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Detection of carbamic and organophosphorous pesticides in water samples using a cholinesterase biosensor based on Prussian Blue-modified screen-printed electrode

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Abstract

In the present paper, a comparative study using Co-phthalocyanine and Prussian Blue-modified screen-printed electrodes, has been performed. Both the electrodes have demonstrated an easiness of preparation together with high sensitivity towards thicholine (LOD = 5×10^{-7} and 5×10^{-6} M for Co-phthalocyanine and Prussian Blue, respectively) with high potentialities for pesticide measurement. Prussian Blue-modified screen-printed electrodes were then selected for successive enzyme immobilization due to their higher operative stability demonstrated in previous works. AChE and BChE enzymes were used and inhibition effect of different pesticides was studied with both the enzymes. AChE-based biosensors have demonstrated a higher sensitivity towards aldicarb (50% inhibition with 50 ppb) and carbaryl (50% inhibition with 85 ppb) while BChE biosensors have shown a higher affinity towards paraoxon (50% inhibition with 4 ppb) and chlorpyrifos-methyl oxon (50% inhibition with 1 ppb). Real samples were also tested in order to evaluate the matrix effect and recovery values comprised between 79 and 123% were obtained.

Keywords: Pesticide; Acetylcholinesterase; Butyrylcholinesterase; Screen-printed electrodes

1. Introduction

The presence of pesticide residues and metabolites in food, water and soil currently represents one of the major issues for the environmental chemistry. Pesticides are, in fact, among the most important environmental pollutants because of their increasing use in agriculture [1–3]. Among the pesticides, organophosphate and carbamate species are the most used due to their high insecticidal activity and relatively low persistence [4]. Their toxicity is mainly due to their inhibitory effect on acetylcholinesterase, a key enzyme for the nerve transmission. Organophosphate and carbamate pesticides toxicity can vary considerably, depending on the chemical structure of the pesticide [5,6].

Many methods are available for pesticide detection: chromatographic methods such as high performance liquid chromatography (HPLC) and gas chromatography (GC) are used as reference methods but present strong drawbacks such as complex and time-consuming treatments of the samples, i.e. extraction of pesticides, extract cleaning, solvent substitution, etc. [7–10]. Moreover, the analysis usually has to be performed in a specialised laboratory by skilled personnel and is not suitable for in situ application. These issues turn out to be a major problem when rapid and sensitive measurement are needed in order to take the necessary corrective actions in a timely fashion.

To respond to these issues, enzymatic methods have been adopted as an alternative to classical methods (GC and HPLC) for faster and simpler detection of some environmental pollutants [11 and references therein]. Cholinesterase-based biosensors are considered as one of the best alternatives in the context of this strategy. The simplicity and low-cost of the equipment also make possible "in situ" measurement of pesticide.

The quantification of anticholinesterase pesticides is based on the measurement of the decreased enzyme activity after exposure of the enzyme, either in the free form or immobilized on

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an appropriate support, to an inhibitor. This measure can only provide information about the total anticholinesterase toxicity of a given sample without the possibility of selectively detecting and quantifying different pesticides. The method is thus suitable only as a screening tool providing a rapid response and signalling the existence of contaminated samples. Various amperometric [12–15], potentiometric [16–18] and conductimetric biosensors [19] have been developed using this approach. For amperometric detection of cholinesterase activity, both the substrates acetylcholine and acetylthiocholine have been extensively used [20–24]. The latter is preferable because it avoids the use of another enzyme, choline oxidase, which is usually used with acetylcholine. However, the amperometric measure of thiocholine, produced by the enzymatically catalysed hydrolysis of acetylthiocholine, has proved difficult at classic electrode surfaces due to the high overpotential needed as well as the possible problems of surface passivation [25,26]. To overcome these important drawbacks pulsed electrochemical detection, use of mercury electrode and derivatizations have been studied [27]. The use of modified electrode surfaces capable of oxidising thiocholine at low applied potentials and without passivation has been also proposed. 7,7,8,8-Tetracyanoquinodimethane (TCNQ) was used as electrochemical mediator for thiocholine detection. Screen-printed electrodes were adopted and modified by depositing TCNQ in Nafion® solution on the working electrode surface [28]. Also TCNQ was mixed with graphite ink in order to obtain a bulk-modified TCNQ electrode [29–31]. The use of glassy carbon electrode modified with carbon nanotube has been also recently proposed for thiocholine amperometric detection at low applied potential demonstrating good analytical features [21].

