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Development of a portable biosensor for screening neurotoxic agents in water samples

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Abstract

A high sensitive portable biosensor system capable of determining the presence of neurotoxic agents in water has been developed. The system consists of (i) a screen-printed electrode with acetylcholinesterase (AChE) immobilized on it, (ii) a self-developed portable potentiostat with an analog to digital (A/D) converter and a serial interface for transferring data to a portable PC and (iii) an own designed software, developed with Lab-Windows CVI, used to record and process the measurements. The system has been developed to perform high precision amperometrical measurements with low drifts, low noise and a good reproducibility. In the configuration depicted, the percentage of AChE inhibition is proportional to the content of neurotoxic agents in a sample. This type of measurement is performed by the steady-state method from the first steady current (by a phosphate buffer solution) and the second steady current (by an enzymatic reaction produced by the addition of acetylthiocholine chloride to the solution). Validation was performed by analyzing spiked water samples containing pesticides. The design is specially suited for screening purposes, does not need sample preconcentration, is totally autonomous and suitable for the field detection of neurotoxic agents in water.

Keywords: Environmental analysis; Enzyme biosensor; Pesticide; Portable

1. Introduction

Pesticides are neurotoxic agents used in agriculture, industry, housework and as war gases. Neurotoxic pesticides mainly kill by the inhibition of acetylcholinesterase enzymes (AChE) [1], an essential enzyme that permits the transmission of electrical signals in the nervous system of most animal beings. Most common types are organophosphorus, but also carbamates, which are considered less toxic since the reversibility of their coupling with AChE. Due to their high acute toxicity and risk towards the population, some neurotoxic pesticides are included in Directives 76/464/CEE and in 60/2000/EU or in food regulations [2]. However, in the recent years there is a fear towards the poisoning of water tanks and drinking water reservoirs by neurotoxic agents which at very low concentration can produce acute poisoning to the general population, being then a great need to have tools to quickly determine with high reliability and preferably at low cost the presence of compounds which can affect the nervous transmission in humans or fauna.

Several analytical methods are used to detect neurotoxic pesticides in water matrices. Routine methods are based on extraction, clean up and analysis normally performed by gas chromatography (GC) or liquid chromatography (LC) coupled to sensitive and specific detectors such as nitrogen–phosphorus detectors (NPD) [3–5], flame ionization detectors (FID) [4,5], ultraviolet detectors (UV) or diode array detectors (DAD) [6–8], although recently most methods involve the use of mass spectrometry (MS) as detector to provide ultimate confirmation [9,10]. These methods are highly sensitive and are capable to determine a large number of compounds but they are costly, long and/or complex.

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Biosensors are alternative methods capable to monitor the total concentration of a chemical family in terms of neurotoxicity and if properly designed, are very helpful tools for environmental/food monitoring [11,12]. AChE biosensors permit to detect the concentration of total neurotoxic agents by measuring the inhibition of AChE [13–15]. By performing an adequate calibration, biosensors can quantify the concentration of total neurotoxic compounds in a sample. The main advantage of AChE-based biosensor is that above a threshold value, they can discern between positive and negative samples with high precision, thus reducing the number of samples to be monitored by analytical tools to determine the exact composition or concentration.

Among different configurations, basically AChE biosensors consist in the enzyme immobilized on a graphite working electrode, and the measurement is performed with 2-electrode amperometry using a potentiostat. Commercial potentiostats are able to perform a wide range of measurements [16] but the main inconvenient is that they are bulky, expensive and cannot be transported to the field/facility for *in situ* measurements. Laboratory biosensors permit to determine neurotoxic agents at the ppb level with reproducibility below 10% and high precision [17]. Their main problem, for early warning stages, is that the sample has to be transported to the laboratory and thus, in an alarm situation, decisions cannot be established with enough effectiveness.

Portable biosensors for pesticide analysis have already been reported in the literatures [18,19] with similar analytical characteristics but with fiber-optics technology or expensive multi-parametric features which would increase the price of the instrument. Also, some portable potentiostats have been developed using electrochemical detection [20,21]. The biosensor herein reported is tailored in such a way that it has the advantage that the system is simplified and analyte specific, while still fulfils the specifications of high-quality commercial potentiostats. Therefore, the goal of this study was to develop a tailored portable biosensor, which permits the measurement of neurotoxic agents in water with high sensitivity. The own designed potentiostat only performs amperometric measurements with two- or three-electrode sensor and has user friendly features like the adjustment of the applied voltage between the electrodes and an internal calibration system. In this study we describe the design and performance of the biosensor in terms of noise, sensitivity, reproducibility, portability and validation.

