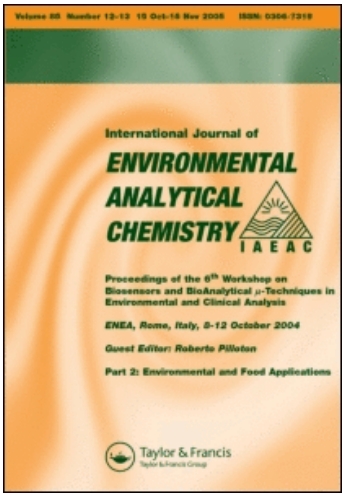

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A preliminary study on electrochemical biosensors for the determination of total cholinesterase inhibitors in strawberries

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Organophosphorous (OP) insecticides reveal acute toxicity because of their capability to affect the nervous system through the inhibition of acetyl cholinesterase function in regulating the neurotransmitter acetylcholine. The present work shows an example of an easy to be handled inhibition electrochemical biosensor, based on thick film technology for low cost production of screen printed electrodes. *Anti-cholinesterase activity* in specific fruits was determined measuring the inhibition of acetyl cholinesterase enzyme owing to the presence of OP pesticides. Paraoxon was taken as *reference* pesticide for each measurement. The main fluidic critical parameters were investigated under flow injection analysis, through the comparison of different enzymatic immobilisation methods. Analytical features were evaluated as a function of experimental parameters. The analytical detection was developed in a three step procedure and the pesticides content was measured in strawberries samples taken from the local market. The separation between the acetyl cholinesterase inhibition and the electrochemical detection with the choline oxidase biosensor decreases the total analysis time, allowing improvements in reproducibility and stability of the system. A comparison with reference materials and standard analytical procedures for pesticides will be required in the future for evaluating the reliability of the method.

Keywords: Cholinesterase inhibitors; Paraoxon; Screen printed electrodes biosensor; Strawberries

1. Introduction

Several organophosphorous (OP) compounds are still used as insecticides and chemical warfare agents [1, 2]. Organophosphorous monitoring in food and water is necessary, due to their acute toxicity as they act affecting the nervous system through the inhibition of acetyl cholinesterase (AChE) function in regulating the neurotransmitter acetylcholine.

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Many biosensors have been developed for *in situ* determination of OP compounds, especially those based on amperometric detection of the AChE activity, as supporting tool of the classical analytical techniques. Single use or disposable biosensors based on thick film technology, are attractive in on-field measurements wherein irreversible processes take place such as in this case. Screen printed electrodes (SPEs) are particularly suitable in low cost, easy to be used instrumentation, even compatible with hand-held analysers [3].

A novel procedure has been recently achieved in our laboratories to determine the acetyl cholinesterase inhibition, using diethyl *p*-nitrophenyl phosphate (Paraoxon) as a reference pesticide. AChE activity was measured with a double-enzymatic amperometric sensor by measuring the re-oxidation current of hydrogen peroxide generated as a final product of the following enzymatic sequence:

(a) AChE catalyzed hydrolysis of acetylcoline to choline;
(b) oxidation of choline to betaine by choline oxidase (ChOx).

AChE was immobilised on a nylon membrane for the measurement of residual cholinesterase activity after inhibition (the incubation steps were performed both into the inhibitor and substrate solutions). Effective measurement of the residual enzyme activity was therefore performed after the incubation in a standardised solution with a ChOx electrochemical biosensor. Homemade SPE were used for amperometric detection. Because the detection of the AChE activity was performed in a standard solution of the substrate, the proposed method is unaffected by interferences, avoiding the use of protecting selective membranes which greatly affect the sensitivity of biosensors. Incubation simultaneously performed on a number of samples enabled to lower the analysis time per sample. High sensitivity and reproducibility were then obtained for the analysis of Paraoxon. The possibility to regenerate AChE after treatment with Paraoxon was envisaged and fully investigated in a previous work [4], using AChE based biosensors and obtaining 86% recovery of the initiual activity. For this reason, such a regeneration was not taken into consideration in this article.

