme kinetic measurements according to Ellman's method, using different pesticide concentrations and by varying the incubation time of the enzyme with the pesticide. The data were analysed with the software GOSA from Bio-log company (Toulouse, France). Table 1 shows a comparison between the inhibition constants of B394 recombinant AChE, wild-type *Drosophila* (*B1*), and the com-

Table 1
Inhibition constants, k_i ($\mu M^{-1}\ min^{-1}$) of the three enzymes for methylparaoxon and dichlorvos

	Electric eel	B1	B394
Methylparaoxon	3.52	0.41	13.6
Dichlorvos	0.026	1.9	224

mercial *E. eel* AChE. The ultrasensitivity of B394 was obvious when comparing it with the widely used *E. eel* enzyme, the inhibition constant of the recombinant enzyme being 10,000-fold higher for dichlorvos. Moreover, dichlorvos shows greater inhibition effect to *E. eel* enzyme than to that of *Drosophila*, while the effect with methylparaoxon is opposite. These cross-response characteristics are a desirable feature for proper operation with the electronic tongue concept.

3.2. FIA manifold

The three pinch valves in the FIA manifold defined the inhibition procedure, controlling the entrance of water, pesticides and substrate solutions at precise timings. At any given time of the analysis, only one of the three-pinch valves is opened and thus the different solutions pass through the system alternatively. Each one of these solutions are merged with the buffer flow and homogeneously mixed in the knotted reactor. After that the mixture is divided into three identical flows, each one is directed towards the potentiostats to pass through the biosensors and finally discarded as waste.

The peristaltic pump regulated the flow rate on each detection channel of the FIA system, and also the flow rate of water/sample/substrate that enters the manifold. The buffer stream is regulated as the difference between these two flow rates. As incoming flow rate for each solution is 1.5 mL min^{-1} and the outgoing flow rate for each one of the three channels is 1 mL min^{-1}, the buffer results fixed at 1.5 mL min^{-1}. These flow rates showed satisfactory operational conditions for the FIA manifold and the flowing amperometric detection.

At the FIA system, the biosensors are kept in a vertical position in the electrochemical cell, in each position a biosensor with different enzyme were placed. The input tube and the WE are located at the low part and the waste at the upper part, this configuration and the continuous upward flow allow to eliminate the air bubbles that could be accidentally aspirated into the system.

The suitable operation of the FIA manifold was checked using SPEs without enzyme and cystamine as thiol-containing analyte employed instead of the enzymatic substrate; the purpose was to verify the reproducibility of the mixture of the aspirated flows in the reactor and its division into three identical parts (one for each detector) plus the ability of the transducer to respond to the thiol substrate. The peaks obtained after injection of 1 mM cystamine (substrate for this test) showed a comparable shape and magnitude for the three channels, the relative standard deviation, R.S.D. being 2.14% (I = 1825 nA ± 39.6 nA, n = 5). The results obtained with four other concentration 1.78, 1.08, 0.59 and 0.28 mM allows to obtain a good calibration curve with a standard deviation lower than 3%.

For biosensors, the operational stability was tested by measuring the analytical response to eight successive determinations of ATChCl. Each experiment was performed at 1 mM ATChCl. The amperometric signal average after substrate injection at AChE *B394* biosensor was 406 ± 6 nA (n = 8) (Relative standard deviation (R.S.D.) = 1.5%). For biosensors with AChE *B1* the average amperometric signal obtained was 399 ± 6 nA (n = 8), (R.S.D.) = 1.5%. For biosensors with AChE from *E. eel* the average amperometric signal was 401 ± 6 nA (n = 8), (R.S.D.) = 1.5%.

Substrate injection time was studied in the 10–60 s range. The height of the FIA peaks increased when the substrate injection time was enlarged, until a plateau was reached. This expected phenomenon could be explained by the longitudinal diffusion of the injected segment characteristic of the FIA system and the time necessary for the substrate to diffuse through the PVA-AWP matrix to the enzyme. The optimum time injection used, where a maximum analytical signal was firstly obtained, was 38 s.

3.3. Amperometric inhibition measurements

In order to model the response of the three biosensor array to the mixtures of dichlorvos and methylparaoxon, a number of standards containing both substances were manually prepared and measured by triplicate according to the automated methodology. As previously specified, a total of 25 pesticides mixed solutions were used to perform the experiment, 17 of them for training and 8 for testing; these were useful to visualize the predictive ability of the model. The solutions were carefully designed to cover the concentrations range for both pesticides; the concentration range for dichlorvos was 0.0001–0.1 μM, and for methylparaoxon the range concentration was 0.001–2 μM.

The used ANN architecture was exhaustively studied (Calvo et al., 2007; Cortina et al., 2008) from an initial configuration using a single hidden layer. In each case, the percentage of inhibition after 15 min insecticide incubation for the three biosensors was used as the input information, thus using an input layer with three neurons. Analogously, as there were two concentrations sought, the output layer was formed by two output neurons. In order to have the best results, 32 ANNs with different configurations were tested; in each, the number of neurons at the hidden layer were varied among 3, 5, 7, and 9, and different combinations (8) of linear and non-linear transfer functions at the hidden and output layers were tested. As criteria to choose the best ANN configuration, we considered that one with minimum root mean squared error (RMSE) and, simultaneously, showing good predictive ability. This was checked through visualizing the obtained vs. expected concentration comparison line—the ideal case being with correlation coefficient close to 1, intercept close to 0 and slope close to 1 (the theoretical diagonal line). From the different tests, the best ANN configuration had 3 neurons in its hidden layer using the *tansig* transfer function, while the transfer function at the output layer was *purelin*.

