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Cholinesterase sensors based on screen-printed electrodes for detection of organophosphorus and carbamic pesticides

Abstract Cholinesterase sensors based on screen-printed electrodes modified with polyaniline, 7,7′,8,8′-tetracyano-quinodimethane (TCNQ), and Prussian blue have been developed and tested for detection of anticholinesterase pesticides in aqueous solution and in spiked grape juice. The influence of enzyme source and detection mode on biosensor performance was explored. It was shown that modification of the electrodes results in significant improvement of their analytical characteristics for pesticide determination. Thus, the slopes of the calibration curves obtained with modified electrodes were increased twofold and the detection limits of the pesticides were reduced by factors of 1.6 to 1.8 in comparison with the use of unmodified transducers. The biosensors developed make it possible to detect down to 2×10^{-8} mol L^{-1} chloropyrifos-methyl, 5×10^{-8} mol L^{-1} coumaphos, and 8×10^{-9} mol L^{-1} carbofuran in aqueous solution and grape juice. The optimal conditions for grape juice pretreatment were determined to diminish interference from the sample matrix.

Keywords Cholinesterase sensor · Biosensor · Pesticide detection · Grapes testing

Abbreviations *ChE* Cholinesterase ·
TCNQ 7,7′,8,8′-Tetracyanoquinodimethane ·
ChO Choline oxidase · *AChE* Acetylcholinesterase ·
BChE Butyrylcholinesterase · *BSA* Bovine serum
albumin · *2-PAM* 2-Pyridine aldoxime methiodide

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Introduction

The growing use of pesticides in agriculture and in daily life requires further progress in the development of novel methods for their rapid and sensitive determination, especially in the field. Most commercially available pesticides are considered hazardous for human health because they inhibit main metabolic pathways. This is particularly true for organophosphorus and carbamic pesticides which can inhibit acetylcholinesterase, the key enzyme in the transmission of nerve impulses (acute cholinergic syndrome) [1]. Exposure to low levels of some organophosphates also results in long-term (chronic) neurotoxic effects related to the inhibition of neuropathy target esterase (delayed polyneuropathy) [2]. Anticholinesterase pesticides cause frequent poisoning of agricultural workers mainly in developing countries [3].

Anticholinesterase pesticides are recommended for use 14 days before harvesting. Nevertheless, their residues can be detected in the crops a month after their application [4]. Moreover, some procedures for food processing, i.e. oil refinery, can result in additional accumulation of the pesticides. For these reasons, the intake of commercial pesticides in the environment and their residues in food are strongly regulated within limited threshold values at the ppm–ppb level [5].

Anticholinesterase pesticides are widely used in the production of table and wine grapes [6]. More than 20 organophosphates and carbamates are allowed for application in wine growing in EU countries with the average application rate of about 1 kg ha^{-1} and higher. Pesticides are applied for treatment of leafhoppers, mealy-bugs, spider mites, phylloxera and other insect pests all the year round including the dormant period but preferably from autumn to late spring. The maximum permissible concentrations of pesticides in wine grapes in harvesting are established at the level of 0.1–0.5 mg kg^{-1}. In 1997, more than 25% of all the grape samples from the vineyards of Michigan, USA, were contaminated with abundant pesticide residues [7]. Although more than 80% of pesticides are decomposed in maceration and wine maturing, the influence of

grape contaminants on the fermentation and wine quality is a matter of concern [8].

Present methods for the quantitative determination of anticholinesterase pesticides with chromatographic techniques involve complex and time-consuming treatment of the samples, i.e. extraction of pesticides, extract cleaning, solvent substitution or analyte modification [9].

Cholinesterase-based biosensors are considered as an alternative to the conventional chromatography techniques for fast and inexpensive detection of dangerous pesticide levels in the samples tested in the field (for reviews see Refs. [9, 10, 11]). The quantification of anticholinesterase pesticides is based on the measurement of the decay of enzyme activity after exposure to the enzyme, in the free form or immobilized on an appropriate support, to an inhibitor. Various amperometric [12, 13, 14, 15], potentiometric [16, 17, 18] and conductimetric biosensors [19] have been developed for this purpose and some of them applied to test food products.

For amperometric detection of cholinesterase activity, synthetic substrates, e.g. thiocholine ethers [5, 13, 14, 15] or *p*-aminophenyl acetate [12] are used. They form electrochemically active species oxidized on the electrode surface (Scheme 1).

However, these reactions are often complicated by the formation of by-products deposited on the electrode or by spontaneous hydrolysis of the substrate. The high working potential necessary for anodic oxidation of the thiocholine limits the use of biosensors especially for testing of samples containing electrochemically active species.

The reduction of the working potential is of critical importance when the biosensor is used for the determination of pesticide residues in soil and plants where phenolic compounds are present. In fact, they mask the oxidation of the products of the enzymatic reaction due to their own oxidation on the electrode. Thus, the amounts of phenols in grapes, especially of rosé and red varieties, are significantly high, up to $10\,mg\,kg^{-1}$, and interfere with detection of pesticides in musts and home produced wines.