Cobalt phthalocyanine (Co-phthalocyanine), after its first demonstrated use as thiocholine mediator, remains one of the most used electrocatalysts for this purpose. Hart and Hartley [32] was the first to show the advantages of such mediator and its possible application for environmental monitoring of pesticides. The best example of the use of such mediator, in terms of easiness of production and sensitivity towards thiocholine, still remains the bulk-modified Co-phthalocyanine electrode, which has been extensively used for pesticide detection purposes [22,32,33].

Recently, our group has demonstrated the possibility to detect some important thiols at low applied potential (200 mV versus Ag/AgCl) with the use of a Prussian Blue-modified screen-printed electrode (SPE) [34]. High amperometric signals, with a corresponding low detection limit, were obtained for thiocholine. The good sensitivity of SPE modified with Prussian Blue, the easiness of surface modification together with the high stability of the Prussian Blue layer led us to investigate in more detail the possibility of applying these sensors for pesticide analysis. In this paper, we present a comparison between our Prussian Blue-modified sensors and cobalt phthalocyanine-modified electrodes in terms of thiocholine detection. A practical application of Prussian Blue-modified electrodes with immobilized cholinesterase is also presented, which demonstrates the suitability of the Prussian Blue sensors for the proposed application. The detection method is achieved via a two steps measurement designated as "medium exchange" method. The first step involve the inhibition, which is performed in a solution obtained with the working buffer and the sample mixed in a 1:1 ratio. The second step is performed after the washing of the electrode and in a working buffer solution where the enzymatic substrate acetylthiocholine is added and the thiocholine is amperometrically detected. In this way, the enzyme acts as a capture agent for the pesticide, and, because of the irreversibility of the inhibition, the successive enzymatic reaction can be carried out in a clean buffer solution, avoiding the effect of any interfering compound eventually present in real samples (i.e. SDS or heavy metals). Real water samples were then analysed with this system demonstrating the suitability of the method.

2. Experimental

2.1. Materials

2.1.1. Reagents

All chemicals from commercial sources were of analytical grade. Cobalt phthalocyanine wad purchased from Fluka (St. Louis, USA) and potassium ferricyanide from Carlo Erba (Milano). Acetylcholinesterase (AChE) from electric eel, butyrylcholinesterase (BChE) from horse, bovine serum albumine, *S*-butyrylthiocholine chloride, acetylthiocholine chloride and glutaraldehyde were purchased from Sigma Chemical Company (St. Louis, USA). Cadmium nitrates from Carlo Erba (Milano); cupric and zinc sulfate from Sigma; sodium dodecyl sulfate (SDS) and sodium fluoride from Fluka. Nafion® (perfluorinated ion-exchange resin, 5% (v/v) solution in lower alcohols/water) was obtained from Aldrich (Steinheim, Germany). Chloropyrifos-methyl(*O*,*O*-dimethyl-*O*-(3,5,6-trichloro-pyridyl)phosphoro-thioate), aldicarb (2-methyl-2-(methylthio)propionaldehyde-*O*-methylcarbamoyloxime), carbaryl (1-naphthyl methylcarbamate) were purchased from Riedel-de-Haen (Seelze, Germany) and paraoxon from Sigma Chemical Company (St. Louis, USA).

2.1.2. Electrodes

Screen-printed electrodes (SPEs) were home produced with a 245 DEK (Weymouth, England) screen-printing machine. Graphite-based ink (Elettrodag 421) from Acheson (Milan, Italy) was used to print the working and counter electrode [35]. The substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The electrodes were produced in foils of 20. The diameter of the working electrode was 0.2 cm resulting in an apparent geometric area of 0.03 cm^2. A silver ink was used to print the reference electrode. Before thiol measurements, the reference electrode was chlorurated. To do this, a potential of 0.6 V was applied between the silver ink and an external Ag/AgCl electrode for 20 s in a phosphate buffer solution in the presence of 0.1 M KCl [36].

2.2. Apparatus

Amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to an *X-t* recorder (L250E, Linseis, Selb, Germany).

Cyclic voltammetry (CV) was performed using an Autolab electrochemical system (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands).