The ANN selected showed satisfactory correlation degree in the graphics for predicted vs. expected pesticide concentrations in the mixed standards; the comparative results for the training and external test set for both pesticides are shown in Fig. 3. Table 2 summarizes the values of the slope and intercept of the fitted comparison lines for the training set and for the external test data by using the optimal ANN configuration. In both cases good correlation was obtained, with the comparison lines close to ideal case. Therefore, prediction capability of the model could be considered as satisfactory for both considered pesticides.

Typical errors of the proposed determination, estimated from the comparison regression for an average interpolated value, were 0.0093 and 0.18 μM (expressed as standard deviation) for dichlorvos and methylparaoxon, respectively.

Table 2
Summary of the goodness of fits obtained for the two pesticides with training (n = 17) and external test sets (n = 8) by using amperometric measurements; r is the correlation coefficient

	Slope	Intercept (μM)
Training		
Dichlorvos, r = 0.962	0.90 ± 0.15	0.007 ± 0.008
Methylparaoxon, r = 0.969	0.92 ± 0.13	0.07 ± 0.15
Test		
Dichlorvos, r = 0.879	0.98 ± 0.61	0.005 ± 0.035
Methylparaoxon, r = 0.889	0.91 ± 0.54	−0.09 ± 0.60

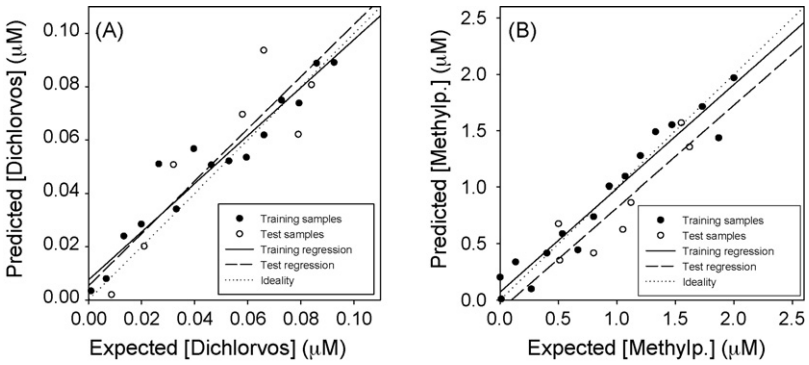


Fig. 3. Correlation results between expected and obtained concentration for (A) dichlorvos and (B) methylparaoxon pesticides.

Table 3
Results obtained in the determination of dichlorvos and methylparaoxon pesticides in real samples and spiked real samples

Sample	Spiked concentration		Found, ANN	
	[Dichlorvos] (μM)	[Methylparaoxon] (μM)	[Dichlorvos] (μM)	[Methylparaoxon] (μM)
Bottled water	–	–	0.008	<LD
	0.03	1.00	0.033	1.401
River water	–	–	<LD	<LD
	0.05	0.50	0.049	0.480

3.4. Application to real samples

The proposed method was carried out in two real samples, river water and bottled mineral water. Each sample was divided in two, with the first portions analysed directly with the FIA system, and the second portions spiked with different amounts of dichlorvos and methylparaoxon. Both samples without added pesticide revealed an inhibition lower than 10% with all kind of biosensors except for the biosensor employing B394-modified enzyme. This fact, since B394 enzyme is the most sensitive to organophosphorous pesticides among the three enzymes tested, suggested the presence of pesticide residues. The results obtained according to the ANN response model are shown in Table 3. As can be seen, bottle water contained traces of the pesticide dichlorvos, and its concentration was estimated as 0.008 μM. On the other hand, the interpolated values for methylparaoxon in these samples were lower than the low level of the training space (<0.001). This fact made us believe the absence of this organophosphate, although the only assertion is that its concentration is below the detection limit. On the other hand, when the samples were spiked with the two considered pesticides, the bottled water sample analysis correctly found evidence for the presence of both pesticides, dichlorvos and methylparaoxon, in this case the amount of pesticide found were 0.0033 μM of dichlorvos and 1.40 μM of methylparaoxon. The found values with the river water sample were also consistent with the spiked concentrations, as can be observed from the results in Table 3. Average recoveries were 104% for dichlorvos and 118% for methylparaoxon. These results suggest that the proposed method is able to resolve the considered pesticide mixture in the real samples tested.

4. Conclusions

A bioelectronic tongue using an array of amperometric biosensors based on three different AChE enzymes capable of differentiating among different pesticides have been used in a Flow Injection Analysis system, permitting to perform automatically the inhibition assay of a pesticide mixture. After modelling the inhibitory response with an optimized Artificial Neural Network, it was possible to resolve binary mixtures of the two pesticides dichlorvos and methylparaoxon at the nM concentration level.

With an automated FIA system like the one presented here, certain advantages can be envisaged in comparison with standard (HPLC) laboratory procedures: the use of screen-printed biosensors permits a reduced operation cost, the reproducibility of operation is highly improved and the analysis time is greatly reduced.

When applied to real samples, the two pesticides could be determined with low errors using an extremely simple procedure that can be the principle to develop an on-site screening methodology. In comparison with previous works (Bachmann and Schmid, 1999; Bachmann et al., 2000), the proposed alternative is fast, portable and automated, and resolves a mixture with two organophosphate pesticides, a challenging case.

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