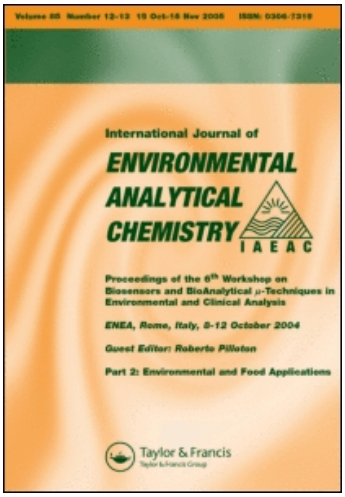

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DETERMINATION OF ANTICHOLINESTERASE ACTIVITY FOR PESTICIDES MONITORING USING A THIOCHOLINE SENSOR

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An acetylthiocholine sensor based on a disposable screen-printed carbon electrode has been assembled for measuring organophosphorus and carbamate pesticides in river water samples through the degree of inhibition of the enzyme acetylcholinesterase (AChE). The carbon working electrode surface was modified by deposition of a mediator, tetracyanoquinodimethane (TCNQ), and Nafion. Acetylcholinesterase catalyses the cleavage of acetylthiocholine to thiocholine, which is measured by differential pulse voltammetry and directly related to the enzyme activity. The scan speed, the pulse amplitude of the differential pulse voltammetry and several parameters in the procedure were optimised. An inhibition calibration curve was obtained using carbofuran. The method was also applied to water samples, showing its suitability as a rapid screening assay (15 min per test) for anticholinesterase activity detection.

Keywords: Tetracyanoquinodimethane (TCNQ); Nafion; acetylcholinesterase; screen-printed electrodes; acetylthiocholine; pesticides

INTRODUCTION

Anticholinesterase inhibitors (organophosphorus and carbamate), have come into widespread use in the last decades, because they are less persistent in the environment than other pesticides, such as organochlorine pesticides[1]. However, their presence in water and food is a potential hazard to human health and there is a growing interest in their rapid and accurate determination. Standard methods, based on gas chromatography (GC), are very reliable but there is the need for fast and innovative methods.

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The use of enzymatic biosensors, and especially electrochemical biosensors, for organophosphorus and carbamate pesticides detection has been reported by many authors [2–6].

These are based mainly on the use of the enzyme acetylcholinesterase (AChE); it hydrolyses the acetylcholine to choline and acetic acid which are not electroactive; therefore another enzyme has to be added in the reaction scheme such as choline oxidase which oxidises the choline to produce hydrogen peroxide easily detected by amperometry[7]. Some other authors report the use of acetylthiocholine as substrate, whose product (thiocholine) can be oxidised directly at the electrode surface[8,9].

The major problem associated with the use the acetylthiocholine as electroactive substrate is the high potential required to oxidise the thiocoline. This can be overcome using an electron transfer mediator[10–14]. Kulys[15,16] has shown that electrocatalytic oxidation of sulphydryl compounds can be achieved at low potentials at graphite electrodes modified with a wide range of redox mediators. Among these, tetracyanoquinodimethane (TCNQ) appeared to exhibit the most suitable characteristics for this application. Due to the presence of four cyano groups and the relative large π conjugation, TCNQ serves as an excellent electron donor and acceptor[17,18]. Even if many theories are reported in literature[19–23], the mechanism of electron transfer is not completely clear.

Among the different electrochemical techniques, amperometry has been the most used technique in acetylcholinesterase activity measurement using TCNQ as electron transfer mediator[2,24,25] or even when choline oxidase is exploited.

However, the disadvantage in the use of amperometry for in situ environmental analysis is the long working electrode polarisation step, (sometimes there is the need to wait 20–30 min to obtain a stable baseline). This problem can be overcome in some cases using a fast voltammetric technique.

The method proposed in the present work is based on the electrochemical determination of cholinesterase enzymatic activity with differential pulse voltammetry (DPV) using a disposable TCNQ-Nafion modified screen-printed electrode.

The combination of voltammetric techniques with screen-printed disposable electrodes is very attractive for the development of in situ analytical tools. Among these techniques DPV is one of the most sensitive and rapid. With this technique we avoid the long period for attaining a stable baseline. Therefore the procedure is really very fast and hence very attractive.

The sensor was used to test River Arno water samples. The results obtained were compared with a standard procedure for GC determination. Pesticides inhibition activity is defined as Total Anticholinesterase Activity (TAA) and is expressed as "equivalent μg/L" of carbofuran as reference pesticide.

