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Multienzyme electrochemical array sensor for determination of phenols and pesticides

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Abstract

The screen-printed four-electrode system was used as the amperometric transducer for determination of phenols and pesticides using immobilised tyrosinase, peroxidase, acetylcholinesterase and butyrylcholinesterase. Acetylthiocholine chloride was chosen as substrate for cholinesterases to measure inhibition by pesticides, hydrogen peroxide served as co-substrate for peroxidase to measure phenols. The compatibility of hydrolases and oxidoreductases working in the same array was studied. The detection of *p*-cresol, catechol and phenol as well as of pesticides including carbaryl, heptenophos and fenitrothion was carried out in flow-through and steady state arrangements. In addition, the effects of heavy metals (Cu^{2+}, Cd^{2+}, Fe^{3+}), fluoride (NaF), benzene and dimethylsulphoxide on cholinesterase activities were evaluated. It was demonstrated that electrodes modified with hydrolases and oxidoreductases can function in the same array.

The achieved R.S.D. values obtained for the flow system were below 4% for the same sensor and less than 10% within a group of five sensors. For the steady state system, R.S.D.s were approximately twice higher. One assay was completed in less than 6 min. The limit of detection for catechol using tyrosinase was equal to 0.35 and 1.7 μM in the flow and steady state systems, respectively. On the contrary, lower limits of detection for pesticides were achieved in the steady state system—carbaryl 26 nM, heptenophos 14 nM and fenitrothion 0.58 μM.

Keywords: Amperometric screen-printed biosensor; Flow-injection analysis; Steady state system; Tyrosinase; Peroxidase; Cholinesterase; Phenols; Pesticides; Wastewater

1. Introduction

Phenols are involved in many industrial processes and consequently, these substances appear widespread in industrial waste [1]. Many of them, especially chlorophenols, exhibit toxic effects on animals and plants [2,3]. Organophosphates and carbamates represent two groups of the most commonly applied pesticides. They are preferred in agriculture because of their relatively low persistence in the environment, but some of them exhibit fairly high acute toxicity. For these reasons, the determination of phenols, organophosphates and carbamates posses a high environmental importance.

Current analytical techniques for these toxic compounds, such as gas and liquid chromatography [4–10], are very sensitive and reliable; on the other hand, the instrumental methods are time-consuming, expensive and not suitable for field use. Bioanalytical assays including biochemical sensors represent a promising alternative to the classical methods due to their relatively low-cost of operation, the potential of miniaturisa-

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tion and rapid and simple detection procedures suitable for fast screening purposes.

Numerous biosensors for detection of phenols based on tyrosinase (EC 1.14.18.1) [1,11–22] and peroxidase (EC 1.11.1.7) [23–31] have been developed. The advantage of peroxidase-based biosensors is an improved sensitivity for several phenolic compounds, which are poor substrates for tyrosinase. The determination of organophosphates and carbamates is usually based on inhibition of cholinesterases. Various electrode configurations based on different transduction systems have been reported for these enzyme sensors. Among these, amperometric detection methods have been one of the most commonly employed [17–27,32–39]. Amperometric biosensors using cholinesterases (acetylcholinesterase, EC 3.1.1.7, and butyrylcholinesterase, EC 3.1.1.8) employ thiocholine esters as enzyme substrates, which are enzymatically hydrolysed producing thiocholine. The last is directly electrochemically oxidised [40]. The principle of such a biosensor is presented in Fig. 1, A. The mechanism involved in peroxidase or tyrosinase-based biosensors (Fig. 1, B) for detection of phenols consists of their enzymatic oxidation to quinones (or free radicals), which are eletroactive and can be reduced back at the electrode surface [18,26]. In the first case, peroxidase is oxidised by hydrogen peroxide followed by its reduction to native form by phenolic compounds. In the second case, the conversion of phenols by tyrosinase proceeds in two consecutive steps with molecular oxygen as the oxidising compound. Monophenol is hydroxylated to produce *o*-diphenol, which is further oxidised within the second step to *o*-quinone. Finally, tyrosinase is oxidised by oxygen to its native form with production of water.