For cholinesterase biosensors, a decrease of the working potential can be achieved by modification of the transducers with mediators, e.g. Co-phthalocyanine [5] and TCNQ [15]. A similar effect is obtained by the use of choline oxidase catalyzing the conversion of choline to betaine (Scheme 2) [20, 21, 22]. This allows the use of native substrate, acetylcholine, in inhibition measurement and hence provides better correlation of the results with ecotoxicity data. As reported below, the use of choline oxidase leads to the production of H_2O_2 (Scheme 2) which can be amperometrically detected in direct or mediated oxidation/reduction on the electrode.

Recently the use of the Prussian blue (ferric hexacyanoferrate), an "artificial peroxidase", was described as a selective mediator for H_2O_2 reduction [23]. Prussian blue-modified screen-printed electrodes were used in the assembly of various enzyme sensors for the detection of oxidase substrates [24, 25, 26, 27].

Optimization of the assembly of cholinesterase sensors is commonly directed toward improvement of substrate detection, i.e. to achieve maximum signal, prolonged shelf-life, stable and reliable response, and lower pH-sensitivity of the signal. In general, this does not assume imperative optimization of inhibitor determination.

From the kinetic point of view, the irreversible inhibition observed must be insensitive to the detection mode. The degree of irreversible inhibition measured with the biosensor depends on the ratio of the enzyme active sites able to bind with the substrate prior to and after inhibition. However, there is much evidence of dramatic changes in the detection limits and concentration ranges of the pesticides determined with biosensors of similar assembly (for reviews see Refs. [5, 9, 10]). The difference in the results is commonly referred to the variation of specific activity of cholinesterase immobilized or to the peculiarities of the sample treatment. The role of mass transfer of substrates/inhibitor as well as the influence of a transducer and enzyme immobilization should be also taken into account.

In this work, we have compared the features of various cholinesterase sensors based on screen-printed carbon electrodes differing in the detection mode and modifier to establish factors affecting the sensitivity of pesticide determination. The biosensors developed were tested for the determination of pesticide traces in grapes.

$$(CH_3)_3N^+CH_2CH_2SC(O)CH_3 \xrightarrow{\text{H}_2\text{O, ChE}} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH$$

Acetylthiocholine → Thiocholine

$$H_2N-\!\!\!\bigcirc\!\!\!-O-\overset{\text{O}}{\underset{\parallel}{C}}-CH_3 \xrightarrow{\text{H}_2\text{O, ChE}} H_2N-\!\!\!\bigcirc\!\!\!-OH + CH_3COOH$$

p-Aminophenyl acetate → p-Aminophenol

Scheme 1

$$(CH_3)_3N^+CH_2CH_2OC(O)CH_3 \xrightarrow{\text{H}_2\text{O, ChE}} (CH_3)_3N^+CH_2CH_2OH + CH_3COOH$$

Acetylcholine

$$(CH_3)_3N^+CH_2CH_2OH \xrightarrow{2\text{ O}_2\text{, H}_2\text{O, ChO}} (CH_3)_3N^+CH_2C\overset{\text{OH}}{\underset{\text{O}}{\diagdown}} + 2\,H_2O_2$$

Choline → Betaine
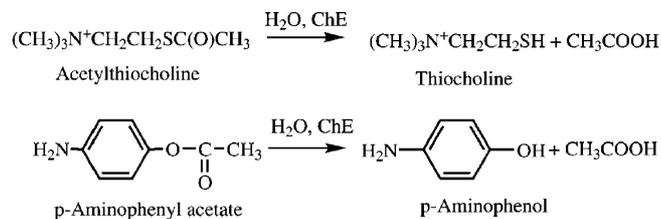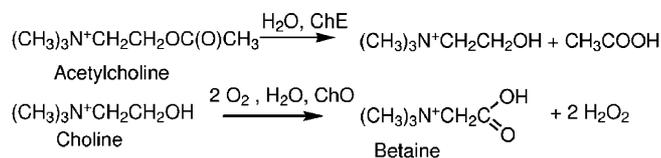
Scheme 2

Experimental

Reagents

Acetylcholinesterase from the electric eel (AChE, EC 3.1.1.7), specific activity $463\,U\,mg^{-1}$ protein, butyrylcholinesterase from horse serum (BChE, EC 3.1.1.8), specific activity 580 and $16\,U\,mg^{-1}$ protein, bovine serum albumin, *S*-butyrylthiocholine chloride, acetylcholine iodide and chloride, and glutaraldehyde were purchased from Sigma (St Louis, USA). Nafion (perfluorinated ion-exchange resin, 5% *w/v* solution in lower alcohols/water) was obtained from Aldrich (Steinheim, Germany). Coumaphos (*O,O*-diethyl-*O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl)phosphorothioate), chloropyrifos-methyl (*O,O*-dimethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate), and carbofuran (2,2-dimethyl-2,3-dihydro-7-benzofuranyl-*N*-methylcarbamate) were purchased from Riedel–de-Haen (Seelze, Germany) and Chem Service (West Chester, UK). All pesticide preparations contained at least 99% of active ingredient

and were used without additional purification. All the other reagents used were of analytical grade (Reakhim, Russia and Fluka, Neu-Ulm, Switzerland).