EXPERIMENTAL

Materials and reagents

Electrodes were printed with a high performance multi-purpose precision screen printer DEK 245 (DEK; Weymounth, UK). Inks were from Acheson Italia (Milan, Italy). The plastic substrate for printing was a polyester film (Autostat HT5) purchased by Autotype Italia (Milan, Italy). Acetylcholinesterase (AChE) from Electric Eel (EC 3.1.1.7, 530 U/mg protein), acetylthiocholine (ATCh chloride salt), and the mediator 7,7,8,8, tetracyanoquinodimethane (TCNQ) were purchased from Sigma (St. Louis, MO). Carbofuran was obtained from Polyscience Corporation (USA). Standard solutions were prepared daily by dissolving the pesticide in acetonitrile purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Water was from a reagent grade ion-exchange Milli-Q system (Millipore Inc). All the electrochemical measurements were performed using a polarographic analyser computer controlled model 433 A (Amel, Milan, Italy).

Sensor construction

The electrodes were printed according to the procedure previously described[4]. The screen printed device consists of a graphite working electrode, an Ag pseudo-reference electrode and an Ag counter electrode (Figure 1). The surface of the working electrode was then completely covered with 2 µl of a solution of 1.10^{-4}mol/L TCNQ in acetonitrile or with 2 µL of a suspension obtained by mixing 5 µL of 1.10^{-3}mol/L TCNQ solution in acetonitrile and 50 µl Nafion. Sensors were stored overnight at room temperature, in the presence of desiccant and used as disposable devices. The sensor can be used up to 7 days by storing it refrigerated in a desiccant. Longer storage time gave an irreproducible behaviour.

Electrochemical procedures

Cyclic voltammetry

Preliminary investigations of the TCNQ modified and of the TCNQ-Nafion modified screen-printed electrodes (SPE) were performed by DC cyclic voltammetry. For this, 100 µL of 0.1 mol/L phosphate buffer (pH 7.5) and 0.1 mol/L KCl were placed onto the SPE and the potential was scanned between –0.4 to 0.7V vs. Ag as pseudo-reference electrode.

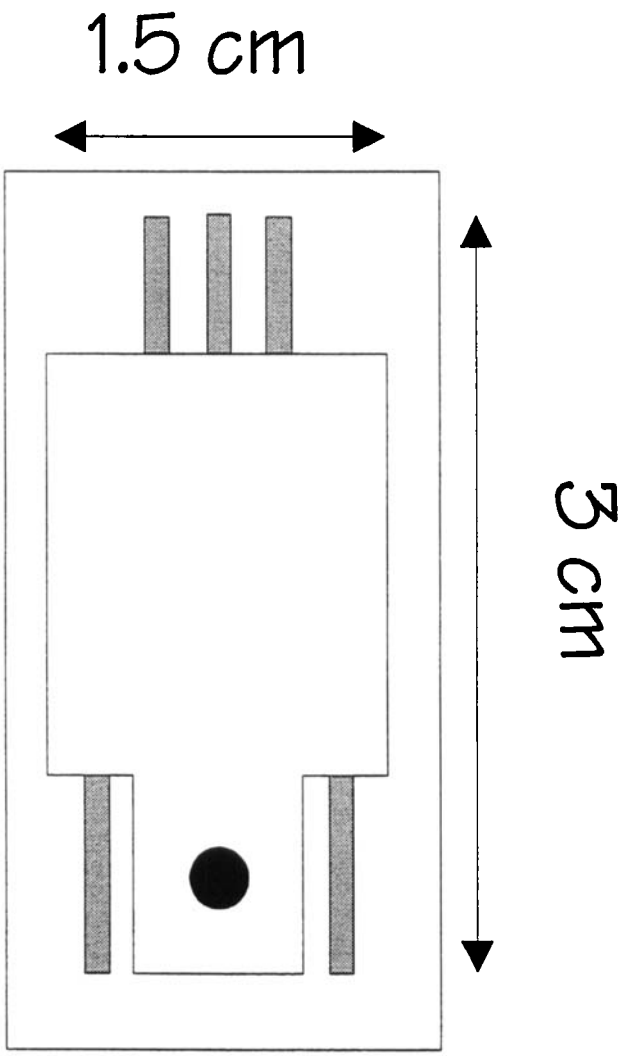

FIGURE 1 Scheme of the Screen-Printed Electrodes (SPE)

Differential pulse voltammetry

First, to optimise the procedure with TCNQ – Nafion modified SPE, DPV experiments were performed in the potential range –0.1 to +0.7V vs. Ag screen-printed pseudo-reference electrode, with a pulse width of 60 ms. Scan

speed and pulse amplitude were experimentally optimised. 100 µL of 0.1 mol/L phosphate buffer (pH 7.5) with 0.1 mol/L KC1, were deposited onto the screen-printed electrodes.