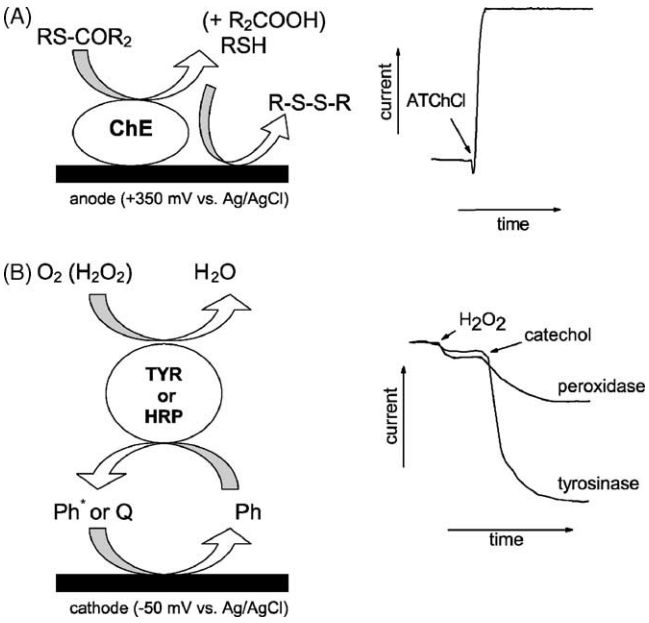

Fig. 1. The mechanism of enzyme-based biosensors. Current time dependence usually obtained in the case of steady state measurements: (A) with cholinesterase; and (B) with tyrosinase- or peroxidase-modified electrodes. Arrows indicate addition of appropriate substrate into electrochemical cell. A–thiocholine ester (~RS-COR_2) is hydrolysed by cholinesterase to produce acid (R_2COOH) and thiol (RSH), which is directly oxidised on electrode surface. The resulting current is proportional to activities of ChEs. B–phenolic compounds (Ph) are converted to quinones (Q) or free radicals (Ph*) by tyrosinase or peroxidase in the presence of molecular oxygene or hydrogene peroxide, respectively. Quinones are then reduced back on electrode resulting into current proportional to concentration of phenols.

The multienzyme electrochemical sensors including immobilised cholinesterase, tyrosinase or peroxidase already exist; the amperometric bi-enzyme sensors with co-immobilised i.e., cholinesterase together with choline oxidase [41,42], peroxidase with glucose oxidase (using screen-printed electrodes) [43], and potentiometric biosensors based on the semiconductor structures using cholinesterase, urease and glucose oxidase [44,45]. All these biosensor arrays were directed at pesticide determination (i.e., carbamates, organophosphates, triazines). For determination of phenols, especially amperometric peroxidase–tyrosinase systems were developed [46,47]. This paper describes the biosensor array based on four electrodes with immobilised peroxidase, tyrosinase, acetylcholinesterase and butyrylcholinesterase as members representing different enzyme groups (oxidoreductases and hydrolases). The performance of the developed enzyme array system was tested using both steady state and flow-through measurements and finally verified by monitoring some real wastewater samples from industrial sources in the steady state system.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA), glutaraldehyde, catechol, acetylthiocholine chloride (ATChCl), acetylcellulose (Mn ~ 30,000), acetylcholinesterase (AChE—electric eel, Type VI-S, 292 IU mg^{-1}) and tyrosinase (TYR—mushroom, 6050 U (ΔA_{280}) mg^{-1}) were purchased from Sigma (St. Louis, MO, USA), Carbopack C (80/100 mesh) from Supelco (Bellefonte, PA, USA), poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) was from Polysciences (Warrington, PA, USA), poly(1-vinylimidazolyl){osmium(1,4′-dimethylbipyridine)$_2$Cl}$^{+/2+}$ (PVI_n-Os, n = 13) from TheraScience (Alameda, CA, USA), butyrylcholinesterase (BChE—horse serum, 65 IU mg^{-1}) was from Fluka (Buchs, Switzerland) and peroxidase (HRP—horseradish, 250 IU mg^{-1}) was obtained from Biozyme (Biozyme Laboratories, Gwent, UK). All pesticides were provided by Dr. B. Šafář (Military Research Institute of Protection, Brno). All other chemicals were obtained from Merck (Darmstadt, Germany). Water purified with the Milli Q system (Millipore, Bedford, MA, USA) was used to prepare all solutions.

The real wastewater samples, containing possible pesticides and phenols, were received from European industries. The samples were marked for confidentiality reasons as S01–S10 (S01 and S02 from pesticides-producing industry,

S03–S05 from pharmaceutical industry and S06–S10 from pulp and paper industry). The tap water was sampled at Lund University (Lund).

2.2. Preparation of biosensors

The four-channel screen-printed sensors (strips 8 mm × 50 mm, electrode diameter 1 mm) were supplied by BVT (http://www.bvt.cz, Tišnov, Czech Republic). The original screen-printed platinum working electrodes were used for immobilisation of cholinesterases. For immobilisation of tyrosinase and peroxidase, the screen-printed platinum electrodes were coated by manual deposition of the carbopack-based carbon ink. The ink was prepared by mixing 100 mg carbopack C with 0.32 ml of a solution containing 1.5% acetylcelullose dissolved in a mixture of cyclohexanone and acetone (ratio 1:1) [48]. The resulting carbopack layer was allowed to dry for 1 h.