Cholinesterase sensor design

Cholinesterase biosensor based on epoxy-graphite electrode

Thick-film epoxy-carbon electrodes (IVA, Ekaterinburg, Russia [27]) were used for biosensor development. The working area of the electrodes was adjusted with an insulating layer to be about 0.06–0.08 cm². The biosensor response was measured against an external Ag/AgCl reference electrode with a Pt auxiliary electrode. AChE or BChE solution (10 μL) was pipetted on the working area of electrodes to the final enzyme activity of 8 U cm⁻². The solution was left to evaporate until a wet protein layer was formed. Then BSA solution (5 μL) was added to a final concentration of 12.5 μg cm⁻² for enzyme stabilization. The electrodes were then treated with glutaraldehyde vapors for 2 min under the vacuum produced by a water-jet pump. Biosensors were washed with deionised water and 2×10^{-3} mol L⁻¹ phosphate buffer, pH 7.8.

Cholinesterase biosensor based on screen-printed carbon electrode modified with TCNQ

The screen-printed carbon-paste electrodes were obtained from the Institute for Technical Biochemistry, University of Stuttgart, Germany [28]. TCNQ and graphite were implemented in a hydroxy-ethyl cellulose matrix and printed on the plastic support together with internal pseudoreference Ag/AgCl electrode, four pairs per plate. The immobilization of enzyme was performed as described above for epoxy-graphite transducer.

AChE-ChO biosensor based on Prussian blue-modified electrode

In this case screen-printed carbon electrodes were obtained from the Biosensors Laboratory of the University of Florence (Italy). Electrodes were printed with a 245 DEK (Weymouth, UK) screen printing machine using Elektrodag inks obtained from Acheson Italiana (Milan, Italy) and polyester plates (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The electrodes were produced in foils of 20 strips, each containing three printed electrodes, a carbon working electrode, and two silver electrodes, acting as pseudo-reference and counter, respectively. The diameter of the working surface of the electrode was 0.3 cm resulting in an apparent geometric area of 0.07 cm². Screen-printed electrodes were electrochemically pre-activated for 3 min at 1.7 V vs. Ag/AgCl in 0.05 mol L⁻¹ phosphate buffer containing 0.1 mol L⁻¹ KCl.

For electrode modification, the chemical deposition of Prussian blue optimized in previous work [29] was adopted. A mixture of 0.05 mol L⁻¹ FeCl₃ and 0.05 mol L⁻¹ K₄Fe(CN)₆ in 0.1 mol L⁻¹ hydrochloric acid (40 μL) was placed on the working electrode area. After 10 min electrodes were washed with 0.1 mol L⁻¹ HCl and incubated at 100 °C for one hour to stabilize the Prussian blue layer.

ChO and AChE were immobilized on the electrode surface as follows. BSA (10 mg) and ChO (2.5 mg) were dissolved in 200 μL of 5×10^{-2} mol L⁻¹ phosphate buffer containing 0.1 mol L⁻¹ KCl, pH 7.0. After that, 40 μL of the solution was mixed with 10 μL AChE solution to obtain the enzyme ratio required. The resulting solution was mixed with 10 μL of 2.5% glutaraldehyde and 15 μL Nafion (5% *v/v* in ethanol) and spread on the working area (7 μL per electrode). The resulting amounts corresponded to 0.11 U AChE and 3.4 U ChO per electrode and about 1.5 mg cm⁻² BSA. Biosensors were left to dry for 45 min at room temperature and then put for 45 min into 0.1 mol L⁻¹ glycine solution to saturate free aldehyde groups.

Cholinesterase biosensors based on a polyaniline-modified electrode were prepared using screen-printed electrodes produced in the Biosensors Laboratory of the University of Florence (Italy)

and by IVA (Ekaterinburg, Russia). No significant changes in the characteristics of substrate and inhibitor determination were found for these two transducers. Polyaniline was synthesized by low-temperature oxidation of aniline as described elsewhere [30], washed with aqueous NH₃, dried and ground with camphorsulfonic acid and phenol in 2:1:1 molar ratio (calculated per one *p*-phenylene imine unit). The mixture was dissolved in chloroform. For electrode modification, 5 μL of 0.15% polyaniline solution was spread on the electrode surface and then the electrode was dried at room temperature. After that, BSA and AChE were dissolved in a 5×10^{-2} mol L⁻¹ phosphate buffer solution containing 0.1 mol L⁻¹ KCl, pH 7.4, to a final concentration of 0.65 mg mL⁻¹ protein. Then 3 μL of the solution was placed on the working area of the electrode and treated with glutaraldehyde vapors as described above for the epoxy-carbon transducer.