In order to optimise the determination of the acetylcholinesterase activity we develop the following procedure: 5–50 microliters of a stock solution of enzyme (80 U/µL) and 20 µL of a 5 10^{-2} mol/L acetylthiocholine were mixed in 1 mL of buffer phosphate 0.1mol/L at pH 7.5; the reaction was allowed to proceed for 4 min at room temperature. Then 100 µL of this solution were deposited onto the planar surface of the TCNQ-Nafion® SPE and after 1 min. DPV measurement was performed from + 0.35 to + 0.7V vs. Ag pseudo-reference electrode; with a pulse amplitude of 50 mV, a scan rate 25 mV s^{-1} and a pulse width of 60 ms. Following this procedure we obtained a current peak at 0.5 V which was exploited as analytical signal.

Determination of pesticides

The procedure described by Cagnini et al.[4,5], was used to measure the total anticholinesterase activity using the acetylthiocholine sensor. 50 µL of pesticide standard solutions or river water samples were added to 1 mL of 0.1 mol/L phosphate buffer solution pH 7.5 with acetylcholinesterase (1U/mL) and incubated for 10 min. Then 20 µL of the 5.10^{-2} mol/L acetylthiocholine solution was added. After 5 min, 100 µl of this solution was directly dropped on the surface of the sensor.

The oxidation current peak obtained by DPV after 5 min was measured (I_2) and compared with the oxidation current value obtained without pesticide (I_1).

The percentage of inhibition (I%) was obtained according to the following formula:

$$I\% = 100[(I_1 - I_2)/I_1]$$

The total anticholinesterase activity of a sample is expressed as the amount of a known pesticide (carbofuran, a carbamate pesticide) producing the same enzymatic inhibition to acetylcholinesterase. Carbofuran was chosen as the reference pesticide because it is widely used for crop protection and it is one of the most frequently found pesticide in the Arno river water samples[26].

Analysis of preconcentrated river water samples

A preconcentration step was found necessary for river water samples. The water samples were prefiltered through a 0.45 µm Millipore filter to clarify the water. Then 1 L of the sample water was passed through an Isolute™ solid-phase extraction column. The organic compounds extracted were then eluted using 5 mL ethyl acetate. The samples obtained were dried; then the dried extract was diluted

in 10 mL of phosphate buffer (pH 7.5). The analysis was carried out as described before. The extracts of the water samples were also analyzed using a standard method with a Varian 3400 gas chromatograph coupled to a Finningan Mat 800 ion trap detector mass spectrometer (GC-ITDMS).

RESULTS AND DISCUSSION

Cyclic voltammetry (CV)

CV was performed to investigate the electrochemical behaviour of the TCNQ at the carbon working electrode surface of the screen-printed strip. Figure 2 shows two well defined peaks due to the oxidation and reduction of the TCNQ in the potential range examined. The voltammograms exhibit also a remarkable 'inert zone' (a range of the application potential in which no faradaic reaction occurs[17]) between the reduction and oxidation peaks, that, in our experience, is not influenced by the scan rate. Although this inert zone is quite wide, the principal peaks ($I_{red}$and I_{ox}) are equal in area, which indicates a chemical reversibility, as reported in literature[17].

It is reported that the TCNQ have two electronic reactions:

$$TCNQ + e^- \rightarrow TCNQ^- (\text{process I})$$

$$TCNQ^- + e^- \rightarrow TCNQ^{2-} (\text{process II})$$

As shown in Figure 2, in our experimental conditions only one process is evident, and from the peak potential values it has been attributed[17] to process (I). Moreover, Figure 2 shows how reproducible signal can be reached only after three scans.

As reported in Figure 3a and 3b, it was experimentally observed that the presence of Nafion increases the sensor stability. We found that by such procedure the sensors can be safely stored up to 1 week. After this period of time unreproduceable results can be expected. This point needs to be extensively addressed however the screen printed electrodes are used only for a single measurement as disposable devices. The CV measurements were carried out on TCNQ modified SPE and TCNQ-Nafion modified SPE stored at room temperature for three days, in the presence of desiccant.