The individual electrodes were subsequently covered by 1 μl of the immobilisation mixtures. The following compositions were used for individual electrodes:

1) 10 μl AChE (20 nkat μl^{-1}), 12 μl BSA (50 mg ml^{-1}), 132 μl phosphate buffer (50 mM, pH 7.0) and 13 μl 1% glutaraldehyde [32];
2) the same as before but BChE used instead of AChE;
3) 30 μl tyrosinase (10 mg ml^{-1}), 6 μl PEGDGE (5 mg ml^{-1}) and 24 μl PVI_{13}-Os (5 mg ml^{-1}) [48];
4) 22 μl HRP (38 mg ml^{-1}), 132 μl phosphate buffer and 13 μl 1% glutaraldehyde;
5) mixture with only BSA–12 μl BSA (50 mg ml^{-1}), 132 μl phosphate buffer (50 mM, pH 7.0) and 13 μl 1% glutaraldehyde.

The electrode arrays covered with appropriate mixtures described above were placed in a closed vessel overnight in a refrigerator (this step is not necessary for tyrosinase immobilisation which is ready after 3–4 h drying at room temperature), and stored in the dry state or covered by a drop of buffer (required for the tyrosinase array as protection of the hydrogel polymer-layer against damage) in the refrigerator.

The initial enzyme combination, which was tested in the flow system, included either acetylcholinesterase or butyrylcholinesterase together with tyrosinase immobilised on platinum and on carbopack layer modified electrodes, respectively. The albumin layer for evaluation of any non-specific response covered the fourth working electrode. For steady state measurements in a stirred vessel, four different enzymes were immobilised on one sensor—AChE, BChE, HRP and tyrosinase (Scheme 1).

2.3. Electrochemical measurements

Scheme 1 briefly summarises, the plan of measurements, the combinations of immobilised enzymes on two types of sensors is given and the analysed compounds in the flow and steady state system are presented. The initial part of experiment was performed in the flow system. The four-channel biosensor was fixed in the flow-through cell with the inner volume of 10 μl (constructed in-house) containing

SYSTEM	SENSOR SPE / ENZYME / IMMOBILISATION	TESTED COMPOUNDS
⇒ flow-through		
	⇒ CP / TYR / PVI-Os	⇒ Cu^{2-}, Cd^{2+}, Fe^{3+} (heavy metals)
	Pt / AChE / GA	⇒ NaF
	Pt / BChE / GA	⇒ DMSO, benzene (organic solvents)
	Pt / BSA / GA	⇒ phenol, catechol (phenols)
		⇒ carbaryl, heptenophos (pesticides)
⇒ steady state		
	⇒ CP / TYR / PVI-Os	⇒ Cu^{2}, Cd^{2}, Fe^{3}
	CP / HRP / GA	⇒ NaF
	Pt / AChE / GA	⇒ DMSO, benzene
	Pt / BChE / GA	⇒ phenol, catechol, *p*-cresol
		⇒ carbaryl, heptenophos, fenitrothion
		⇒ REAL SAMPLES (spiked and industrial)

Scheme 1. Scheme of measurements in the flow-through or steady state systems using either immobilised tyrosinase with acetyl and butyrylcholinesterase or tyrosinase, peroxidase, acetyl and butyrylcholinesterase on carbopack layer (CP) or free Pt under glutaraldehyde (GA) or PEGDGE/PVI-Os cross-linking conditions. NaF and some compounds from the group of heavy metals, organic solvents, phenols and pesticides were tested with further evaluation of the biosensor for determination of real samples in the steady state system.

pseudoreference silver electrode and connected to the multipotentiostat detector (MEB, Multichannel Electrochemical Biosensor, constructed by Jiří Kitlička, Brno, Czech Republic). The measured signals (electrode currents) were recorded in a computer using the MebTools program (created by P. Skládal). The flow rate (100 μl min^{-1}) of buffer (50 mM phosphate buffer pH 7.0 with 100 mM KCl) and samples was controlled by the Minipuls pump MP-3 (Gilson, Villiers-le-Bel, France) and sample additions (50 μl) were realised by the Gilson 233 XL Autoinjector. The operating potential was +350 mV (at the cholinesterase-modified electrodes) and −50 mV (at the tyrosinase-modified electrodes) versus the internal Ag reference electrode. Determination of analytes was based either on the inhibition action (for ChEs, in the presence of 0.5 mM acetylthiocholine chloride as substrate) or enzymatic generation of oxidised phenols (for tyrosinase- and peroxidase-modified electrodes). For measurements, pesticides were prepared as methanolic solutions [38] and mixed with 0.5 mM ATChCl solution to provide final pesticide concentrations of 1, 10, 100, 1000 and 10000 μg l^{-1}. Samples were always injected after stabilisation of the background current. The differences in the peak current were used for the evaluation of pesticide effect on the biosensors. The initial signal (S_0) of biosensor from substrate (acetylthiocholine chloride) was determined without pesticide and then the signal S_x in the presence of pesticide (without a preincubation steps). Inhibition was calculated as $Inh = 1 - S_x/S_0$.