2.3. Procedures

2.3.1. Preparation of Prussian Blue-modified screen-printed electrodes

Prior to Prussian Blue modification, screen-printed electrodes (SPE) were pre-treated in a 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4 by applying an anodic potential of 1.7 V for 3 min. Prussian Blue modification of SPEs was then accomplished by placing a drop (10 μL total volume) of "precursor solution" onto the working electrode area. This solution is a mixture obtained by adding 5 μL of 0.1 M potassium ferricyanide ($K_3Fe(CN)_6$) in 10 mM HCl to 5 μL of 0.1 M ferric chloride in 10 mM HCl. The drop was carefully applied exclusively on the working electrode area. The electrodes were shaken gently on an orbital shaker for 10 min and then rinsed with a few millilitres of 10 mM HCl. The probes were then left 90 min in the oven at 100 °C to obtain a more stable and active layer of Prussian Blue.

The Prussian Blue-modified electrodes were stored dry at room temperature in the dark.

2.3.2. Preparation of cobalt phthalocyanine bulk-modified screen-printed electrodes

The preparation of cobalt phthalocyanine-modified SPEs was obtained by following the procedure by Hart and Hartley [32]. Before the printing step, the graphite ink was carefully mixed with 5% of cobalt phthalocyanine (CoPC) powder in order to make the paste homogenous. After this, the ink was used to print the working electrode of SPE as for the other electrodes (same geometry and surface area as above).

2.3.3. Thiocholine measurements

Thiocholine was produced enzymatically by AChE using acetylthiocholine as substrate (because thiocholine is not commercially available). For this purpose, 1 mL of 1 M acetylthiocholine solution was prepared in phosphate buffer 0.1 M (pH 8), and 100 units of AChE were added to this solution. After 1 h, the concentration of thiocholine produced by AChE was estimated spectrophotometrically by Ellman's method. For this purpose, 900 μL of phosphate buffer solution (0.1 M, pH 8), 100 μL of 0.1 M DTNB, and 5 μL thiocholine solution (diluted 1:100 in water) were put in a spectrophotometric cells. The absorbance was measured, and the real concentration was evaluated by using the Lambert–Beer law with the known molar extinction coefficient of TNB ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$) [37]. After 1 h, the acetylthiocholine hydrolysis is completed, and 1 mL solution of 1 M thiocholine is obtained. The solution is stable for 1 day at 4 °C.

In the case of PB-modified SPE, thiocholine measurements were performed using amperometric batch analysis in a stirred phosphate buffer solution 0.05 M + 0.1 M KCl, pH 7.4 (10 mL) with an applied potential of +200 mV versus Ag/AgCl.

Cobalt phthalocyanine-modified SPE were used with the same procedure, but the phosphate buffer solution was pH 8 (10 mL) and the applied potential +100 mV versus Ag/AgCl. In both cases, when a stable baseline was reached (1 min), the analyte was added and the response was recorded.

2.3.4. Cholinesterase biosensor based on Prussian Blue-modified SPE

Prussian Blue-modified SPEs were used as substrate for enzyme immobilization. Two different biosensors were produced immobilizing, in one case acetylcholinesterase to create an AChE biosensor, and in the other case butyrylcholinesterase to obtain a BChE biosensor. For this purpose a cross-linking method using two steps was adopted. Two microliters of a glutaraldehyde solution were applied with a syringe exclusively on the working electrode. For the AChE biosensor, a 1% (v/v) solution of glutaraldehyde (diluted in water) was used, while for the BChE biosensor a 0.25% (v/v) solution (diluted in water) was utilised. The solution was then left to evaporate. Then, 2 μL of a mixture of BSA, enzyme and Nafion® were applied on the working electrode. The mixture was obtained by mixing 25 μL of BSA (5% (w/v) prepared in water), 25 μL of Nafion® (1% (v/v) diluted in water) and 25 μL of an enzyme stock. In the case of AChE biosensor, acetylcholinesterase was used in a concentration of 15 U mL^{-1}. For BChE biosensor, 4 U mL^{-1} of butyrylcholinesterase were used.

The total amount of enzyme immobilized onto one single electrode was 0.01 and 0.0025 U for acetylcholinesterase and butyrylcholinesterase, respectively. Once the solution had evaporated, the biosensors were kept in phosphate buffer solution 0.05 M + 0.1 M KCl, pH 7.4 at 4 °C.