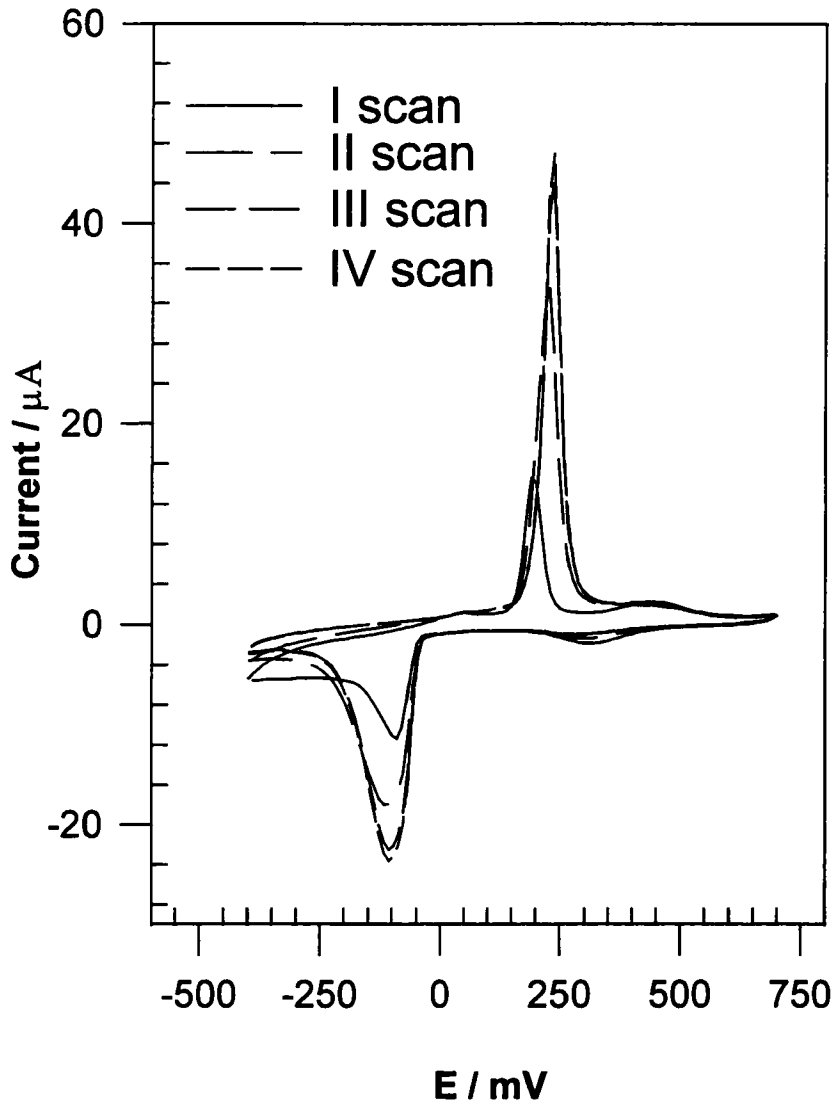

FIGURE 2 Voltammetric response of the TCNQ-Nafion® modified SPE, immersed in 0.1 mol/l phosphate buffer (pH 7.5). Potential range –0.4 to +0.7V; scan rate 50 mV s^{-1}

Optimisation of DPV parameters and sensor realisation

The effect of scan rate and pulse amplitude on the DPV, was investigated over the range 5 to 100 mV s^{-1} for the scan rate and 5 to 100 mV for the pulse ampli-