Procedures

Biosensor response

The cholinesterase activity was measured in DC mode in a three-electrode cell with Ag/AgCl reference electrode either internal or external. Pt wire or screen-printed Ag layer were used as auxiliary electrodes (see description of screen-printed electrodes used). Ecotest-VA (Econix-Expert, Moscow, Russia) and Autolab PGSTAT 10 (Ecochemie, Utrecht, Holland) were used in amperometric measurements. The electrodes were immersed in the 2×10^{-3} mol L⁻¹ phosphate buffer, pH 7.8, containing 0.1 mol L⁻¹ NaCl. Once a stable background current was reached, an appropriate volume of acetylthiocholine solution was injected and the oxidation current was recorded at +560 mV (epoxy-graphite electrode) or +250 mV (TCNQ-modified electrode). In the case of Prussian blue modified electrodes the sensors were immersed in phosphate buffer 5×10^{-2} mol L⁻¹+KCl 0.1 mol L⁻¹, pH 7.8, at an applied potential of –50 mV vs. the internal reference. Once a stable baseline current was reached acetylcholine was added and the response referred to H₂O₂ reduction was measured.

The response of AChE sensors based on polyaniline modified transducers was measured in potentiometric mode with acetylcholine as enzyme substrate. The maximum shift of the potential measured against Ag/AgCl was recorded as a biosensor response with Ecotest-001 digital ionometer (Econix-Expert, Moscow, Russia). The activity of the free enzyme was photometrically determined by the Ellman method [31] in 2×10^{-3} mol L⁻¹ phosphate buffer, pH 7.8, at 37 °C.

Inhibition measurements

Coumaphos and chloropyrifos-methyl in aqueous solutions and spiked grape juice were electrochemically oxidized to their oxygen analogues in accordance with the procedure reported on literature [32]. The electrolysis was performed in the presence of 0.1 mol L⁻¹ NaCl on Pt electrodes (on-load voltage 6 V). Excess chlorine was removed by addition of 100 μL of 2% (v/v) formic acid followed by the pH correction with 10% NaOH. Carbofuran was used in inhibition measurements with no special pretreatment.

The cholinesterase biosensor was incubated in the pesticide solution for 10 min and washed with the working buffer solution. After that, the response toward the substrate was measured as described above and the degree of inhibition was calculated as a relative decay of the biosensor response (Eq. 1):

$$I\,(\%) = \frac{I_0 - I_t}{I_t} \times 100 \qquad (1)$$

where I_o and I_t are the biosensor response (current or potential) before and after the incubation procedure, respectively. After the measurement, the inhibited cholinesterase was reactivated by treatment with 0.1% 2-PAM for 10 min. Each biosensor allowed at least 10 inhibition measurements if the degree of inhibition did not exceed 40%.

**Table 1** Operational characteristics of cholinesterase sensors

Transducer	Modifier	Enzyme	Detection limit (mmol L^{-1})	Concentration range (mmol L^{-1})	Sensitivity	Maximum signal	Response time (min)	Storage time (days)
Epoxy carbon composite	No	AChE	0.05	0.2–5.0	0.50±0.01 μA (mmol L^{-1})	0.80 μA	3	25
		BChE	0.03	0.2–6.0	0.60±0.02 μA (mmol L^{-1})	1.20 μA	2	20
Screen-printed graphite	TCNQ	AChE	0.005	0.01–0.032	8.0±0.1 μA (μmol L^{-1})	40 μA	2.5	15
	Prussian blue	AChE-ChO	0.004	0.005–0.1	7.0±0.5 μA (mmol L^{-1})	5.2 μA	0.3	21
	Polyaniline	AChE	0.05	0.1–1.5	82±6 mV per decade	100 mV	1.5	30
		BChE	0.1	0.2–1.0	99±13 mV per decade	160 mV	3	30

Results and discussion

Response characteristics

All the biosensors developed showed a fast and reliable response to specific substrates, i.e. acetylthiocholine for amperometric sensors and acetylcholine for biosensors based on polyaniline and Prussian blue-modified transducers. The characteristics of substrate determination are summarized in Table 1. The detection limit of the substrate refers to its concentration resulting in a shift of the signal equal to three times the standard deviation of the background signal (0.03 μA for amperometric sensors and 6 mV for potentiometric sensors). The concentration range corresponds to the linear part of the calibration curve in plots of response (mV or μA) against log[substrate, mol L^{-1}]. The sensitivity of response measurement was calculated from the slope of the linear part of the calibration curve. The response time corresponds to a 95% change in the shift of the current or potential observed after substrate addition. Storage time is the period in which the response is decreased by 20% of its initial value. The biosensors were stored under dry conditions at room temperature.

The dependence of the response on the amounts of the enzyme taken for the immobilization is shown in Fig. 1 for sensors modified with polyaniline as an example. The saturation observed at a high enzyme loading corresponds to the decrease of the rate of diffusion of the substrate in a protein layer. The achievement of high stability and reproducibility of the response is important for a reliable inhibition measurement, so the enzyme loading corresponding to the plateau on the above curves was used for biosensor development. In this case, minor variations in the initial enzyme activity did not result in significant changes in the signal of biosensors prepared in different periods of time.