2.3.5. Acetylthiocholine and butyrylthiocholine measurement with AChE and BChE biosensors

Acetylthiocholine measurements were performed using an amperometric "drop" procedure in phosphate buffer solution (0.05 M + 0.1 M KCl, pH 7.4) with an applied potential of +200 mV versus Ag/AgCl. The drop (50 μL) of buffer containing different amount of acetylthiocholine was placed onto the AChE biosensor in such a way that the counter and reference electrodes were also covered. After applying the potential, the signal was recorded continuously and the current value at the steady state was taken. The time needed for the stabilisation of the current was found to be between 2 and 10 min depending on substrate concentration. For butyrylthiocholine measurement the same procedure was carried out using a BChE biosensor.

2.3.6. Inhibition measurement using AChE and BChE biosensors

The inhibitory effect of different pesticides on AChE and BChE biosensors was evaluated by determining the decrease in the current obtained for the oxidation of thiocholine that was produced by the enzymes. To do this, the cholinesterase biosensor was first incubated in the pesticide solution for a certain period (incubation time) and then rinsed three times with distilled water. After that, the response toward the substrate

Table 1
Analytical parameters for the cobalt phthalocyanine and Prussian Blue-modified electrodes

Transducer	Modifier	Applied potential vs. Ag/AgCl (mV)	Time for background stabilisation (s)	Sensitivity (mA M^{-1} cm^{-2})	Noise (nA)	Detection limit (M)	Linear range (M)	Working stability
Screen-printed graphite	Cobalt phthalocyanine	+100	30	24	0.2	5×10^{-7}	1×10^{-6}/7×10^{-5}	Fair
	Prussian Blue	+200	30	143	4	5×10^{-6}	5×10^{-6}/5×10^{-4}	High

For details about measurement conditions see text.

was measured as described above and the degree of inhibition was calculated as a relative decay of the biosensor response (Eq. (1)).

$$I\% = \frac{I_0 - I_i}{I_0} \times 100 \quad (1)$$

where I_0 and I_i represent the biosensor response before and after the incubation procedure, respectively.

Four standard pesticides were tested in this work. Aldicarb and carbaryl were tested as carbamate pesticides while paraoxon and chlorpyrifos-methyl oxon were used as example of standard organophosphate pesticides. Chlorpyrifos-methyl oxon was obtained from chlorpyrifos-methyl via an oxidation step [38]. The oxidation was carried out using the procedure described by Ivanov et al. [39]. Real samples were first diluted 1:1 with phosphate buffer 0.1 M + 0.2 M KCl, pH 7.4 before the inhibition step. After the dilution, pH was verified and, when necessary, adjusted to pH 7.4. To study the effect of different salt contents, the inhibition with 5 ppb of paraoxon was studied using 0.05 M phosphate buffer with different KCl concentrations (i.e. 0.1, 0.2 and 0.5 M).

To evaluate the possible interference effect due to the presence of detergent in the sample, two different experiments were carried out. The biosensor was incubated in phosphate buffer solutions containing sodium dodecyl sulfate (SDS). In the first experiment, the biosensor was rinsed after the incubation time and the residual activity was measured in a new phosphate buffer solution.

In a second experiment, in the other hand, the substrate was added into the same buffer solution containing the interference specie without any washing step after the incubation time.

The same procedure was also adopted to evaluate the possible interference effect due to heavy metals and fluoride. In this case copper, fluoride, zinc and cadmium were tested. Concentrations used for both detergent (SDS) and heavy metals are those reported as the maximum admissible value for wastewater [40].

2.3.7. Sample collection

Wastewater samples were supplied by ACEA (municipal water company) and collected in different days from input to the water softener. Tiber river samples were also collected. All the samples were tested before and after the spiking with pesticide. Each measurement was also performed before and after a filtration step, using a 20 μm filter.

3. Result and discussion

3.1. Thiocholine sensors

As already stated in Section 1, the measurement of thiocholine is of central importance for pesticide measurement with ChE biosensors in order to avoid the use of a bienzymatic system. In this work, we have investigated the response of Prussian Blue and Co-phthalocyanine-modified SPE as thiocholine sensors. The comparison of their performances in terms of thiocholine signal, stability and response time would allow us to select the most suitable sensor for the successive ChE enzyme immobilization. The Co-phthalocyanine-modified sensor is well known and many examples of its use can be found in literatures [22,32,33]. In the case of Prussian Blue-modified electrodes, their first application for this purpose was recently proposed by our group [34].