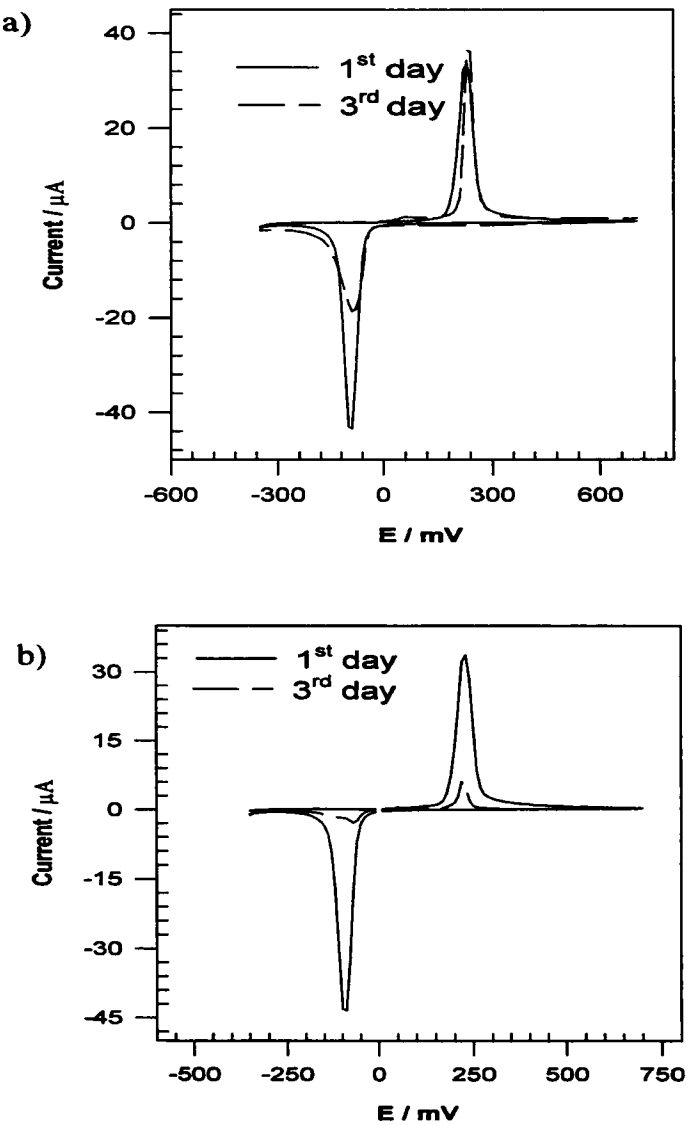

FIGURE 3 Effect of Nafion® on sensor stability: Cyclic voltammogram of TCNQ-Nafion® modified SPE (a) and cyclic voltammogram of TCNQ (without Nafion) modified SPE (b). The sensors were stored at room temperature for three days, in presence of dessicant

tude. Each scan was examined in triplicate and the mean was used for the evaluation of the current (Figure 4). A pulse amplitude of 25 mV and a scan rate of 50

mVs^{-1} are chosen as good compromise between sensitivity and reproducibility (signal to noise ratio).

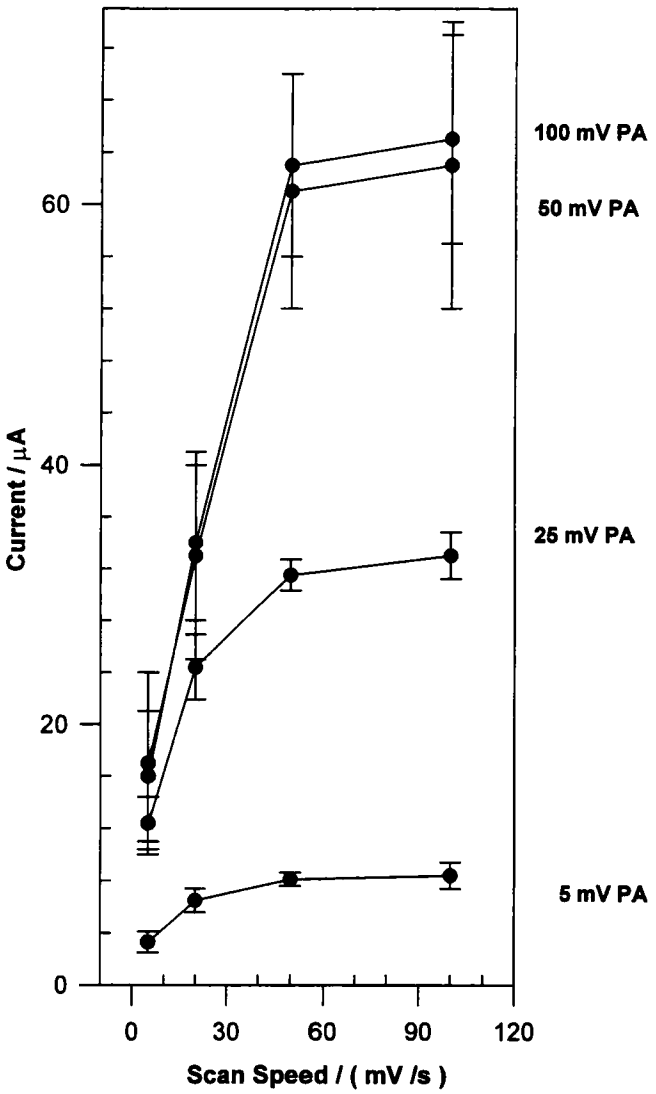

FIGURE 4 Effect of the Scan Speed (mV/s) (a) and of Pulse Amplitude (mV) on sensor response, pulse width 60 ms

The effect of the TCNQ loading was investigated over the range 10 to 100 ng TCNQ per electrode. The current values range from 20 μA to 80 μA but the best signal to noise value were obtained at 50 ng TCNQ per electrode. This value reflects the reproducibility of the electrochemical scan.

Acetylcholinesterase measurements

$TCNQ_{ox.}$ immobilized on the graphite surface is reduced by the thiocholine produced in the enzymatic reaction to $TCNQ_{red.}$; this form is reoxidized at the electrode surface in a potential window ranging from 0.35 to 0.7 V vs. Ag pseudo reference electrode. Therefore the potential scan was fixed between the two values, obtaining an oxidation peak which is our analytical signal. In absence of TCNQ no peak can be observed even up to 1 V under the same conditions. The height of this value is proportional to the thiocholine concentration produced, therefore to the activity of the enzyme.