All the biosensors investigated did not dramatically differ from each other in the conditions of substrate diffusion, which are determined both by enzyme loading and duration of cross-binding with glutaraldehyde. Maximum signals corresponding to saturation of the enzyme layer and the detection limits of pesticides depended on the detection system. The use of mediators provided higher signal but did not significantly alter the detection limits of acetylcholine and acetylthiocholine. Unfortunately, modification of thick-film epoxy-carbon electrodes did not afford reliable and stable response and extended life-time. Their manufacture involved mechanical polishing of the surface followed by an increase in porosity. This required higher loading of modifiers in comparison with screen-printed electrodes. As a result, the protein film obtained in immobilization flaked away together with the polymer

Fig. 1 The effect of enzyme loading on the response of an AChE sensor based on graphite screen-printed electrode modified with polyaniline. Acetylcholine chloride 1.5×10^{-3} mol L^{-1}
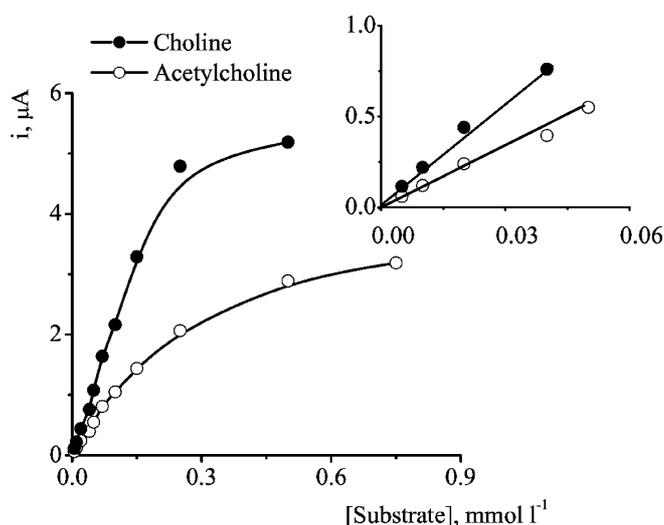
Fig. 2 Determination of acetylcholine and choline with and AChE-ChO biosensor based on screen-printed graphite electrode modified with Prussian blue

layer. In contrast with that the stability of the response of biosensors based on unmodified electrodes was higher for epoxy-carbon composites than for screen-printed electrodes. For this reason the effect of modifiers has been established for modified screen-printed electrodes whereas the performance of unmodified biosensors was investigated with epoxy-carbon electrodes. We did not observe any significant difference in the features of screen-printed electrodes produced in Germany and Italy in respect of their stability, response time, and reproducibility.

It should be mentioned that the specific AChE activity of AChE-ChO biosensors was lower than that of other biosensors developed, because of the need to couple formation and subsequent oxidation of choline. Therefore the signal of this sensor was found to be lower than that of the TCNQ-modified biosensor. Comparison of the calibration curves of choline and acetylcholine obtained with AChE-ChO biosensor showed the high efficiency of the consecutive conversion of acetylcholine (Fig. 2). Less than 20% of choline formed from acetylcholine is lost in the second process within the linear region of the calibration curves. This assumes that the rate of ChO reaction does not limit H_2O_2 production and the concentration of these products can be used as a measure of AChE activity.

The amperometric biosensors usually show lower detection limits for the substrate due to the higher accuracy of signal measurement in comparison with potentiometric devices. However, this is not as obvious for biosensors based on polyaniline-modified electrode. The transducer showed a high pH-sensitivity of the signal (about 80 mV per pH unit). This compensated for the lower accuracy of substrate determination caused by the semi-logarithmic form of calibration plot. The amounts of polyaniline and the optimal condition of modification and measurement were determined earlier [33]. In comparison with preliminary results obtained with glassy carbon electrode, the use of screen-printed graphite and epoxy-graphite electrodes slightly diminished the slope of the calibration curve of acetylcholine and the maximum response referred to the saturation conditions. Probably, this is due to the higher porosity of the electrode material followed by the complicated distribution of modifier and enzyme on the electrode surface. Meanwhile, the response reproducibility and the biosensor shelf-life were found to be higher for screen-printed electrodes due to the better adhesion of the components on the electrode material.

The nature of the enzyme (AChE or BChE) and of the substrate used (acetylcholine and acetylthiocholine) affected the response in accordance with their relative activity in the enzymatic reaction. Thus substitution of acetylcholine with acetylthiocholine decreased the rate of hydrolysis in the presence of native AChE by a factor of 1.7. A similar ratio was reported for the hydrolysis of acetylcholine with AChE and BChE [34]. The immobilization did not change the pH optimum of enzymatic reactions, for the AChE-ChO biosensor pH 7.0 was chosen for response measurement as a compromise between the optimum pH of both enzymes.

For inhibitor measurements, saturating concentrations of the substrates are preferable. They provide maximum sensitivity for inhibitor determination. As mentioned above, pesticide residues are quantified from the decrease in the response measured before and after inhibition. If the enzyme layer is not saturated with the substrate, the decrease in enzyme activity caused by irreversible inhibition is partially compensated for by the involvement of the active sites that remained free during the first measurement. Thus the changes in the biosensor signal will be lower than the real decrease of the enzyme activity.