Table 1 shows the analytical parameters obtained for amperometric thiocholine detection using the modified SPEs at their optimised conditions.

In the case of Co-phthalocyanine-modified SPE, the sensors were able to detect thiocholine at a concentration of 5×10^{-7} M with a linearity up to 7×10^{-5} M. Our results are in agreement with Hart's works [32] in terms of linear range and detection limit.

Above the good analytical features demonstrated by these electrodes, it has to be stressed the easiness of production which makes them suitable for mass production. Moreover, the low applied potential and the fast response time make it theoretically possible to achieve selectivity with the avoidance of the majority of the electrochemical interferences. However, under our operative conditions only a few measurement could be performed with the same sensor because of their limited stability.

The performance of Prussian Blue as a mediator of thiocholine oxidation has been investigated by our group in a previous work [34]. This mediator shows a good stability (when stored several months dry at room temperature in the dark) [41] and reproducibility (7%). The modification of the electrode surface is easy to perform as well as being amenable to mass production. Moreover, the Prussian Blue-modified sensors allow the measurement of thiocholine at micromolar levels, with high sensitivity and a broad linear range. The sensitivity is higher for Prussian Blue electrode than Co-phthalocyanine electrode. However, a lower detection limit was obtained with Co-phthalocyanine electrode due to the lower noise of these electrodes (see Table 1). The major advantage associated with

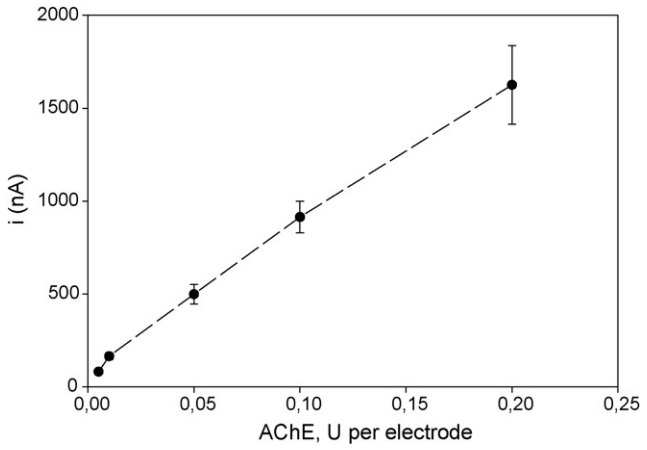

Fig. 1. Effect of enzyme concentration on the response of the AChE biosensor. Applied potential = +200 mV vs. Ag/AgCl. ATChCl = 5 mM, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4.

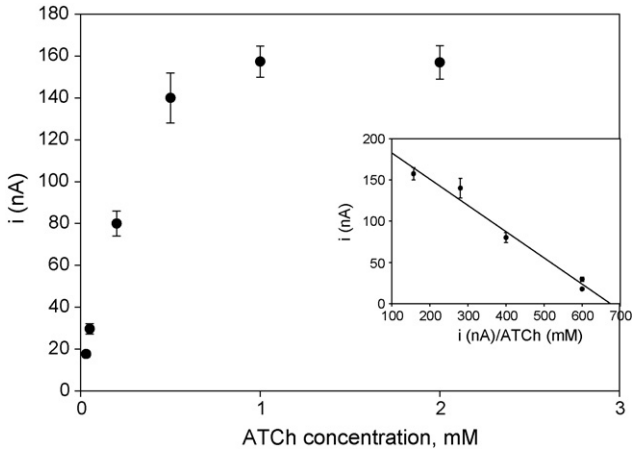

Fig. 2. Calibration plot of acetylthiocholine using an AChE biosensor. Applied potential: +200 mV vs. Ag/AgCl. AChE = 0.01 U, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. Inset: Eadie–Hofstee plot.

the use of PB-modified sensor is the very high stability even under drastic conditions, thus making it very useful for practical applications. Also, no effect due to thiol passivation has been observed, thus allowing several measurements without loss of sensitivity. For these reasons, and given our long experience in the use of Prussian Blue-modified SPEs, these sensors were selected as probes for thiocholine detection to develop a biosensor for pesticide detection.