The procedure was extended to the determination of cholinesterase inhibitors in strawberries, adopting a very simple preparation of fruit sample, at the beginning of the above described analytical sequence. These samples were tested to verify any residual presence of cholinesterase inhibitor.

2. Experimental

2.1 *Materials and reagents*

ChOx from *alcaligenes species* (EC 1.1.3.17) 14.0 units per mg, AChE from *electric eel* (EC 3.1.1.7, lyophilized powder) with specific activity of 1170 units per mg of solid, bovine serum albumin (BSA, stock solution: 4% w/v), hydrogen peroxide, acetylcholine chloride (AcChCl), choline chloride (ChCl), glutaraldehyde (GA) 25% aqueous solution, polyazetidine (PAP) solution, amino-propyl trietoxy silane (APTES), Paraoxon-ethyl (pure yellow powder), potassium chloride, potassium and sodium phosphate salts were purchased from Sigma. All reagents, buffer and electrolyte supporting solution, e.g. 0.1 M phosphate buffer (PB) solutions, were prepared from

deionized (Millipore, mQ Synergy 170 apparatus). All other chemicals were of analytical grade and used without further purification.

2.2 *Screen printed probes preparation*

Conducting and insulators inks were printed on 0.3–0.5 mm thick polyvinyl-chloride (PVC) substrate using a HT10 Fleischle^{TM} screen printer (Brackenheim, Germany). Silver and carbon-graphite pastes for the conducting paths and working electrode (WE), Ag/AgCl for a pseudo-reference and insulator pastes were all from GWENT Electronics Materials Inc^{TM} (Polypool, GB). The 3-electrodes configuration of home produced SPE was reported in previously [5]. Active area of 2.54 mm^2 was measured for the graphite working electrode.

2.3 *Immobilisation of enzymes (choline oxidase and cholinesterase)*

AChE enzyme was covalently immobilised on different nylon pre-activated membranes by means of glutaraldehyde (GA). In order to achieve the covalent bonding via imide formation, amine-based membranes from PALL^{TM} (VWR International S.r.l.) were used in all the following experiments, owing to their stability and mechanical flexibility. The PALL membrane was immersed for 1 h in a solution of GA 12.5%, then washed in PB (pH 6.8). The amount of enzyme strictly influence the resulting limits of detection of the biosensing methods, therefore the AChE concentration was optimized at the 25 U mL^{-1} in PB; 5 μL of enzyme were dropped on each cm^2 of nylon membrane, thus allowing the covalent bond between aldehyde and free amino-group of enzyme. Each AChE membrane was fixed into a single needle of 6 × 4 homemade rubber support for simultaneous incubation of a number of samples in a 24 wells plate.

Different immobilisation procedures were tested with respect to ChOx enzyme:

(1) *ChOx entrapment in diglyceryl-silane (DGS)*. The electrode was firstly treated in APTES (10% in 0.1 M PB containing 0.1 M KCl) for 1 h and accurately washed. Simple covalent immobilisation was performed by dissolving in DGS aqueous solution the right amount of enzyme (from 10–50 mg mL^{-1}), then 5 μl of ChOx was dropped onto the functionalized WE.
(2) *Entrapment and crosslinking in polyazetidine (PAP)*. ChOx (10–50 mg mL^{-1}) was simply dissolved in PAP buffered solution and 5 μl dropped onto WE.
(3) *Crosslinking in a BSA-GA matrix*. The electrode was left in a basculating APTES 10% solution (0.1 M PB containing 0.1 M KCl) for 1 h, then washed and treated with 10 μl of crosslinked ChOx. In order to crosslink the enzyme (10–50 mg mL^{-1}), 5 μl of enzymatic solution were rapidly mixed to 20 μl of BSA (0.2 mg μl^{-1}) and 10 μl of 12.5% GA. The exceeding unreacted enzyme was removed by a following washing step.

In all the cases, the enzymatic solutions used for immobilisations were saturated with high concentrations of substrate (1 mM ChCl). In fact the application of the above reported immobilisation techniques without substrate saturation, did not give significant response, especially in the case of crosslinking in a BSA + GA matrix. During the immobilisation a chemical interaction between matrix and enzymatic active sites may take place in competition with the specific analytical reaction

enzyme/substrate, resulting in lowering of the biosensor duration. Hence, saturation with choline substrate represents a good solution to replace the role of the matrix in a such interaction with the available enzyme.