Pesticide determination

Thionic pesticides were first oxidized to their phosphoryl analogs by chlorine generated in the electrolysis (Scheme 3). The efficiency of oxidation and the inhibitory effect of the products depended both on the pesticide and sample tested. For free enzymes and aqueous pesticide solutions the maximum inhibition is obtained in approx. 10 min electrolysis of chloropyrifos-methyl and coumaphos, with an electrolysis voltage of about 3 V. Increasing the voltage to 6 V reduced the time of electrolysis to 3–5 min. However, excessive oxidation of the pesticides diminished the inhibition observed (Fig. 3). This can lead to an underestimation of pesticide residues in real samples. The effect of oxidation time on immobilized enzymes is higher than on free cholinesterases. Oligomeric products, e.g. polyphosphoric acids, can probably be formed and prevent substrate access by adsorptive blocking of the electrode surface.

The white and rosé grape juices did not affect the response of the biosensors toward the substrates used. Elec-
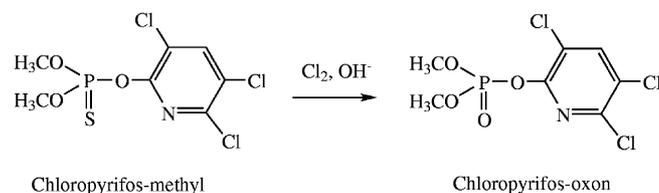
Scheme 3
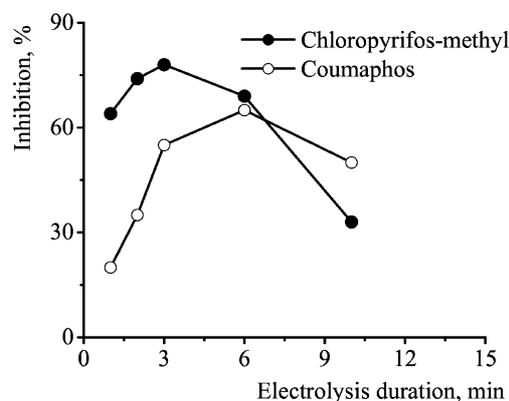
Fig. 3 Dependence of the inhibitory effect of chloropyrifos-methyl (6×10^{-7} mol L^{-1}) and coumaphos (8×10^{-7} mol L^{-1}) on the duration of their preliminary electrochemical oxidation. Outer voltage 6 V; AChE-ChO biosensor based on screen-printed electrode modified with Prussian blue
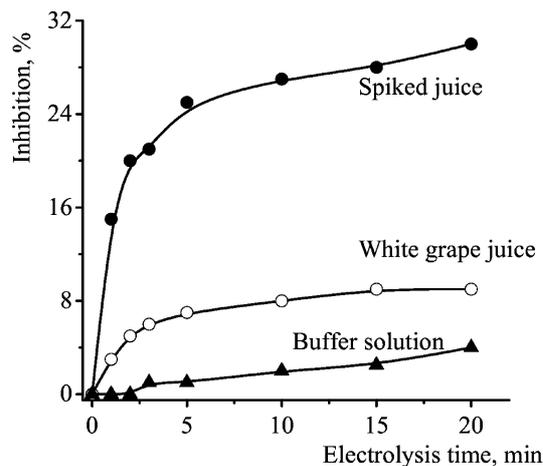
Fig. 4 The effect of oxidation of white grape juice and juice spiked with 1×10^{-8} mol L^{-1} chloropyrifos-methyl on its inhibitory effect on free AChE (1 U mL^{-1}). Incubation 10 min

trochemical oxidation resulted in minor changes in the response. The oxidative conversion of thionic pesticides in spiked grape juice is less effective than in aqueous solutions. Thus, with free AChE and chloropyrifos-methyl it was shown that the oxidation of thionic pesticide started from the fifth minute of electrolysis (Fig. 4). Part of the chlorine generated is probably consumed by oxidation of phenolic compounds present in the sample.

All the pesticides investigated resulted in strongly irreversible inhibition of AChE and BChE. The degree of inhibition increased with incubation time up to 15 min and leveled-off at 60–80% inhibition. For carbofuran the enzyme activity was partially reactivated spontaneously af-

ter 20 min incubation, if the initial inhibition did not exceed 40%. The analytical parameters of pesticide determination are summarized in Table 2. All the calibration curves are reported as plots of % inhibition against log[Inhibitor, mol L^{-1}]. The minimum inhibition level reliably detected, and hence the detection limit of a pesticide, corresponded to the decay of the biosensor response by 6%. The sensitivity was calculated as the slope of the linear parts of calibration curves.