3.2. Cholinesterase enzyme biosensor based on Prussian Blue-modified SPE

In this work two enzymes, belonging to the same family of cholinesterase (EC 3.1.1.8), were considered to evaluate the sensitivity towards various pesticides. Thus, two different biosensors have been prepared, one based on AChE enzyme and another using BChE. The performance of these biosensors with respect to their respective enzymatic substrates have then been studied in order to evaluate the most important analytical parameters of the sensor itself: detection limit, linear range, response time, and biochemical parameters such as K_M and V_{max} of enzymes.

3.2.1. AChE biosensor

The dependence of the response on the amount of the enzyme immobilized on the electrode surface is shown in Fig. 1 for the AChE biosensor. Due to the fact that pesticide detection involve an irreversible inhibition of the enzyme, the lowest feasible concentration of enzyme is necessary to reach a low detection limit. A value of 0.01 U was chosen as the best compromise between a low enzyme loading and sufficiently high substrate signal. The reproducibility of the biosensors obtained was quite good. A R.S.D.% of 3% was observed for five replicates using the same biosensor (R.S.D.% intra-electrode). Five different biosensors were also tested with the same concentration of ATChCl resulting in a R.S.D.% value of 10% (inter-electrodes R.S.D.%).

Finally, Fig. 2 shows the calibration curve for different concentrations of substrate (i.e. ATChCl).

The apparent Michaelis–Menten constant, K_M^{app}, for acetylthiocholine can be determined using the Eadie–Hofstee form of Michaelis–Menten Eq. (2):

$$V = \frac{V_{max} - (VK_M)}{[S]} \quad (2)$$

Thus, resulting in a K_M^{app} of 0.33 mM (Fig. 2 inset). This value appears to be in accordance with the K_M (0.20–0.22 mM) [34,42] determined for the enzyme free in solution, demonstrating that the immobilization procedure does not lead to a significant decrease in affinity towards the substrate. A substrate concentration of 1.0 mM ATCh was then chosen for the inhibition measurement, given that it was the lowest concentration of substrate still giving the maximum saturated rate of reaction.

3.2.2. BChE biosensor

The amount of BChE to be incorporated on the electrode surface was optimized in the same manner as for AChE (data not shown) and resulted in a choice of 0.0025 U of BChE per electrode. Fig. 3 shows the calibration curve obtained for different concentrations of substrate (i.e. BTChCl). The K_M^{app} for

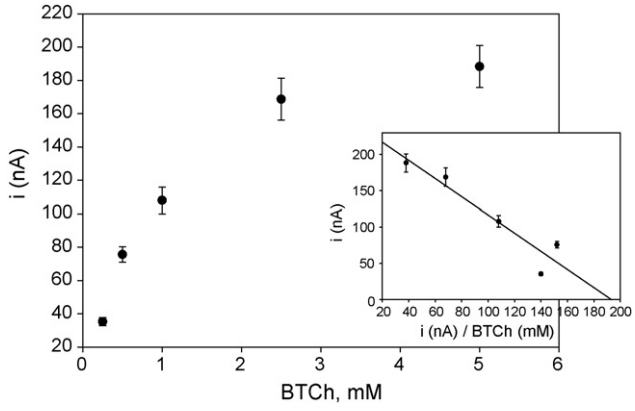

Fig. 3. Calibration plot of butyrylthiocholine chloride using a BChE biosensor. Applied potential: +200 mV vs. Ag/AgCl. BChE = 0.0025 U, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. Inset: Eadie–Hofstee plot.

Table 2
Concentration of pesticide giving 50% enzyme inhibition

Class of pesticide	Pesticide	AChE biosensor 50% of inhibition (ppb)	BChE biosensor 50% of inhibition (ppb)
Carbamates	Aldicarb	50	500
	Carbaryl	85	130
Organophosphates	Paraoxon	25	4
	Chlorpyrifos-methyl oxon	10	1

ATChCl (1 mM) and BTChCl (5 mM) were used a substrates for AChE and BChE, respectively. Applied potential: +200 mV vs. Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. Incubation time = 30 min.

BChE using BTChCl as substrate was calculated as described above and is equal to 1.4 mM. In this case, it appears that the immobilization procedure causes some distortion of the enzyme structure such that there is a strong decrease in its affinity for BTCh. The value of the K_M for the enzyme free in solution is in fact much lower (ca. 0.010 mM) [43] than that estimated for the enzyme immobilized on the biosensor. A substrate concentration of 5.0 mM was then chosen for the inhibition measurements.