2.4 *Measuring set-up*

A Perspex homemade flow cell (volume about 38 µl) was used along a flow injection analysis (FIA) system in connection with a Gilson™ Minipuls-3 peristaltic pump and and sample loop of 115 µL. PB at the above optimized pH was used as a carrier in all the experiments. Amperometric measurements were carried out by means of Autolab™ potentiostat PGSTAT10 provided with GPES electrochemical analysis system (ECOchemie), applying the desired potential and allowing for steady state current to be reached.

Choline determination were performed separately from the AChE incubation step using the corresponding ChOx sensor, because of the two-enzymatic sequence here reported, generating as a by-product hydrogen peroxide:

$$\text{acetylcholine} + H_2O \rightarrow \text{acetic acid} + \text{choline}\,(\text{enzyme I : AChE}) \quad (1)$$

$$\text{choline} + O_2 + H_2O \rightarrow \text{betaine} + 2H_2O_2(\text{enzyme II : ChOx}) \quad (2)$$

The inhibitory effect was evaluated measuring the current decrease by following the oxidation of H_2O_2 at the electrode. The average current of the blank sample (no inhibitor, I_0) and average current of the contaminated sample (I_1) were used for calculating the inhibition percentage according to the following equation:

$$I(\%) = 100 \times \frac{(I_0 - I_1)}{I_0}. \quad (3)$$

3. Results

3.1 *Optimization of flow parameters in the inhibition biosensor*

Although many publications have been reported on the application of amperometric biosensors for measuring cholinesterase inhibition, few of them contain an exhaustive study on the optimization of the analytical performances of such biosensors (e.g. linearity range of calibration, limit of detection, sensitivity to the substrate, sensor lifetime). In the case of our disposable ChOx SPE-based sensor, shelf stability results much more important than operational stability. The main flow and chemico-physical parameters to be investigated throught the comparison of the immobilisation methods chosen for the ChOx biosensor were: injection time, flow rate, buffer pH and applied potential. At room temperature with PB solution at pH 6.8, similar sensor responses were obtained by varying the flow rate in the range 0.05–0.5 mL min^{-1}. In order to reduce the acquisition time, a high value of 0.40 mL min^{-1} was chosen for all the following experiments.

Figure 1 shows the dependence of the signal on the buffer pH in the enzyme working range after FIA injection of 0.1 mM ChCl, with respect to the immobilisation method nr.3 reported in the experimental section 2.3. Current response poorly varied along the pH range, excepting below 6.6. Similar responses were obtained using the remaining