The detection limits and sensitivities of pesticide determination depended on enzyme nature and on biosensor assembly much more than do the characteristics of substrate determination (Tables 1 and 2). Modification of transducer resulted in a remarkable advantage in the sensitivity of pesticide determination. Appropriate values of the slopes were 1.5–1.8 times higher than those obtained with unmodified biosensors. It should be mentioned that the sensitivity of inhibition refers to the relative changes in the biosensor response and cannot be directly related to the characteristics of absolute response. The precision of response measurement and the high sensitivity of substrate determination obtained with modified biosensors seems more important for the pesticide detection limits and do not completely explain the changes in the inhibition observed. This calls for consideration of additional factors altering the efficiency of the inhibitor detection, so changes in the distribution of hydrophobic pesticides between the bulk solution and enzyme layer can be considered for these reasons. Electrode modification can change both the hydrophobic/hydrophilic balance on the electrode sensor and the specific features of the protein layers obtained on these surfaces. This stimulates the accumulation of organophosphates in the proximity of the immobilized cholinesterase

Table 2 Analytical characteristics of pesticide determination

Transducer	Modifier	Enzyme	Detection limit (mol L^{-1})	Concentration range (mol L^{-1})	Sensitivity (% per decade)
Chloropyrifos-methyl					
Epoxy carbon composite	No	AChE	8×10^{-8}	3×10^{-7}–9×10^{-7}	42 ± 3
		BChE	7×10^{-8}	7×10^{-8}–6×10^{-6}	33 ± 3
Screen-printed graphite	TCNQ	AChE	7×10^{-8}	8×10^{-8}–4×10^{-7}	64 ± 5
		BChE	2×10^{-8}	3×10^{-8}–3×10^{-7}	69 ± 3
	Prussian blue	AChE-ChO	3×10^{-8}	4×10^{-8}–5×10^{-7}	69 ± 3
	Polyaniline	AChE	9×10^{-8}	1×10^{-7}–1×10^{-6}	71 ± 3
Coumaphos					
Epoxy carbon composite	No	AChE	2×10^{-7}	1×10^{-7}–4×10^{-6}	45 ± 4
		BChE	1.5×10^{-7}	2×10^{-7}–5.5×10^{-6}	37 ± 3
Screen-printed graphite	TCNQ	AChE	1×10^{-7}	3×10^{-7}–5×10^{-6}	55 ± 4
		BChE	5×10^{-8}	1×10^{-7}–4×10^{-6}	34 ± 3
	Polyaniline	AChE	9×10^{-8}	1×10^{-7}–9×10^{-7}	59 ± 4
Carbofuran					
Epoxy carbon composite	No	AChE	5×10^{-8}	6×10^{-8}–8×10^{-7}	34 ± 5
		BChE	4×10^{-8}	7×10^{-8}–9×10^{-7}	34 ± 3
Screen-printed graphite	TCNQ	AChE	2×10^{-8}	4×10^{-8}–1×10^{-7}	64 ± 5
		BChE	1×10^{-8}	3×10^{-8}–1×10^{-7}	44 ± 3
	Prussian blue	AChE-ChO	8×10^{-9}	1×10^{-8}–1×10^{-7}	54 ± 5
	Polyaniline	AChE	9×10^{-9}	1×10^{-8}–1×10^{-7}	54 ± 3

and hence the apparent degree of inhibition. The influence of surface modification is less pronounced for more polar carbofuran although the main trends in the analytical performance of the biosensors are also the same for this carbamate pesticide.

Similar phenomena have previously been observed for potentiometric BChE sensors treated with non-ionic surfactants [35]. Addition of polyethylene glycols resulted in a decrease of the detection limits of diazinon followed by the suppression of the effect of ionic species (fluorides and copper(II) ions). A similar effect can be related to the polyaniline that increases the sensitivity (slope of calibration curve) of pesticide determination in comparison with the use of an unmodified electrode. The charge of the surface layer caused by deposition of polyionic substances (positively charged polyaniline and negatively charged Nafion added to the Prussian blue-modified sensor) did not affect the sensitivity of pesticide determination.

The modification of cholinesterase sensors leads to substantial improvement of the analytical characteristics of pesticide detection in comparison with literature data [36, 37, 38]. Thus detection limits of 4×10^{-8} mol L^{-1} for coumaphos and 1×10^{-6} mol L^{-1} for chloropyrifos-methyl were obtained with unmodified BChE sensors based on screen-printed electrodes [36] and of 1×10^{-8} mol L^{-1} for carbofuran with an AChE biosensor based on a graphite-epoxy composite [37]. Only pre-accumulation of the pesticide in the flow-through enzyme reactor provided higher sensitivity of carbofuran detection (2×10^{-10} mol L^{-1} with AChE-ChO detection system [38]).

Spiked juice testing

For determination of pesticide residues in wine grapes, the white and red grape varieties available in local markets were used. The freshly pressed juice was tested with cholinesterase before and after addition of known amounts of the pesticides. Chloropyrifos-methyl is widely used in grape cultivation so we tested this pesticide only. Carbofuran was used as a representative of carbamate pesticides which do not need any special oxidative treatment. The biosensors were incubated in the juice and the response was then measured under the standard conditions described above.

It was shown that white grapes exert very small reversible inhibition of free cholinesterases. The decay of the response did not exceed 5% in 20 min exposure and the initial signal was re-established by 10 min washing in the working buffer solution. However, red grapes resulted in an irreproducible increase in the background current in the potential range of about 200–500 mV. This prevents measurement of the response of unmodified cholinesterase sensors directly in spiked grape juice.