Figure 5 presents DP voltammograms of the TCNQ-Nafion modified SPE performed under different conditions. In phosphate buffer solution pH 7.5, no peak was observed in the applied voltage range, because the TCNQ is oxidized under these conditions (a), in 5.10^{-3} mol/L acetylthiocholine solution the differential pulse voltammetric curve changed slightly, but no peak occurred, indicating that the acetylthiocholine is not oxidised, under these experimental conditions (b); in the presence of acetylthiocholine and the enzyme AChE, the curve displayed a peak at +0,5V (c). The peak current increases with increasing the time of the enzymatic reaction and attained a constant value after 5 min. The peak current increases with the enzyme activity and it is exploited for inhibition detection.

The measurements of enzymatic inhibitors have to follow a zero order kinetic ($[S] \gg K_M$, where [S] is the concentration of the substrate and $K_M$is the Michaelis-Menten constant). For this reason the enzyme activity has to be optimized. This parameter affects the detection limit, with higher enzymatic activity resulting in higher detection limits, while lower enzymatic activity results in a higher coefficient of variation due to the low reproducibility of the analytical signal [6]. The enzyme concentration was varied in the range 0.2 – 4 U/mL and best results in term of sensitivity and reproducibility were obtained with 1 U/mL AChE (Figure 6). These concentrations were used for the further work. Such values ensure that the enzymatic reaction is complete. It is important to consider that the amount of thiocholine obtained is not dependent from the acetylcholine concentration but upon the enzyme activity. We must assume that in our conditions a steady state is reached and the concentration value of thiocholine depends on the activity of enzyme.

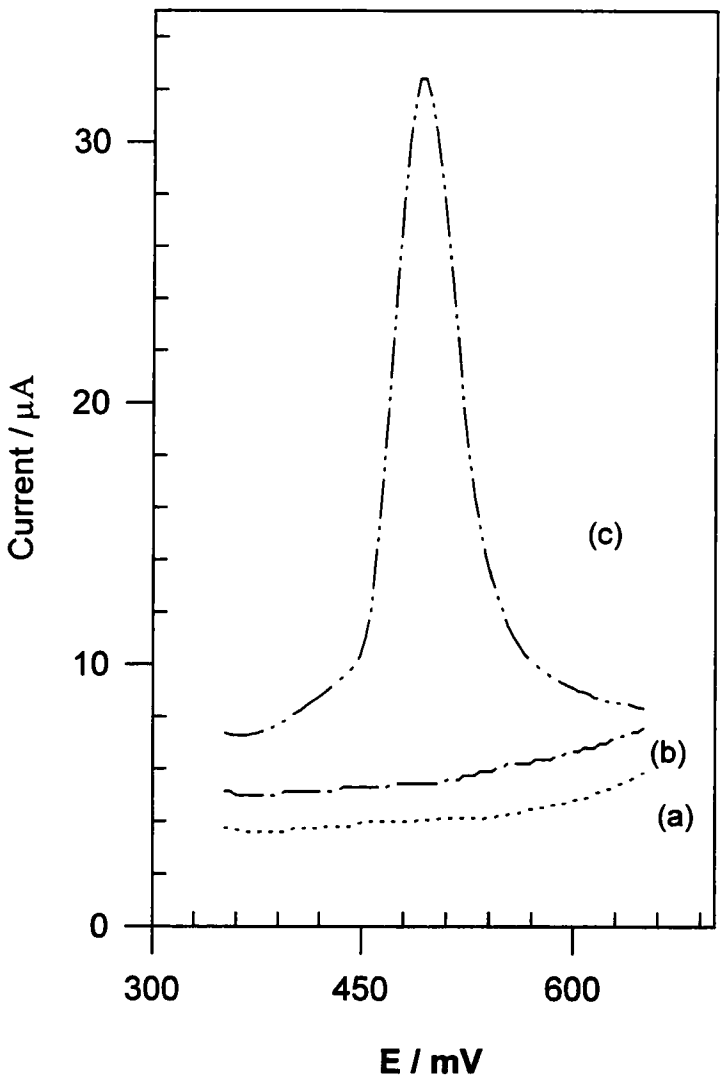

FIGURE 5 Differential Pulse Voltammogram in different conditions: a) phosphate buffer 0.1M pH 7.5, b) phosphate buffer 0.1M pH 7.5 + 5 mM acetylthiocholine, c) phosphate buffer 0.1M pH 7.5 + 5 mM acetylthiocholine + AchE

The influence of the solution pH was analyzed in the range 4–10. As it is well known, the pH of the solution greatly affects the enzyme activity. When the pH range is 4–6 the current responses change slightly, when the pH is more than 8