2. Experimental

2.1. Chemicals and reagents

The pesticides carbaryl (CA), chlorpyrifos (OP) and chlorfenvinphos (OP) (99% purity pesticides from Sigma–Aldrich, St. Louis, U.S.A.) were used as model AChE toxic compounds belonging to the carbamate (CA) and organophosphorus (OP) family. Stock standard solutions at 1000 μg/mL were prepared in methanol and kept at −20 °C. Chlorpyrifos oxo-derivative was prepared by oxidizing the parental compound with *N*-bromosuccinimide, which rendered chlorpyrifos-oxon of 99% purity. Chlorpyrifos is applied in the environment in the sulfured form ($C_9H_{11}Cl_3NO_3P{=}S$) but its oxidation is very fast giving the more active and toxic metabolite chlorpyrifos-oxon ($C_9H_{11}Cl_3NO_3P{=}O$) [22]. Working standard solutions were prepared daily by successive dilutions in HPLC grade water. A 0.05-M phosphate buffer solution with 0.1 M KCl (reagents from Merck, Darmstadt, Germany) at pH 8.0 was used to give a stable aqueous medium for enzymatic reaction. Acetylthiocholine chloride (substrate of AChE) was obtained from Sigma–Aldrich.

2.2. System description

The developed portable amperometric biosensor consists of a recognition element, in this case a sensor strip or screen-printed electrode where AChE has been immobilized, a transducer based in a thiol oxidation at a potential of 100 mV and a detection element which measures the total circulating current. The system is formed by three parts:

(i) A screen-printed electrode, with a graphite working electrode and an Ag/AgCl reference electrode. The sensor strip was manufactured by successive screen-printing of six layers upon a polyvinyl chloride (PVC) sheet. The first layer is a silver track used as the conductor. The second layer is a graphite film used to isolate the silver conducting track from solution. The reference electrode (RE), made of Ag/AgCl, is printed in the third layer. The working electrode (WE) is printed in the fourth layer; it is made of graphite and 7,7,8,8-tetracyano quinodimethane as a mediator. The fifth layer is an insulating ink to fix the working surface. The sixth and last layers are manually deposited on the WE; it is *Drosophila melanogaster* acetylcholinesterase enzyme entrapped in a polyvinyl alcohol photopolymer – named PVA-SbQ – with a polymerization degree of 2300 from Toyo Gosei Kogyo Co., Ltd. Photopolymers are easy to handle and have shown excellent performance when immobilizing enzymes over screen-printed electrodes; the procedure used is based with that described previously [23]. The electrodes are designed for single use.

(ii) Potentiostat: the potentiostat is a circuit designed in such a way that it ensures that the voltage between the working and reference electrodes of the sensor is set at a predetermined value, and also gives a path for electron flow around the sensor. The potentiostat includes a current to voltage converter that yields an output voltage proportional to the sensor current. This output voltage is converted to a digital format using an analog to digital converter (A/D). Data is transmitted to a PC via a serial port and displayed with a customized software.

(iii) The circuitry needed for analog to digital conversion and serial port interface with a personal computer (PC) and a program in C for Virtual Instrumentation (Lab-Windows), designed to control the biosensor: the user can have a graph of the current plotted, autoscale the graph, make calibrations and store data and information about current waveforms in files.

2.3. Experimental setup

The system has been characterized by testing the noise (both electronic and overall), drift, temperature dependence and repeatability of the measurements. Calibration curve has been constructed using hydrogen peroxide. Only the simple and common 2-electrode configuration has been tested.

2.3.1. Electronic noise

There are two main sources of noise: the electronics and the biosensor. Electronic noise has been measured for three different full-scale ranges (5 μA, 500 nA and 50 nA, which correspond to $R_{feedback}$ = 1 MΩ, 10 MΩ and 100 MΩ, respectively). To perform the measurements, the sensor was replaced with a resistance equal to $R_{feedback}$ placed between auxiliary and working electrodes. The applied voltage was 600 mV. Measurement of the noise was performed without any further processing.