For steady state measurements, the biosensor was placed in a stirred (2000 rpm) vessel containing 8 ml of 50 mM phosphate buffer with 100 mM KCl (pH 7.0) together with the pseudoreference silver electrode. As signals, the changes of currents from electrodes in array reflecting enzymatic oxidation or reduction processes or inhibition effects from the chemical compounds in samples were recorded. ATChCl (0.5 mM) as a substrate for cholinesterases and hydrogen peroxide (100 μM) as co-substrate for peroxidase were used. The pesticide solutions and calculation of percentual inhibition were prepared in the same way as for the flow system.

The developed biosensor was tested using real samples in the above-mentioned steady state system. The pre-treatment included only filtration step for separation the biggest particles (Swedish filter paper Class 2 No. 3; A3-90-700; Grycksbo Papperbruk, Sweden). Two types of real samples have been measured using the biosensor arrays. First, the responses for pesticides and phenols were recorded in tap water. These samples were spiked with pesticides (carbaryl and heptenophos) and phenols (phenol and catechol) to get final concentrations of 7, 15 and 125 μg l^{-1} and 5, 20 and 50 μM for pesticides and phenols, respectively. The spiked samples in the volume of 20 μl were added into 8 ml of buffer used in the measurement cell. The second set of the real samples was provided by European industries. The effects of these samples on the responses of the array biosensor electrodes to 0.5 mM ATChCl and 20 μM catechol were evaluated.

3. Results and discussion

Cholinesterases and phenol oxidase enzymes have been frequently used for the development of biosensors for detection of pesticides and phenolic compounds in environmental or wastewater samples. The current trend in development of parallel measurement systems brings the questions if these biosensors proven to be potentially useful in rapid analysis of environmental hazards could be combined into arrays. No principal limitations for combining several enzymes in one array seem to exist; however, the requirements of different co-substrates for functioning of the mentioned enzyme-modified electrodes make the answer less straightforward. For example, the amperometric cholinesterase-based biosensors require acetylthiocholine- and peroxidase-modified electrodes require hydrogen peroxide for their function. To properly address the compatibility question, the effects of peroxide on the action of esterase and tyrosinase-modified electrodes as well as the effects of acetylthiocholine on peroxidase- and tyrosinase-modified electrodes should be evaluated. The discussion below briefly summarises the results, which have been carried out to better understand the compatibility problem arising from combination of two classes of biosensors in one sensing array.

3.1. Cholinesterase-modified electrodes in biosensor arrays

The calibration curves for several pesticides based on the determination of acetylcholinesterase and butyrylcholinesterase activity in flow-through and steady state systems are shown in Figs. 2 and 3. Limits of detection (corresponding to 10% inhibition) are summarised in Table 1. From

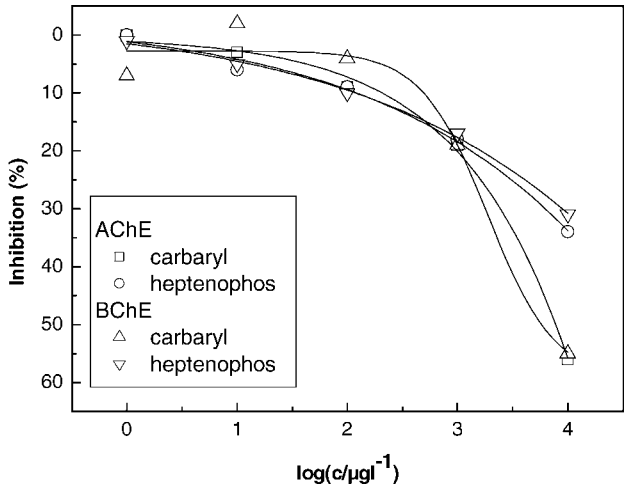

Fig. 2. Calibration curves for carbaryl and heptenophos obtained with the ChE-based arrays in the flow system without pre-incubation steps; percentual inhibition was calculated as $Inh = 100(1 - S_x/S_0)$; S_0 and S_x represent the signals for substrate without or with pesticide, respectively. As substrate 0.5 mM ATChCl was used and +350 mV potential was applied.