Even in this case, the reproducibility of the biosensors was quite good: R.S.D.% value of 4% and 10% were obtained for intra-electrode ($n = 5$) and inter-electrode ($n = 5$) reproducibility. For both biosensors, however, a washing step is required between measurements. This is probably due to the fact that thiocholine is likely to be entrapped between the electrode surface and the membrane, thus affecting the successive measurement. To avoid this, a 2 min-washing step in a stirring buffer solution is required between measurements.

When stored at 4 °C in buffer solution the biosensors maintained their activity for 3 weeks for AChE biosensor and for 2 weeks for BChE biosensor, after which there was a rapid decrease for both enzymes.

3.3. Pesticide determination using AChE and BChE biosensors

The use of two different enzymes (i.e. AChE and BChE) for biosensors construction could potentially provide important information about the relative inhibitory effect of carbamate or organophosphate pesticides in relation to the type of cholinesterase adopted. An incubation time of 30 min was selected as a good compromise between the requirement for a rapid assay and an achievement of high degree of inhibition. The BChE and AChE biosensors were tested using standard solutions of organophosphate and carbamate pesticides. In the case of chlorpyrifos-methyl, an organophosphate with a thione group, it was first oxidized to its phosphoryl analogue by chlorine generated in the electrolysis [39]. This procedure led to an increased sensitivity towards the pesticide due to the higher inhibitory effect of the organophosphate with the oxo group [38].

Table 2 shows the concentration of pesticide, which gave rise to a 50% of inhibition using either an AChE or a BChE biosensor. In our experimental conditions, AChE seems to be more strongly inhibited than the BChE by carbaryl and aldicarb. In fact, in the case of AChE, a 50% inhibition was observed with concentrations of carbaryl and aldicarb of 85 and 50 ppb, respectively, while for the BChE biosensor, the same inhibition (i.e. 50%) was obtained in the presence of 130 ppb of carbaryl and 500 ppb of aldicarb.

Differential behaviour of AChE and BChE has been also observed with paraoxon and chlorpyrifos-methyl oxon. In this case BChE immobilized in our condition, shows a higher affinity towards the organophosphates tested. Fifty percent inhibition was obtained with a paraoxon and chlorpyrifos-methyl oxon concentrations of 4 and 1 ppb, respectively, while for the AChE biosensor, 25 ppb of paraoxon and 10 ppb of chlorpyrifos-methyl oxon were needed to obtain the same degree of inhibition.

Analytical parameters such as linear range, limit of detection (LOD) and reproducibility (RDS) of the AChE and BChE biosensors were then selectively investigated. The AChE biosensor was tested with aldicarb and carbaryl due to its higher sensitivity toward these compounds. For the same reason, paraoxon and chlorpyrifos-methyl oxon were tested with the BChE biosensor, which have shown a higher sensitivity towards these pesticides. Table 3 shows the results obtained. The detection limit (LOD), defined as the concentration giving an inhibition of 20% [11], was found to be 24 and 25 ppb, respectively for aldicarb and carbaryl using the AChE biosensor. With BChE biosensor, 2 and 0.5 ppb were the LOD values obtained for paraoxon and chlorpyrifos-methyl oxon, respectively. Both reproducibility and linear range (see Table 3) were satisfactory with AChE and BChE biosensors. The analytical parameters (such as detection limit (LOD) and linear range) obtained using

Table 3
Analytical parameters for AChE and BChE biosensors relative to different pesticides

Class of pesticide	Pesticide	Treatment before measurement	Type of biosensor	Linear range (ppb)	LOD (ppb)	R.S.D. (%)
Carbamates	Aldicarb	–	AChE	12–60	24	11
	Carbaryl	–	AChE	25–100	25	9
Organophosphates	Paraoxon	–	BChE	2–5	2	7
	Chlorpyrifos-methyl oxon	Oxidation	BChE	0.5–2	0.5	8

ATChCl (1 mM) and BTChCl (5 mM) were used a substrates for AChE and BChE. Applied potential: +200 mV vs. Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. Incubation time = 30 min.