2.3.2. Overall system noise and drifts

The overall system noise included the noise generated by the electronics and the biosensor. The measurements were performed by dipping the sensor into 500 mL of phosphate buffer kept at constant 25 °C with stirring. Full-scale range was set to 5 μA. Data was analyzed in raw and filtered with a moving average to demonstrate the benefits of this signal processing technique. Low frequency noise, which includes drifts, was measured for 400 sample points.

2.3.3. Temperature dependence

Temperature coefficient of the biosensor has been calculated measuring the steady-state current by dipping the sensor into 500 mL of phosphate buffer with a small temperature change between 22.6 °C and 25.1 °C. This range has been chosen because only small changes in temperature should be expected between consecutive measurements.

2.3.4. Repeatability and repetitivity of the measurements

To certify the reliability of the self-made electrodes, repeatability tests were carried out during three consecutive days with hydrogen peroxide. Additions of 10 μL of hydrogen peroxide solutions into 10 mL of fresh phosphate buffer were performed for a final concentration of 7.5×10^{-4} M using the same electrode without an enzyme. Four measurements were as well completed to check the repetitivity of the biosensor to 10 μL substrate addition into 10 mL of fresh phosphate buffer, using the same electrode with 5 mU of AChE enzyme. The temperature remained mainly constant during these experiments.

2.3.5. Calibration

Calibration curve has been done using hydrogen peroxide with nine calibration points from 7.50×10^{-4} to 6.75×10^{-3} M using electrodes without an enzyme. Hydrogen peroxide was added to the biosensor's reaction cell and a potential of 600 mV (increasing this value to 1 V which corresponds to the maximum response of hydrogen peroxide at electrode based on carbon would represents extra power consumption from (limited) batteries and besides, extra noise) was applied for its oxidation into oxygen following the reaction:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

This reaction is usually used to determine the efficiency of electrodes in amperometrical analysis.

To investigate the dynamic range of the AChE electrodes, HPLC grade water was spiked at different levels from 0 μg/L to 16 μg/L with chlorpyrifos-oxon, known to be one of the most toxic compounds to *D. melanogaster* AChE [24]. Experimental was conducted as depicted in Section 2.4.

2.4. Experimental procedure

Measurement of neurotoxic agent is achieved in a two-step ratiometric measurement. In the first step the amperometric response of the biosensor is measured as the current increases in relation with the base current when the substrate is added to the buffer ($\Delta I_1 = I_{\text{buffer with substrate}} - I_{\text{buffer}}$). After that, the biosensor is placed for 10 min in contact with the sample containing the neurotoxic agents, which inhibit the AChE and the same measurement is repeated (ΔI_2). The relative inhibition is calculated as follows:

$$\text{Inhibition}\,(\%) = \frac{\Delta I_1 - \Delta I_2}{\Delta I_1} \times 100$$

Concentration of neurotoxic agents is proportional to the inhibition of AChE and, if quantification is required, it can be measured using linear regression calibration.

Samples with inhibition lower than 10% are considered free of neurotoxic agents. If the inhibition is greater than 90%, the concentration of neurotoxic agents cannot be measured because of the sensor saturation and thus sample should be diluted.

2.5. Application to water samples

The biosensor was used to determine total neurotoxic agents from waters. Carbaryl, chlorpyrifos and chlorfenvinphos were used as inhibitors. Four wastewater samples from different wastewater treatment plants were collected using ambar glass bottles. These samples were filtered and spiked with carbaryl to concentrations from 0 μg/L to 4.1 μg/L. Electrodes with 1 mU of enzyme were used for this experiment. Four other samples of groundwater and bottled water were tested spiked with chlorpyriphos, chlorfenvinphos or a mix of organophosphorus and carbamate pesticides. As these pesticides are the greater inhibitors of AChE, electrodes with 10 mU of enzyme were used allowing a greater linear range. Spiked concentrations are shown in Table 1.