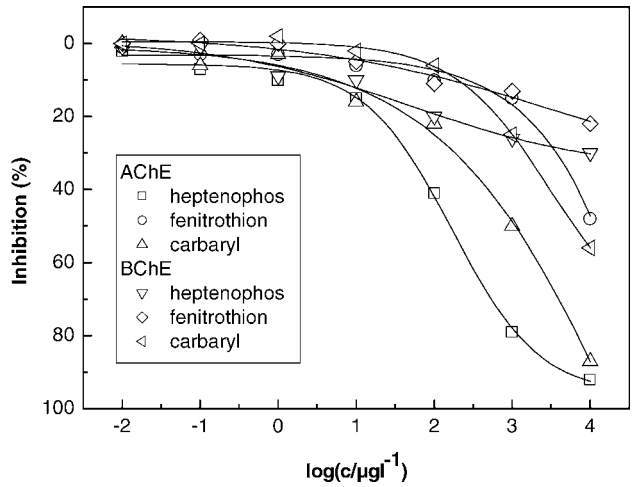

Fig. 3. Calibration curves for carbaryl, heptenophos and fenitrothion obtained in the steady state system; inhibition has the same meaning as already mentioned in Fig. 2.

Table 1
Limits of detection of pesticides determined in the flow and steady state systems; the values represent concentrations required to obtain 10% inhibition of cholinesterase activity

System	LOD ($\mu g\,l^{-1}$)					
	Carbaryl		Heptenophos		Fenitrothion	
	AChE	BChE	AChE	BChE	AChE	BChE
Flow	208	492	121	118	–	–
Steady state	5.3	201	3.6	4.7	266	160

0.5 mM ATChCl as cholinesterases substrate was used.

this table it is clear that using steady state measurements it is possible to determine pesticides at lower concentration; probably due to the longer (10 min) interaction with the enzymes at electrodes if compared with flow-injection measurements.

In addition to pesticides, cholinesterases are inhibited by heavy metals and by fluoride [49,50]. The effects and limits of detection of some heavy metals (Cu^{2+}, Cd^{2+}, Fe^{3+}) and NaF on the biosensor system are summarised in Tables 2 and 3.

Table 2
The effect of some heavy metals and NaF on cholinesterase activity determined in the flow and steady state systems

	Steady state system		Flow system	
	AChE	BChE	AChE	BChE
Inhibition (detection limit, μM)				
Cd^{2+}	4	7	n.o.[a]	n.o.
Fe^{3+}	–	150	n.o.	n.o.
NaF	125	46	290	100
Activation (detection limit, μM)				
Fe^{3+}	600	–	2300	2300
Cu^{2+}	10	13	50	60

Detection limits of inhibition or activation corresponded to ratio signal/noise equal three.

[a] Not observe either activation or inhibition effect up to the concentration of 5 mM.

Table 3
Linear range of detection for the tested heavy metals and NaF with the AChE or BChE-modified electrodes

	Linear range (mM)			
	Steady state system		Flow system	
	AChE	BChE	AChE	BChE
Cu^{2+}	0–0.1	0–0.05	0.01–0.1	0.01–0.1
Cd^{2+}	0–0.4	0–0.05	n.o.[a]	n.o.
Fe^{3+}	0.1–1.3	0.1–2	1–10	0.5–10
NaF	0.05–0.2	0.01–0.2	0.1–1	0.05–1

[a] Not observed (up to 5 mM concentration).

DMSO and benzene as organic solvent slightly decreased the enzyme activity of ChEs. The presence of 0.1% (v/v) DMSO resulted in a 10% loss of activity. Benzene was effective above 0.01% (v/v) with 40% inhibition obtained for 0.1% (v/v) concentration. The effects of organic solvents on ChEs have been studied and published by other researchers [39,51].

To record activity/inactivation of the ChE-modified electrodes, +350 mV applied potential is required for oxidation of thiocholine. In the case that the analysed samples contain phenolic compounds, some of them will be directly electrochemically oxidised. This can be concluded from our observation that catechol in solution contributed to the electrode current similarly as thiocholine. For 0.2 mM catechol, the electrode current was about 300 nA, i.e., about two-times higher than typically observed with the ChE-modified electrodes at 0.5 mM of acetylthiocholine. The presence of phenol didn't have any effect. Catechol also slightly inhibited ChEs. Repeated measurements (10) using 0.1 mM catechol solution decreased the response of ChE-modified electrodes as much as to 30% of the initial its value. Similar inhibition effects were observed in flow-injection and steady-state measurements.

The effects of H_2O_2 at concentration range of 5–200 μM on the ChE-modified electrodes were investigated. No significant effects of H_2O_2 were noticed on the cholinesterase arrays at lower concentrations of peroxide. However, at 100 μM and above the 20–40% decreases of the responses were observed.