Thus, for red grape testing only two biosensors based on polyaniline and Prussian blue-modified electrodes were used. A remarkable decrease of signal reproducibility was observed for TCNQ-modified electrodes, probably as a result of the oxidation of phenolic compounds present in red varieties in amounts about 10 times higher than in white grapes.
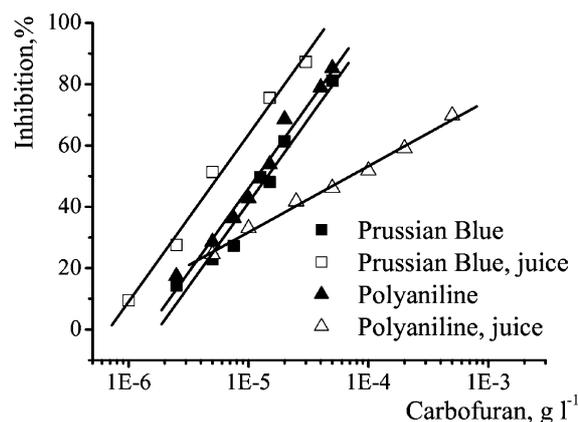
Fig. 5 The effect of carbofuran in aqueous solutions and spiked juice of red grape on AChE sensors modified with polyaniline and Prussian blue. Incubation 10 min
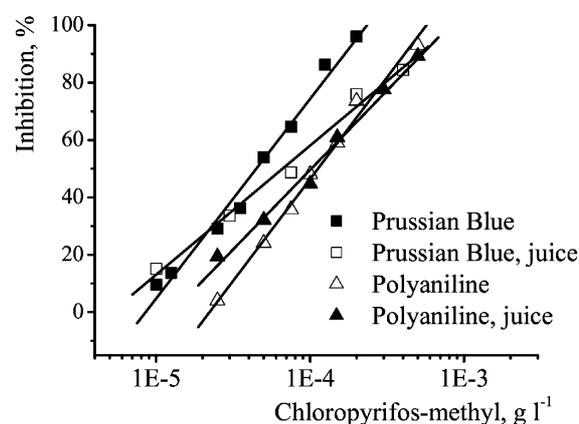
Fig. 6 The effect of chloropyrifos-methyl in aqueous solutions and spiked juice of red grape on AChE sensors modified with polyaniline and Prussian blue. Incubation 10 min

For detection of chloropyrifos, KCl was introduced directly into the pressed juice and electrolysis was performed for 3–5 min to obtain the oxon form in accordance with Scheme 3. Before spiking, oxidation of the juice increases the value of the background signal by about 5% with no effect on immobilized AChE.

Results from the determination of anticholinesterase pesticides in spiked juice, in comparison with aqueous solutions, are presented in Figs. 5 and 6. The slopes of appropriate calibration curves are very similar to each other for both the polyaniline and Prussian blue-modified sensors. The minimum detectable levels correspond to 1×10^{-6} g L^{-1} carbofuran and 1×10^{-5} g L^{-1} chloropyrifos-methyl. Taking into account that no dilution is assumed in sample treatment this is sufficient for reliable detection of pesticide residues at the permissible levels. This is particularly the case when calibration curves obtained with the same modifier are compared in pairs for aqueous solutions of pesticide and for spiked juice. The effect of phenolic compounds and other interferences from the matrix does not lead to underestimation of the pesticide content.

For polyaniline-modified biosensors a slight decrease in the sensitivity of carbofuran detection in red grapes was observed. This might be because of the effect of surface charge caused by the modifiers used or by changes in the buffer capacity of the sample tested. The polyaniline biosensor must be more sensitive to changes on the charged surface due to the potentiometric mode of measurement. Because carbofuran is more polar and hydrophilic than the other pesticides, inhibition on such surfaces was, as discussed above, less dependent on surface modification. Nevertheless, the inhibition observed with these biosensors is similar to each other at low carbofuran levels, which is more important in contamination testing.

Conclusions

Modification of the sensor surface is a powerful tool for improvement of biosensor performance. The influence of polymeric layers and of mediators deposited on the electrode surface or introduced into the electrode materials is commonly discussed in terms of reducing the working potential and suppression of the interference caused by electrochemically active substances. The effect of modification on the sensitivity of inhibitor determination has not yet been reported. It is usually assumed that the effect of irreversible inhibition should not depend on the detection system, because both the responses, i.e. prior to and after incubation, are measured with the same transducer and with the same substrate concentration specified – rather high and constant. In this work we showed that the potential of surface modification is far from being exhausted. The introduction of the mediators considered in this work increases both the response of the biosensors toward the substrates and the sensitivity of pesticide determination. In comparison with unmodified transducers the slopes of the calibration curves obtained with modified electrodes increased twofold and the detection limit was lowered by a factor of 1.6 to 1.8. The effect of modifiers is much more pronounced in the detection of pesticide traces in grapes. The use of polyaniline and Prussian blue for electrode modification makes it possible to detect hazardous contamination of grapes with minimum sample treatment and without any additional dilution of the pressed juice.

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