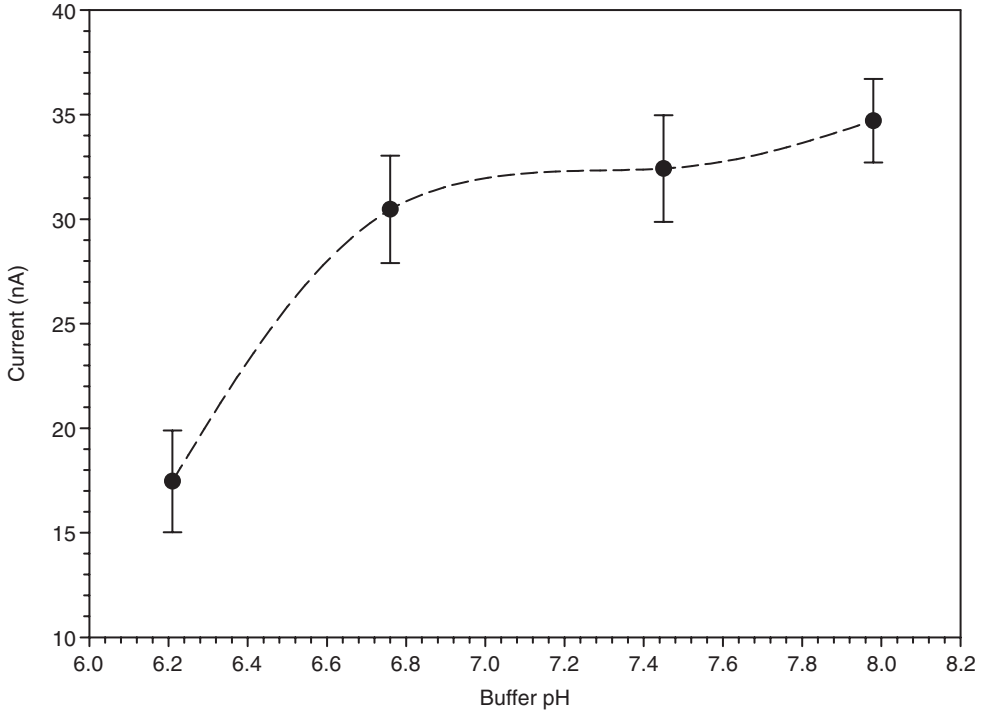

Figure 1. FIA response as a function of buffer pH in a biosensor based on crosslinked ChOx on SPE; flow rate: 0.4 mL min^{-1}, applied potential: +650 mV *vs.* Ag/AgCl pseudo RE, substrate concentration: 1mM choline chloride, sample loop: 115 μL.

immobilisation methods. pH = 6.80 was chosen as a good compromise between high sensitivity and stable working conditions for the enzymes: as a matter of fact, at pH = 8.00 begins to occur the spontaneous hydrolysis of the substrate of AChE.

3.2 *Comparison of the methods for the choline oxidase immobilisation*

Three immobilisation techniques were compared by their own performances under FIA conditions. They were fully described in a previous section: enzyme entrapment in DGS, immobilisation on SPE of enzyme in PAP, covalent immobilisation of enzyme crosslinked in BSA-GA matrix. Figure 2 shows the hydrodynamic voltammogram obtained on ChOx biosensors after sequential application of controlled potential from 0 to +800 mV *versus* Ag/AgCl pseudo reference electrode. Flow rate, substrate concentration (0.1 mM), time and volume of injection were kept as constants, deriving from the FIA optimization. The current and background responses were expressed as a function of the applied potential during each amperometric run, and signal to background ratio was calculated with respect to different immobilisation methods on SPE. In all the cases the highest sensitivity has been reached in the range of potential between +500 and +700 mV *versus* Ag/AgCl; +650 mV *versus* Ag/AgCl were applied in all the following biosensor based measurements. On the other hand, the comparative response suggests the use of covalent immobilisation of enzyme crosslinked in BSA-GA matrix.