3. Results and discussion

3.1. Performance of the portable potentiostat

A portable potentiostat was designed and tested to determine neurotoxic agents at the low microgram/liter levels, which are the lowest limits imposed for individual pesticides in water and food. The initial requirement was to develop a portable,

Table 1
Matrix and pesticides studied, concentrations spiked and inhibitions measured with the biosensor

Matrix	Compound	Units of AChE (mU)	Concentration spiked (in μg/L)	Biosensor inhibition (%)
Wastewater 1	None	1	0.0	0
Wastewater 2	Carbaryl	1	0.3	11
Wastewater 3	Carbaryl	1	3.4	60
Wastewater 4	Carbaryl	1	4.1	72
Groundwater	Chlorfenvinphos	10	34.0	29
Groundwater	Chlorpyrifos-oxon	10	10.0	44
Bottled water	None	10	0.0	3
Bottled water	Mix of OP and CA	10	100.0	96

autonomous system capable to be used in the field. The first step was then to reduce the size of the potentiostat and the weight, which by the present design is kept at only 45 g, making it light enough for field use. A picture of the developed potentiostat is shown in Fig. 1.

The main requirement of the potentiostat for low level pesticide measurements was to achieve high sensitivity. This can only be obtained with a wide dynamic range, which in turn calls for a low noise system. The voltage applied between electrodes is obtained from a low noise voltage source integrated circuit and can be adjusted between −1.2 V and 1.2 V. The current-to-voltage converter uses a low-noise operational amplifier (OA) combined with a single high-quality, high-value, $R_{feedback}$ resistor in the feedback loop of the current-to-voltage converter. Increasing the value of $R_{feedback}$ increases the signal and the noise. Signal is increased proportionally to the value of $R_{feedback}$. Noise can be split into two main sources: thermal noise, which increases with the square root of $R_{feedback}$ and the noise of the OA which in turn can be split into voltage and current noise. Both noises are independent of the resistance, but current noise is converted into voltage in $R_{feedback}$ and therefore it also increases linearly with its value. The aggregated signal-to-noise ratio increases with $R_{feedback}$ up to a certain value, which is mainly given by the noise figures of the OA. The use of a low-noise OA allows the use of very high-value resistors, which permits low level measurements. Power supply is obtained from two batteries, which also helps to lower the overall noise of the system. With ±7.2 V and 1500 mA batteries, an autonomy of 3 weeks was achieved using it daily.

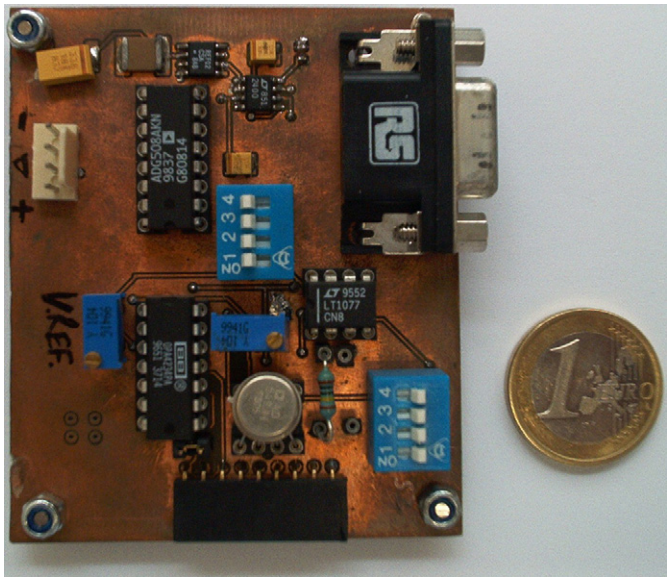

Fig. 1. Picture of the miniaturized electronic plate that functions as a potentiostat.

In order to process the response of the biosensor the voltage signal is digitized using a 24-bit A/D delta-sigma converter. This A/D was chosen because of its very high resolution and low noise permitting to obtain a very wide dynamic range.

The software was designed to collect real-time data. In order to record the response of the biosensor, the digitized signal is transmitted to a PC via an RS-232 serial port. The signal is plot in a strip-chart by means of a customized software made with Lab-Windows CVI. This program permits to select several parameters like sampling rate, number of samples averaged in each time slice and value of the feedback resistance. A 3-point calibration algorithm [25] can be applied to reduce linear and non-linear errors. Data can be filtered with a user-selectable moving average to further reduce the noise. Raw data can be saved in ASCII files and retrieved for display.

3.2. Electronic noise

The electronic noise measurements for the tested ranges are shown in Table 2. In each case, the noise was approximately gaussian. As it can be seen, there is a noise reduction as $R_{feedback}$ increases, which permits us to conclude that noise is mainly due to voltage sources and theoretically higher resolution could be achieved by further increasing the $R_{feedback}$. In practice, this is not feasible as will be shown in the following section.