Table 4
Recovery studies of spiked real water samples

Type of biosensor	Source of water	Pesticides	Expected (ppb)	Obtained (ppb)	Recovery (%)
AChE	Tiber filtered	Aldicarb	48	40	83
	Tiber		48	38	79
	Entrance water-softener filtered		48	51	106
	Entrance water-softener		48	60	125
BChE	Tiber filtered	Chlorpyrifos-methyl oxon	1.5	1.6	107
	Tiber		1.5	1.7	113

ATChCl (1 mM) and BTChCl (5 mM) were used a substrates for AChE and BChE. Applied potential: +200 mV vs. Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4. Incubation time = 30 min. All the values are mean of triplicate measurements.

biosensors based on Prussian Blue-modified SPE are comparable to those previously shown with Co-phthalocyanine.

In order to test these biosensor in real samples of wastewater, the influence of salt concentration on the degree of inhibition was also evaluated. The same degree of inhibition ($60 \pm 4\%$) with 5 ppb of paraoxon was obtained in phosphate buffer 0.05 M with different concentration of KCl (0.1, 0.2 and 0.5 M) demonstrating that under our experimental conditions the AChE biosensors are not affected by ionic strength; a comparable result was obtained with BChE biosensors.

The need for adopting a medium exchange method in the protocol for pesticide measurement has been demonstrated by testing the sensors with various interfering species such as fluoride (well known reversible cholinesterase inhibitors) [44,45], detergents [46,47] and heavy metals. The test was performed, as reported in Section 2, by measuring the residual enzymatic activity in the same buffer solution (with no washing step after the incubation time) and also by using the medium exchange method. It is known that heavy metals are not good ChE inhibitor [48], but their interference with thiocholine-based pesticide detection is mainly due to the fact that they can easily react with the thiocholine enzymatically produced and thus hampers its electrochemical oxidation. This was recently studied and reported by recent papers [42,49]. In the case of heavy metals no interference effect was observed with both methods adopted. This is probably due to the fact that the thiocholine is produced by the immobilized enzyme in the vicinity of the electrode surface and so is rapidly electrochemically oxidized and cannot react with heavy metals present in solution.

SDS was also tested as possible interfering specie [47]. When SDS (200 ppb, the limit value for wastewater [40]) was present in the buffer during the incubation time, measurement of the residual enzymatic activity in the same solution (with no washing step) resulted in an inhibition of 88%. On the contrary, when the medium exchange method was adopted, and a washing step was carried out between the incubation step and the measurement of residual enzymatic activity, no enzyme decay was observed. In our opinion, this demonstrated the need for adopting the medium exchange method, especially when handling wastewater samples, which are usually highly contaminated with detergents. With this procedure, the enzyme acts as a high affinity capture agent for the pesticide, and, because of the irreversibility of the inhibition, the successive enzymatic reaction can be carried out in a fresh buffer solution, thereby circumventing the effect of reversible inhibitors present in real samples.

3.4. Waste and river water measurements

BChE and AChE biosensors were then tested using both wastewater and river water samples from Tiber River, obtained from ACEA laboratory (municipal water company). Despite the fact that samples were taken from input tube of the softener, no inhibition was observed for either AChE or BChE biosensors. Finally, the samples were spiked with various amounts of pesticides. About 48 ppb of aldicarb were spiked in wastewater and river samples. Table 4 shows the results obtained for analysis of these spiked samples. A recovery of 79 and 125% was observed respectively for river samples and wastewater thus demonstrating low matrix effect on the biosensor signal. In the case of BChE biosensor, the river sample was spiked with chlorpyrifos-methyl oxon at a concentration of 1.5 ppb. Also in this case a good recovery was observed (i.e. 113%).

4. Conclusions

Co-phthalocyanine and Prussian Blue-modified screen-printed electrodes (SPE) for thiocholine detection were studied and their analytical features compared. An easiness of preparation together with a high sensitivity were observed for both sensors. Even if in the case of co-phthalocyanine electrodes a lower detection limit was observed, the Prussian Blue-modified SPEs was ultimately selected because of their superior operative stability. The Prussian Blue-modified SPEs were then used as substrate for the successive immobilization of two different ChE enzymes. AChE-based biosensors have shown a higher sensitivity towards aldicarb and carbaryl (LODs 12 and 25 ppb, respectively), while BChE demonstrated a higher affinity towards paraoxon and chlorpyrifos-methyl oxon (LODs 2 and 0.5 ppb, respectively). The response of the two biosensors were then characterised with different pesticides considering their different affinity. Real samples were also used in order to evaluate the matrix effect and applicability of the sensors to analytical problems giving encouraging results. Further studies are in progress to deeper investigate the different behaviour of the AChE and BChE towards the pesticide tested.

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