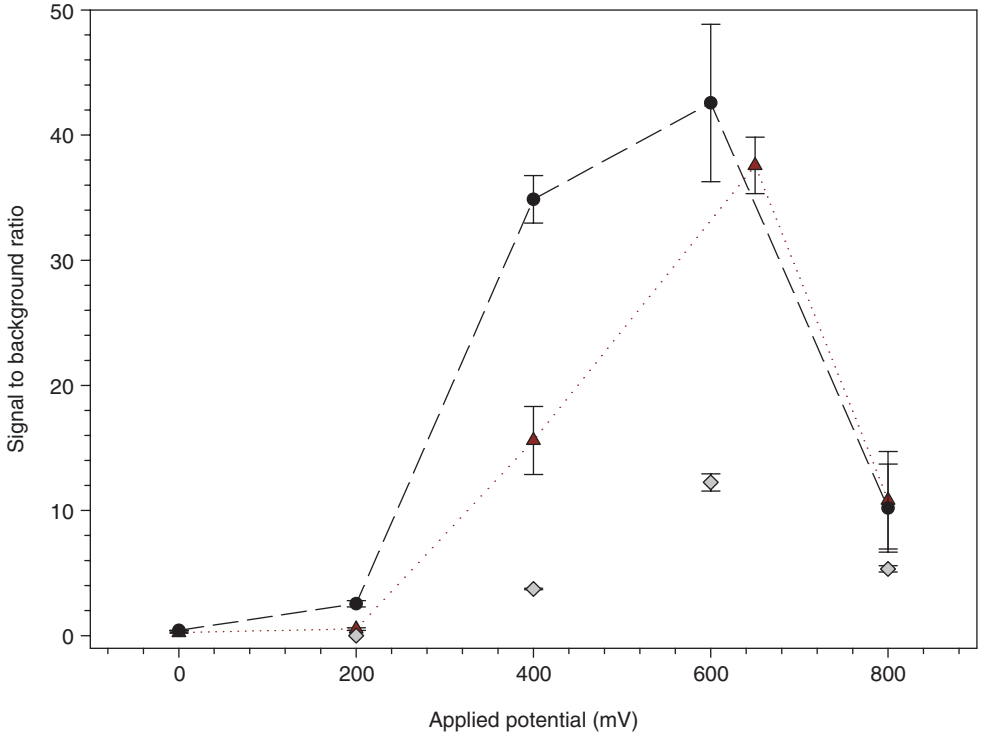

Figure 2. Signal to background ratio as a function of the applied potential for ChOx biosensors according to different immobilisation methods on SPE; flow rate: 0.4 mL min^{-1}, substrate concentration: 1mM ChCl in 0.1 M phosphate buffer at pH 6.8, sample loop: 115 µL. Black dots with dashed line: ChOx crosslinked in a BSA-GA matrix; dark triangles with dotted line: ChOx in polyazetidine blocked on SPE; grey diamonds: ChOx entrapped in diglycerylsilane.

Calibration curves of choline were also plotted to compare the main analytical performances of the proposed immobilisation techniques. In table 1 all results are summarised. Linear dynamic range between 3 µmol L^{-1} and 1 mmol L^{-1} was observed in the case of ChOx entrapped in DGS and ChOx immobilised with polyazetidine, but in the case of ChOx crosslinked with BSA GA matrix a very high correlation and sensitivity were achieved ($r^2 = 0.9999$).

Stability under flow conditions were evaluated by injection of fixed concentration of freshly prepared ChCl in PB 0.1M a pH 6.8 (100 µM). Lifetime of about 60 days was reached in the case of ChOx crosslinked in BSA GA matrix. Lifetime at a half of the first peak signal t_{L50} is less than one week in the remaining cases. Especially for these analytical features the preferred method for immobilising ChOx was the crosslinked BSA GA matrix.

3.3 *Evaluation of the cholinesterase inhibition*

The bi-enzymatic sensors for measuring the content of OP pesticides can be realized according to different ways and analytical formats, each of them taking

Table 1. Main analytical performances for ChOx biosensor under FIA conditions. Applied voltage: +650 mV *vs.* Ag/AgCl pseudo RE, flow rate: 0.4 mL min^{-1}, sample loop: 115 µL. *BSA + GA*: immobilisation on SPE of ChOx crosslinked in a matrix of bovine serum albumin and glutaraldehyde; *DGS*: immobilisation on SPE of ChOx entrapped in diglycerylsilane aqueous solution; *PAP:* ChOx immobilisation on SPE in polyazetidine.

Immobilisation technique	BSA + GA	DGS	PAP
Response time (s)[(a)]	40	30	20
Reproducibility (RSD%)[(b)] ($n = 10$)	1.5	5.5	4.6
Sensitivity (µA mM^{-1})	5.49×10^{-2}	9.34×10^{-3}	1.52×10^{-2}
Linearity range (µM)	30 ÷ 1000	10 ÷ 250	30 ÷ 1000
r^2	0.9999	0.9837	0.9954
Plot equation	$y = (0.48 \pm 0.03) + (5.49 \pm 0.03)10^{-2} \times$	$y = (1.22 \pm 0.05) + (9.3 \pm 0.3)10^{-3} \times$	$y = (1.00 \pm 0.04) + (1.52 \pm 0.04)10^{-2} \times$
t_{L50} (50% of initial response)	2 months	6 h	7 h

Notes: (a) assayed using as a substrate 100 µM ChCl, (b) RSD: relative standard deviation calculated at the substrate concentration 100 µM ChCl, n = number of replicates.

limitations in the sensitivity and accuracy of the method that were extensively reported in literature:

- using a biosensor with acetylcholinesterase and choline oxidase co-immobilised on the support [6–11];
- with immobilised ChOx on the electrode and AChE free in solution [12–17];
- using AChE immobilised on a separate membrane upon which inhibition takes place and a ChOx sensor for the amperometric detection into interference-free solutions.