3.3. Overall system noise, drifts and temperature dependence

Overall system noise was found to be around 160 pA and followed a gaussian distribution as shown in Fig. 2. This overall

Table 2
Electronic noise for different $R_{feedback}$ tested

$R_{feedback}$ (MΩ)	Range (nA)	I (mean) (nA)	σ_I (pA)
1	5000	603.99	31
10	500	60.921	1.4
100	50	6.0866	0.19

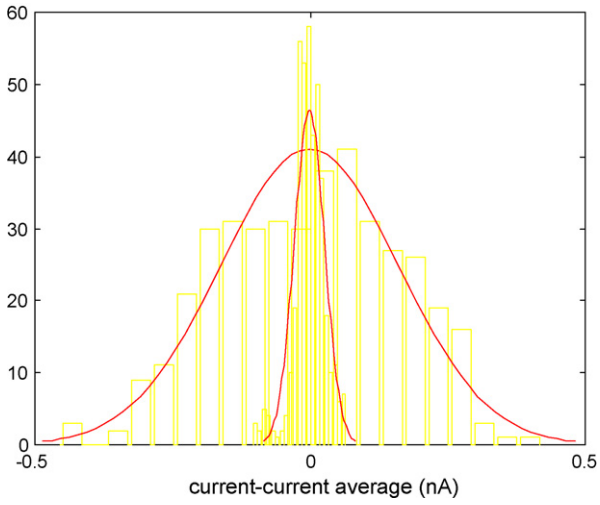

Fig. 2. System noise and its reduction with a moving average. Noise in a steady segment ($n = 400$ samples) $s = 0.16$ nA and 0.5 nA resolution. Applying a moving average tf = 10 s, $s = 0.03$ nA and 0.1 nA resolution.

noise is much higher than the electronic noise and therefore it is due to the biosensor noise, it is the one which determines the resolution of the system. Averaging the output signal it is possible to reduce the noise to 30 pA, which is close to the limit posed by the electronics. It should be noted that averaging reduces electronic noise to 5 pA, so we can still affirm that biosensor is, by far, the main source of noise.

The temperature dependence has been established in 5% $^{\circ}C^{-1}$. Fig. 3 shows the drift of the current intensity with the temperature. The values indicated in Fig. 3 correspond to the amperometric response of the first step before inhibition. Biosensor measurements were performed at laboratory temperature (23–24 °C).

3.4. Calibration and quality parameters of the measurements for AChE electrodes

As a first approach, calibration points were performed with hydrogen peroxide, which showed a good linearity with $R^2 = 0.997$ in the chosen range of 7.50×10^{-4} M to 6.75×10^{-3} M. This performance proves the good proportional response of the electronic system *versus* different analyte concentrations. Moreover different quality parameters tested showed a good performance of the method:

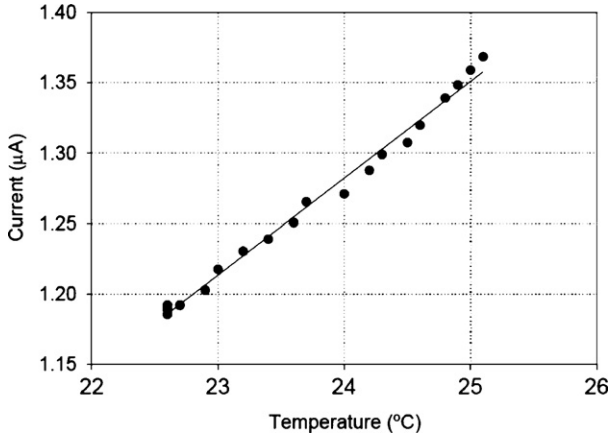

Fig. 3. Temperature dependance on the biosensor's current.

- *Low drifts*: once the reactions have reached the stationary state, the intensity current is constant. The maximum derivative of the averaged intensity is 0.001% s^{-1}.
- *Low noise*: when measuring currents of mean μ (in nA), equivalent noise due to the electronic components present a gaussian distribution with a standard deviation $\sigma < 0.002\mu$.
- *Wide dynamic range*: the dynamic range is higher than four orders of magnitude, specifically from 1 to 50,000, equivalent to a resolution of 15.7 bits.
- *Repeatability*: differences between measurements performed in different days under the same conditions are below 5%.