The main conclusions from testing of the ChE-modified electrodes in array containing also redox enzymes sensitive to phenols can be summarised as follows. The signals on the ChE-modified electrodes are significantly affected by substrates of phenol oxidising enzymes. The effects are well pronounced and might increase as well as decrease the signal, e.g., current from direct oxidation of catechol will increase the current response being not distinguishable from the oxidation current of thiocholine. An opposite contribution from catechol will be due to slow irreversible inhibition (decrease) of response of the ChEs-modified electrodes. Despite these negative effects of phenolic compounds on the specific responses of hydrolase-modified electrodes, the ChE-modified electrodes can still function as pesticide

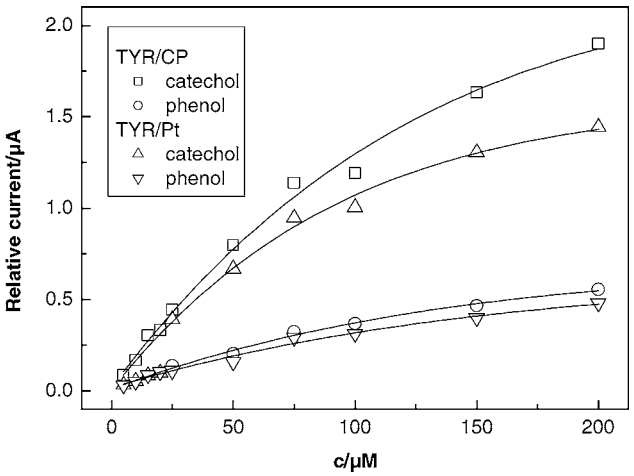

Fig. 4. Calibration curve for catechol and phenol in the flow system based on their detection with biosensors consisting of tyrosinase immobilised either on pure Pt or on the carbopack layer. Potential of −50 mV was applied.

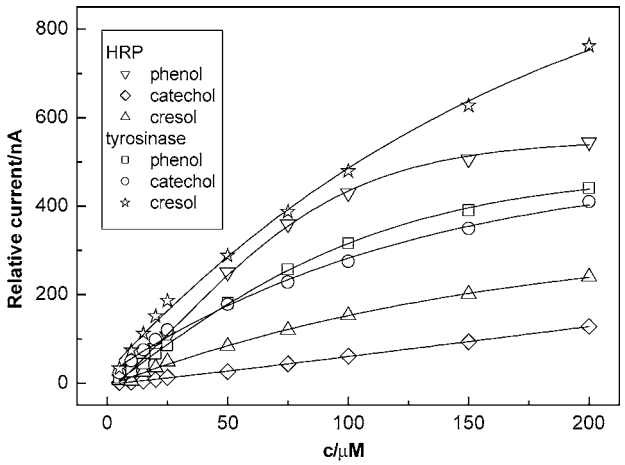

Fig. 5. Calibration curves for phenols (phenol, catechol, *p*-cresol) in combination with 100 μM H_2O_2 and 0.5 mM ATChCl received in steady state system with tyrosinase and peroxidase arrays. Potential of −50 mV was applied.

sensitive biosensors in arrays hosting also other classes of enzymes.

3.2. Tyrosinase- and peroxidase-modified electrodes in biosensor arrays

To understand the compatibility of oxidoreductases with ChEs in the array, the effects of acetylthiocholine on performance of the tyrosinase and peroxidase-modified electrodes have been studied. The calibration curves for tyrosinase-modified electrodes in the absence of acetylthiocholine obtained for catechol and phenol are shown in Fig. 4. The responses of these electrodes decreased in the presence of ATChCl. The decrease depended on design of the tyrosinase-modified electrode and on concentration of ATChCl. At 0.5 mM ATChCl, the signal of the tyrosinase-modified electrodes for 50 μM of catechol or phenol decreased by 5–8%. At higher acetylcholine concentration (1 mM), the response for phenol decreased by up to 25% and for catechol by up to 50%. The limits of detection with tyrosinase-modified carbopack or platinum electrodes were 0.35 and 0.38 for catechol or 1.5 and 1.6 μM for phenol, respectively, in general slightly better in the absence than in the presence of acetylthiocholine. 0.5 mM concentration of ATChCl was chosen for the future measurements since cholinesterases exhibited sufficiently good response already at this concentration and at the same time the influence of ATChCl on the responses of tyrosinase-modified electrodes was relatively low.

Similarly, the signal decreasing effect of ATChCl has been found at the HRP-modified electrodes. The calibration curves for *p*-cresol, catechol and phenol in the presence of 100 μM H_2O_2 are shown in Fig. 5. The sensitivity of peroxidase was as follows: *p*-cresol (100%), catechol (45%) and phenol (30%). With the additional presence of 0.5 mM ATChCl, the signals were lower: *p*-cresol (90%), catechol (70% from the current for catechol in the absence of ATChCl) and phenol (50% from the current for phenol in the absence of ATChCl). For comparison between HRP and tyrosinase-modified electrodes, the calibration plots recorded with the tyrosinase-based biosensor are also included in Fig. 5 and limits of detections are summarised in Table 4. The following order of sensitivities with tyrosinase was found: catechol (100%), *p*-cresol (30%) and phenol (15%). From these results it is clear that in general ATChCl decreases the response of HRP and the tyrosinase-modified electrodes. The degree of signal decrease depends on the type of phenol.