The last approach after development of an optimized analysis protocol measuring the residual activity of AChE immobilised onto nylon membranes with a ChOx modified SPE has been demonstrated to be more efficient and sensitive [18], resulting in a fast affordable, interference-free methods for OP detection. Paraoxon was used as reference pesticide for sensor calibration. The above mentioned analytical procedure was adopted also in this work for measuring cholinesterase inhibitors into strawberry samples. Briefly, the AChE membranes prepared as reported in previous sections, were at first incubated in standard solutions at increasing concentration of Paraoxon for 90 min and secondly in substrate solution (AcChCl 2 mM) for 30 min. The incubation times were optimized and similar analytical protocol was carried out in a previous work [18]. The resulting solution wherein the second incubation took place were injected in a FIA system using a ChOx biosensors with ChOx crosslinked by BSA-GA matrix (as resulting from the optimization in section 3.2).

The inhibition curve was obtained plotting the residual activity percentage as reported in equation (3) as a function of the Paraoxon concentration within the range 5.2–103.3 ng mL^{-1}. Each standard was measured in triplicate and the intraelectrode coefficient of variation was calculated between 2.7 and 11.4%. The curve in figure 3 is described by the following four-parameters equation:

$$y = 33.4 + 1.8 \exp(-0.097x) + 65.0 \exp(-0.037x) \quad (4)$$

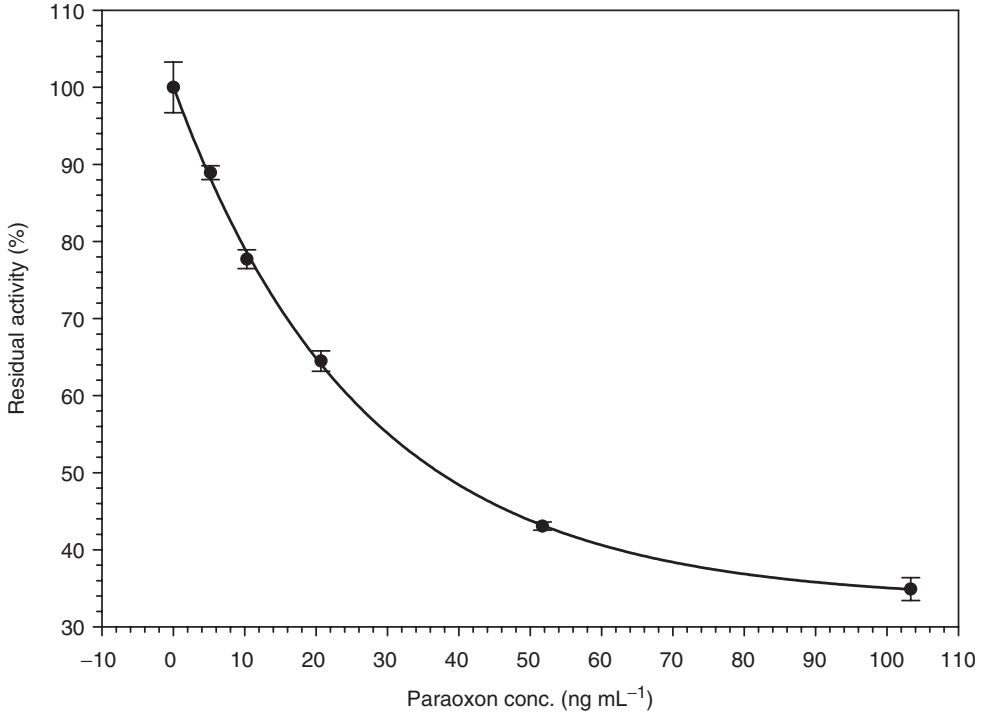

Figure 3. Inhibition curves as a function of the concentration of Paraoxon, using the analytical protocol with incubation for 1 h in Paraoxon standard solutions, 30 min in AcChCl substrate (2 mM) and following amperometric measurement by ChOx biosensor (applied voltage: +650 mV *vs.* Ag/AgCl). Each point was taken in triplicate and was built using the same inhibition process in a 24 wells apparatus.