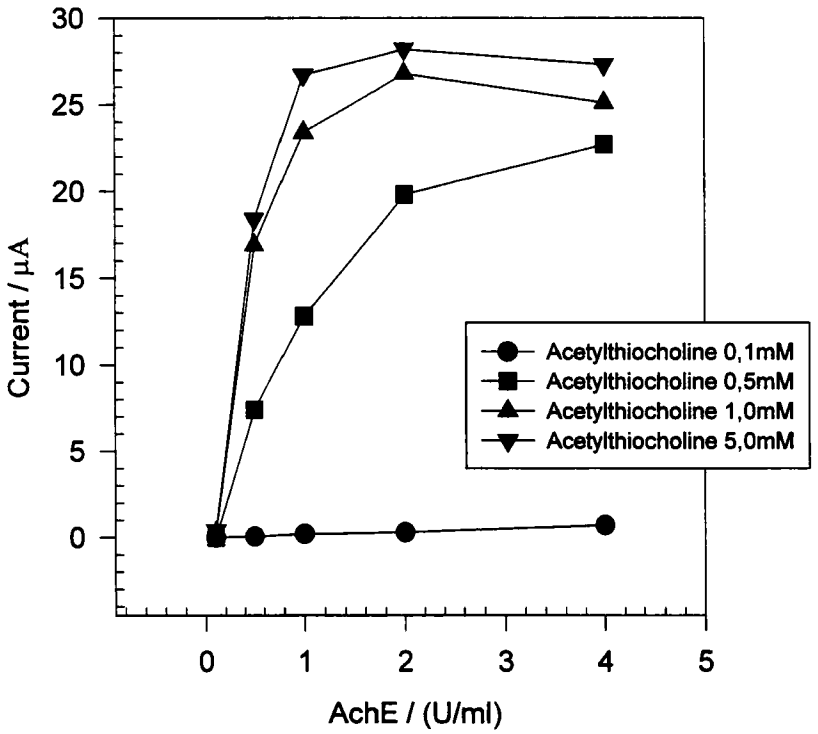

FIGURE 6 Influence of the enzyme activity and of the substrate concentration on sensor response

the voltammetric response is very small. The maximum currents appear at pH 7–8; thus the pH 7.5 was chosen as optimal value.

Then, a calibration curve of acetylthiocholine was obtained with 1 U/mL AChE. The variation coefficients evaluated for four replicates of 10^{-3} mol/L were 22% and 18% using 2 min and 5 min of enzyme-substrate incubation time respectively. The variation coefficients were calculated using different electrodes. The reproducibility of the SPE construction generally contributed to the standard deviation of the final measurement with about 10%. The remaining contribution to the standard deviation can be attributed to the amount of TCNQ, Nafion and the preparation of the enzyme/substrate solution. The test is also performed at room temperature and this can be another factor of inaccuracy. However the use of SPE is only compatible with such kind of procedure.

CARBOFURAN DETERMINATION

Figure 7 shows the calibration curve of Carbofuran, in the range 10^{-9}-10^{-6} mol/L. The detection limit defined as the concentration of the pesticide that produces a detectable decrease of the current peak can be expressed as the concentration required for 20% inhibition of the cholinesterase activity, corresponding to 9.10^{-9} mol/L (2 μg/L) of Carbofuran. This method clearly only allows a semiquantitative determination of pesticide concentration.