Finally, the effects of H_2O_2 concentration in the range of 5–200 μM on tyrosinase-modified electrodes were investigated. Below 100 μM, no effects on response to catechol were observed at the tyrosinase-modified electrodes. Above 100 μM, the presence of peroxide decreased the response of the electrode. In comparison with the previously published results [48], it seems that H_2O_2 can activate or inhibit the function of tyrosinase. The result probably depends on the

Table 4
Limits of detection of phenolic compounds (*p*-cresol, catechol and phenol) received with the tyrosinase- and peroxidase-modified electrodes in biosensor array in the steady state system either for phenols only or in combination with 100 μM H_2O_2 and 0.5 mM ATChCl

Phenolic compounds	Limit of detection (μM)	
	Peroxidase	Tyrosinase
p-cresol	–	5.3
phenol	–	7.0
catechol	–	1.7
H_2O_2/*p*-cresol	0.38	1.0
H_2O_2/phenol	63	3.7
H_2O_2/catechol	13	0.74
ATChCl/H_2O_2/*p*-cresol	9.1	10
ATChCl/H_2O_2/phenol	77	6.1
ATChCl/H_2O_2/catechol	19	4.2

Peroxidase and tyrosinase were immobilised on the carbopack-layer.

Table 5
The results of the enzyme-based sensor for spiked samples of the tap water

Real concentration in spiked sample	Concentration in sample determined by biosensor	
	AChE	BChE
7 μg l^{-1} carbaryl	4 ± 1	5.3 ± 0.6
15	19 ± 1	6 ± 1
125	137 ± 26	150 ± 22
7 μg l^{-1} heptenophos	6.6 ± 0.2	5 ± 1
15	18 ± 3	7 ± 4
125	145 ± 40	162 ± 21
	Tyrosinase	HRP
5 μM catechol	4 ± 1	3.9 ± 0.5
20	17 ± 3	19 ± 3
50	48 ± 10	50 ± 5
5 μM phenol	6 ± 4	4 ± 1
20	25 ± 8	17 ± 2
50	55 ± 4	45 ± 5

AChE, BChE, tyrosinase and HRP stand for the corresponding enzyme-modified electrode in array. The concentrations are means from two measurements.

chemical design (immobilisation matrix, mechanism of registration of signal, etc) of the electrode.

3.3. Precision of measurements and stability of biosensor arrays

The R.S.D. values characterising the measurements of catechol with tyrosinase- and peroxidase-based sensors were less than 10% in the flow system and twice higher in the steady state system, based on 10 measurements carried out using five different similarly prepared sensors. The responses of the sensors have decreased with the number of measurements. Specifically, after 150 measurements conducted during 5 days, ChEs typically lost about 40–60% of the initial activity, peroxidase about 80%, and tyrosinase 50–60%.

3.4. Sample analysis

For evaluation and testing of the suitability of the biosensor array based on two cholinesterases, tyrosinase and peroxidase for detection of pesticides and phenols in real samples, the initial test was made with tap water after fortification with either carbaryl and heptenophos, or catechol and phenol as model compounds representing pesticides and phenols, respectively. The absolute contents of pesticides and phenols were estimated using calibration curves. The responses from the enzyme-modified electrodes are summarised Table 5. The calculated concentration values agreed more precisely on the tyrosinase and HRP-modified electrodes compared to the ChEs electrodes. However, especially at the lower spiked concentrations, they distinguished nearly 20% of originated concentrations. The found values for pesticides were lower for concentration below or close to LODs.

The second part of experiments was directed towards testing real wastewater samples. The effect of dilution was studied using wastewater samples S02 and S06 originating from the pesticide and pulp and paper industries. It was found that the samples provided response at ChEs, HRP and tyrosinase-based electrodes if diluted up to 16-times with buffer. The buffer solution contained 0.5 mM ATChCl and 100 μM hydrogen peroxide as substrates. The equivalent concentrations of heptenophos determined with ChE-modified electrodes and the corresponding catechol concentrations present in these wastewaters determined by tyrosinase- and peroxidase-modified electrodes in array are shown in Table 6. Several conclusions can be made by analysing the obtained results. First, presence of some phenolic compounds were found in samples: S03–S05, S09 and S10, i.e., wastewaters from pharmaceutical and pulp and paper industry, respectively. Secondly, the HRP-modified electrodes responded to all of these samples from both industries, however, tyrosinase only to water from the pharmaceutical industry. The explanation could be, that the samples from pulp and paper industry contain compounds for which HRP is active, but not tyrosinase, e.g., lignin degradation products such as vanillin, guaiacol [52]. These results prove that peroxidase and tyrosinase-based biosensors in array provide complementary information about the chemical composition of wastewater. Almost all samples (except S01, S07 and S02 with S08 for BChE) have inhibiting influences on the ChE-modified electrodes; inhibition was especially pronounced for the S10 sample. The