In a second step, the neurotoxic agent chlorpyrifos-oxon was used as a test compound. With such, the following parameters were measured:

- *Narrow dynamic range*: concentrations between 0 μg/L and 16 μg/L were tested in HPLC grade water with the analysis of six calibration points with five replicates. Four calibration points showed linearity at a narrow concentration range from 2 μg/L to 8 μg/L for electrodes with 10 mU of AChE. Linear regression in this interval gave the following equation:

$$y = 9.646x + 9.576, \quad r^2 = 0.9969$$

- *Detection limits*: the system permitted to detect selected pesticide below 2 μg/L using a 10-mU enzyme screen-printed electrode. The limit was achieved by successive tests at different concentrations with replicates searching for the lower concentration that would give a significant inhibition over blank values (over 10% as described in Section 2.4).
- *Repetitivity*: the repetitivity of the amperometric response was better than 5% for both 1 mU and 10 mU electrodes. The addition of 10 μL of substrate to both types of biosensors was repeated four times washing the sensor before each addition. This performance certifies the correct setup of the electrodes and the efficient entrapment of the enzyme on their surfaces.

3.5. Application to water samples

The level of saturation of the enzyme immobilized in the screen-printed electrode (and then, the linear working range) will depend on the toxicity of the pesticide but it would be generally very short (a range of some μg/L for the most toxic pesticides). So, the use of the biosensor to determine neurotoxic agents permitted to discern between positive (spiked) and negative samples, being an appropriate measurement to discriminate between contaminated and non-contaminated samples. Table 1 reports the samples tested, the type of compound tested, the concentration spiked and the percentage of inhibition determined.

According to Table 1, a high inhibition was observed in "wastewater 3" and "wastewater 4" what was in agreement with the spiked concentrations of carbaryl. 3–4 μg/L of concentration gave an inhibition close to the saturation point (near 85–90%). The low enzyme charge immobilized on the electrode (1 mU) was easily inhibited by carbaryl even though it is a weak inhibitor of the AChE [26]. For other compounds like chlorpyrifos and chlorfenvinphos, a very significant difference was observed in groundwater: 10 μg/L of spiked chlorpyrifos-oxon gave higher inhibition (44%) than 34 μg/L of spiked chlorfenvinfos (29%). In those experiments, the enzymatic charge was of 10 mU to obtain a greater linear range when working with more toxic compounds. The explanation of the different inhibition rates is found in the toxicity of each compound, being the oxidized form of chlorpyrifos much more toxic towards AChE than chlorfenvinphos [22]. Finally, two samples of bottled water were tested without and with 100 μg/L of a mix of pesticides (sum of all) to demonstrate the biosensor capacity to discern between non-contaminated and contaminated samples. The bottled water sample spiked with a high level of pesticides (100 μg/L) gave a complete AChE inhibition while the unspiked sample gave no inhibition at all (lower than 10%).

Sample measurements can be performed *in situ* with a total analysis time of 20 min per sample, no need of sample preparation or processing and thus, a high sample throughput can be envisaged. However, the results indicated in this study are only preliminary and ongoing studies are being performed to determine neurotoxic agents in water and food samples using the proposed biosensor.

4. Conclusions

The portable biosensor depicted in this study is capable to determine the presence of neurotoxic compounds *in situ*. The aim of this biosensors is to be used where high-speed analysis and excellent performance is needed to discern between positive (samples containing neurotoxic agents) and negative samples, without the need of transporting the sample to a laboratory. At this point it can be concluded that (i) the developed potentiostat produces low noise and low drift, (ii) the biosensor shows good amperometric response to buffer tests and the ability to detect low neurotoxic agents concentrations and (iii) the developed biosensor is totally autonomous and suitable for the field detection of pesticides, as has been demonstrated for different types of water samples where a good agreement was found between the biosensor response and pesticide concentration. However, the main feature of the developed biosensor is its versatility and possibility to increase the sensitivity and specificity by assembling different screen-printed electrodes thus providing a multisensor system which has clear technical and competitive advantages in the food and environmental field. In addition, by changing the type of enzyme immobilized, different types of organic pollutants can be measured, thus, enlarging the future applicability of the biosensor.

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