and the inhibition at 50% of activity ($I_{50\%}$) of 38 ng mL^{-1} can be extrapolated. A limit of detection (LOD) of 4.5 ng mL^{-1} (ppb) was also calculated in the same calibration equation using the formula:

$$I\%_{(\mathrm{LOD})} = \frac{2.6\,\mathrm{SD}(I_0)I_{50\%}}{100 - 2.6\,\mathrm{SD}(I_0)}$$

where SD(I_0) is the standard deviation of the signal in absence of inhibition. Taking into account the sampling volume (3 mL) and the weighed amount of sample (0.5 gr), this value corresponds to 27 ppb (μg kg^{-1}) of Paraoxon in the original sample, which is largely below the maximum quantity for single pesticide (500 ppb) allowed by national and european regulation on reward [19], even observing that the chosen reference pesticide in our procedure (Paraoxon) shows one of the higher *anticholinesterase* activity among the different OP products.

Comparable value (4.7 ng mL^{-1}) was obtained taking the points of the same plot at lowest concentrations where linearity is observed and applying the Zund Meier [20] method for LOD extrapolation to the linear curve ($r^2 = 0.9791$). Figure 4 shows the application of such a method.

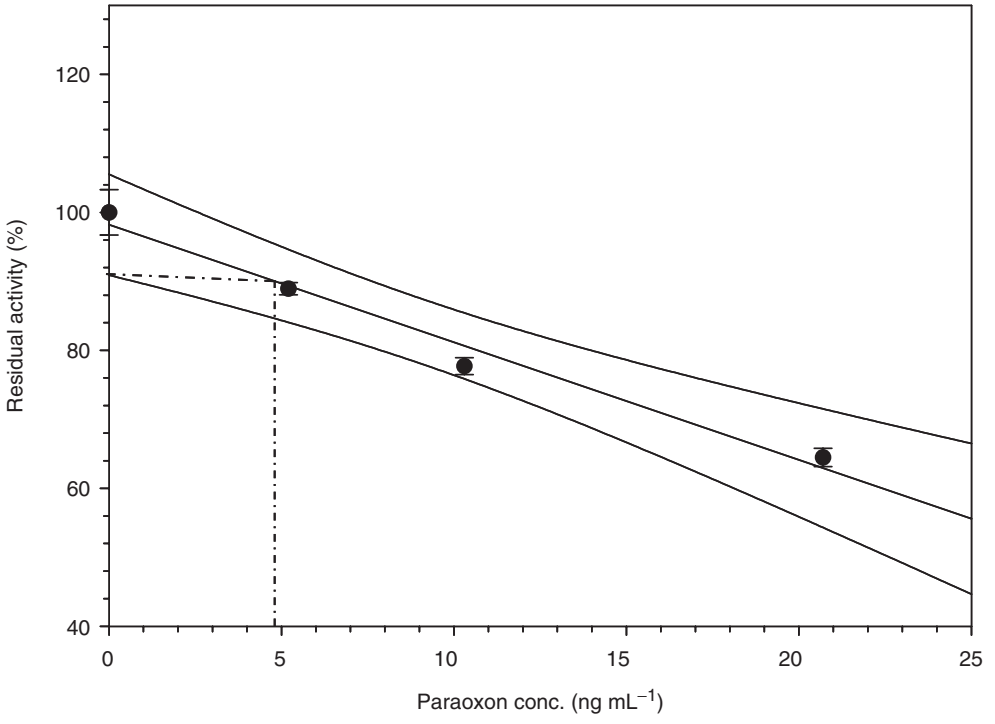

Figure 4. Calibration plot of Paraoxon, using the analytical protocol with incubation for 1 h in Paraoxon standard solutions, 30 min in AcChCl substrate (2 mM) and following amperometric measurement by ChOx biosensor (applied voltage: +650 mV *vs.* Ag/AgCl). Concentration range 0–21 ng mL^{-1}. Each point was taken in triplicate and was built using the same inhibition process in a 24 wells apparatus. LOD was obtained by application of Zund Meier method (4.7 ng mL^{-1}).