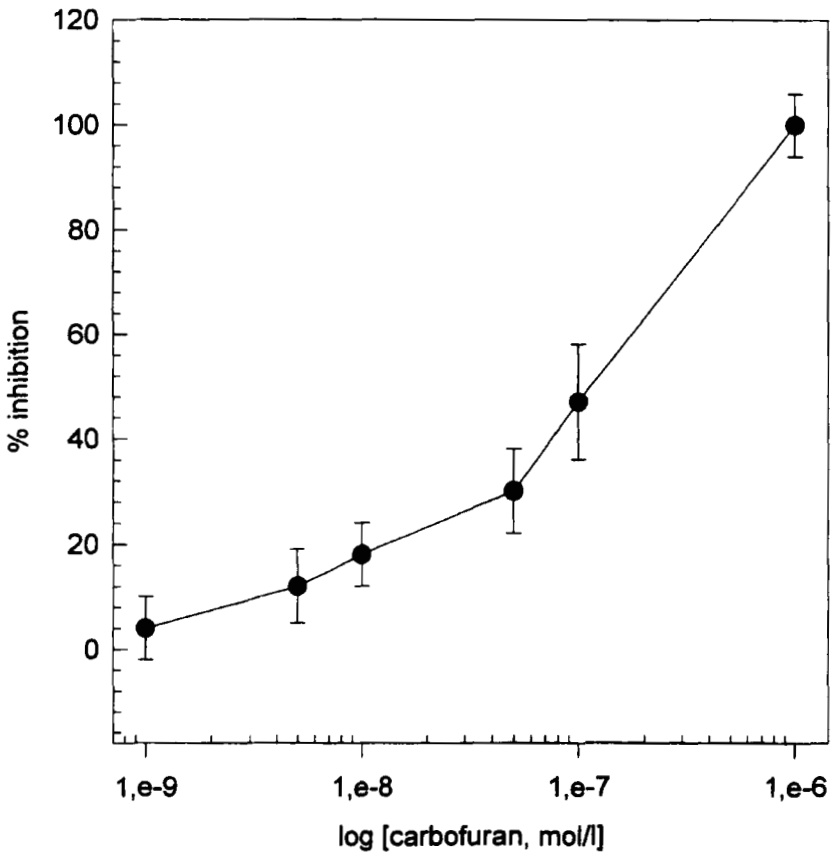

FIGURE 7 Inhibition Calibration Curve of Carbofuran: incubation time 10 min

APPLICATION TO REAL SAMPLES

Preliminary analysis of the Arno river water performed in our laboratory and in the Water Supply Company of Florence[26] during 1997 and 1999 have demonstrated that the presence of pesticides in the Arno river has a seasonal behaviour. Maximum concentrations of each pesticide were observed each year in May and June, showing an obvious relationship with the increase of agricultural activities during this period of each year. These results agree with the use of the pesticides in the Tuscany region, where the application period for pesticides is generally about 10 weeks, starting in the last week of April and lasting until the first week of July. Autumn and winter applications are minimum, and this is reflected in the small concentrations and low detection frequency during autumn and winter months. The cholinesterase inhibitor pesticides, as propoxur, ethoprop, bendiocarb, phorate and carbofuran, were only occasionally detected at very low concentrations (in many cases, <0.1 μg/L, this value is lower than the detection limit of our approach).

Therefore, to detect these low levels of pesticide concentration with our system, the samples have to be preconcentrated by the use of solid-phase extraction (SPE) techniques[27,12](the SPE procedure was optimised previously[26]). The Arno River water samples were preconcentrated 100 times (in this case the detection limit of the procedure becomes 9.10^{-11} mol/L). Table I illustrates the results of the water samples obtained with a standard method (gas chromatography) and by the thiocholine sensor. There is a good correlation between the two sets of data in the period of high pesticide occurrence in the Arno river. However the results obtained with the sensor were slightly greater than those obtained by the reference method, because the sensor determinates a group of cholinesterase inhibitor compounds.

CONCLUSION

This approach proposes the use of a fast and sensitive voltammetric technique as DPV coupled with screen-printed disposable electrodes for pesticides determination. The method is based on a simple measurement procedure with a detection limit of 0.02 μg/L, with 10 min of incubation time; it can be proposed as a rapid screening tool of toxicity of water samples, and can be utilised for in field monitoring. The described sensor has a good analytical performance in comparison with standard method (GC-ITDMS), when it is utilised for the analysis of the preconcentrated samples.

TABLE I Comparison of the results obtained by Gas Chromatography (GC-ITDMS) and Thiocholine Sensor. The Arno River water samples were 100 times concentrated by Solid-Phase Extraction, Carbofuran was the reference pesticide

	Gas Chromatography		*Thiocholine Sensor*	
Samples (date)	*Carbofuran Concentration ng/L*	*Carbofuran Concentration mol/L*	*% Inhibition*	*Carbofuran Concentration Equivalent mol/L*
17/4/99	0.2	$2\ 10^{-13}$	----	$< 9\ 10^{-11}$
21/4/99	0.2	$2\ 10^{-13}$	----	$< 9\ 10^{-11}$
30/4/99	0.2	$2\ 10^{-13}$	----	$< 9\ 10^{-11}$
24/5/99	**26**	$\mathbf{1.2\ 10^{-10}}$	**40 ± 9**	$\mathbf{1\ 10^{-9}}$
25/5/99	**24**	$\mathbf{1.1\ 10^{-10}}$	**30 ± 7**	$\mathbf{4\ 10^{-10}}$
26/5/99	**21**	$\mathbf{1.0\ 10^{-10}}$	**30 ± 7**	$\mathbf{4\ 10^{-10}}$
27/5/99	**13**	$\mathbf{6.0\ 10^{-11}}$	**15 ± 6**	$\mathbf{1\ 10^{-10}}$
7/6/99	**33**	$\mathbf{1.5\ 10^{-10}}$	**40 ± 9**	$\mathbf{1\ 10^{-9}}$
21/6/99	**24**	$\mathbf{1.1\ 10^{-10}}$	**40±9**	$\mathbf{1\ 10^{-9}}$
3/7/99	0.3	$4\ 10^{-13}$	----	$< 9\ 10^{-11}$
9/7/99	0.5	$5\ 10^{-13}$	----	$< 9\ 10^{-11}$
13/7/99	0.2	$2\ 10^{-13}$	----	$< 9\ 10^{-11}$
16/7/99	0.6	$8\ 10^{-13}$	----	$< 9\ 10^{-11}$

Acknowledgements

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