3.4 *Inhibition assay in strawberry samples*

The same experimental procedure and conditions were used to evaluate the inhibition of cholinesterase in fruits coming from the local market. We have previously performed measurements of cholinesterase inhibition on the surface of unpeeled grapes after contamination with a spiked amount of pesticide [18]. Strawberries were chosen because of the lack of the protecting lipophilic skin with respect to other fruits and the resulting capability to isotropically absorb each contaminant, in such a way allowing for to express the measurements in mg of analyte per kg of fruit.

The fruit samples were treated either by homogenization or centrifugation and the content of inhibitors of cholinesterase in strawberries were determined. For this purpose, both the sample homogenized and separated by centrifuge were used as stock solution for incubation without any dilution. The incubation was made into three replicates of unspiked sample solution. The signal obtained from the amperometric measurements under FIA conditions was recorded and residual activity was calculated by using the calibration curve in figure 3 (equation 4). Taking into account the above mentioned sampling volume (3 mL) and the amount of weighed sample, the content of cholinesterase inhibitors expressed in Paraoxon equivalent units, was respectively found of 366 and 374 μg kg^{-1} for homogenized and centrifuged fruit sample.

Table 2. Inhibitors detection in strawberries. The content of cholinesterase inhibitors was expressed as equivalent Paraoxon concentration.

Sample	Added Paraoxon conc. ($ng\,mL^{-1}$)	Found Paraoxon conc. ($ng\,mL^{-1}$)*	Recovery ($R\%$)
Homogenized strawberries	/	61.0 ± 0.6	/
	10.0	72.1 ± 0.2	110.7
	100.0	157 ± 2	95.9
Centrifuged strawberries	/	62.3 ± 0.3	/
	10.0	72.0 ± 0.5	96.7
	100.0	152 ± 3	89.5

*All measurements were performed in triplicates and data were given as value ±SD. Recovery was calculated as $R\% = (C_F - C_0)/C_{add} \times 100$, where C_F is the concentration measured on spiked sample, C_0 is the concentration measured of unspiked sample, C_{add} the known added concentration of inhibitor.

Recovery assay was performed by adding to treated strawberry samples known amounts of Paraoxon in the concentration range of the calibration curve, i.e. 10 e $100\,ng\,mL^{-1}$. The enzyme membranes were incubated into spiked solutions and three replicates were acquired for each added concentration. Table 2 summarizes the results: it worth to be noted that recovery values were between 90 and 111% and the recovery data both on homogenized and centrifuged samples were rather similar.

4. Conclusions

A bi-enzymatic biosensor was realized based on screen printed electrodes for the determination of cholinesterase inhibitors on food samples, i.e. strawberries from local market. High sensitivity, in comparison with other AChE based biosensors, was reached. The analysis protocol is mainly based on a preliminary inhibition process, during which enzymatic membranes were incubated into pesticide solutions and contaminated real samples, then on the amperometric choline oxidase sensors which measures the residual activity of acetyl cholinesterase. Two advantages arise from the separation between the acetyl cholinesterase inhibition (incubation) phase and the effective amperometric measurement:

- possibility to perform simultaneously calibration and incubation steps for a number of samples, with reduction of analysis times (130 min for all calibration and measurements);
- reusability of the amperometric biosensor for several determinations with reduction of the total analysis costs.

Because of the extremely broad class selectivity of cholinesterases, the proposed biosensor methodology allows for the measurement of an overall inhibition of enzyme activity, rather than an accurate determination and quantitation of single compounds. Hence, such a device should be considered as a tool to evaluate a global toxicity index, to be used complementarily with the conventional reliable and certified analytical techniques. On-going efforts in our laboratories are aimed to evaluate the accuracy of this analytical protocol, comparing results obtained by inhibition biosensor on spiked

and unspiked strawberry samples with certified samples and standardised analytical techniques for pesticides.

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