Table 6
The equivalent concentrations of heptenophos (in μg l^{-1}; measured by AChE and BChE-modified electrodes) and catechol (in μM; measured by Tyrosinase and HRP-modified electrodes) in real wastewater samples

Enzyme-modified electrode	Origin of wastewater									
	Pesticide industry		Pharmaceutical industry			Pulp and paper industry				
	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10
AChE	<LOD	120	57	13	280	13	<LOD	26	76	2300
BChE	<LOD	<LOD	410	14	34	210	<LOD	<LOD	34	>10000
tyrosinase	<LOD	<LOD	10	10	6	<LOD	<LOD	<LOD	<LOD	<LOD
HRP	<LOD	<LOD	29	55	26	<LOD	<LOD	<LOD	48	70

The equivalent heptenophos concentrations were determined from percentual inhibition of AChE or BChE on the electrodes by wastewater using calibration curves presented in Fig. 3. Similarly, the equivalent concentrations of catechol were determined relating the response from wastewater observed with tyrosinase or HRP-modified electrodes and calibration plots (Fig. 5). The detection limits for heptenophos and catechol were 3.6 μg l^{-1} (at AChE-modified electrode), 4.7 μg l^{-1} (BChE), 4.2 μM (tyrosinase) and 19 μM (HRP).

inhibition effect was expressed by the corresponding heptenophos concentration equivalents. However, it is hard to conclude if inhibition was due to this compound or any other pesticides and ChE-inhibiting compounds. The inhibition of ChE on electrodes, however, points that some of these samples might be toxic.

4. Conclusions

The amperometric biosensor array was constructed by co-immobilisation of enzymes from different groups, i.e., oxidoreductases and hydrolases on four electrodes in array. Specifically, the array contained acetyl and butyrylcholinesterases-, tyrosinase-, and peroxidase-modified electrodes. The array was tested in flow and steady state amperometric measurement modes. The experiments with measurements of pesticides and phenolic compounds in buffer solutions have shown that the co-substrates of the enzymes in array affect the signals of the neighbour biosensors in array. However, the effects (mostly inhibition) were in all cases below 10%, if the optimal concentrations of substrates were chosen. This proves that there are no principal limitations to combine the two classes of enzymes in one biosensor array. The comparison of flow-injection and steady state measuring methods has shown that advantages in the case of the flow arrangements include improved reproducibility, faster analysis and better limits of detection for phenols. On the other hand, the steady state measurement mode is more suitable for cholinesterase systems providing better limits of detection for pesticides. The detection limits for catechol of tyrosinase and peroxidase-modified electrodes in array were in the range of 0.4–19 μM. The achieved limits of detection for pesticides at ChE-modified electrodes were higher than 0.1 $\mu g\,l^{-1}$ and 0.5 $\mu g\,l^{-1}$ (EU limit for individual and sum of pesticides in drinking water, respectively) [53], however quite satisfactory for a preliminary screening procedure.

Additionally, tyrosinase and peroxidase enzymes, though being from the same class, provided different responses for phenols. Especially important, these two biosensors in array exhibited different current signals from real samples of wastewaters from pulp and paper industry. As could be expected peroxidase-modified electrodes showed much higher responses to real wastewater samples where lignin degradation products might be present.

In general, it would be difficult to expect that biosensor arrays will be used for specific detection of hazards in wastewaters. Our experiments indicate that unknown complexity of the real samples gives responses of biosensor in array, which are hard to explain or predict using calibration methods. Modern statistical processing of the data exploiting principal component analysis and other chemometric methods are currently under studies to evaluate possibilities of biosensor array for pattern recognition of toxicity and degree of bioremediation of wastewater samples.

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References

[1] F. Ortega, E. Domínguez, E. Burestedt, J. Emnéus, L. Gorton, G. Marko-Varga, J. Chromatogr. A 675 (1994) 65.
[2] M.V. Reddy, G.R. Blackburn, C.A. Schreiner, M.A. Mehlman, C.R. Makerer, Environ. Health Perspect. 82 (1989) 253.
[3] J.W. Yager, D.A. Eastmond, M.L. Robertson, W.M. Paradisin, M.T. Smith, Cancer Res. 50 (1990) 393.
[4] A. Di Corcia, S. Marchese, R. Samperi, J. Chromatogr. A 642 (1993) 175.
[5] E.R. Brouwer, U.A.Th. Brinkman, J. Chromatogr. A 678 (1994) 223.
[6] D. Puig, D. Barceló, J. Chromatogr. A 778 (1997) 313.
[7] R. Wissiack, E. Rosenberg, M. Grasserbauer, J. Chromatogr. A 896 (2000) 159.
[8] A. Di Corcia, M. Marchetti, Anal. Chem. 63 (1991) 580.
[9] V. Leoni, A.M. Caricchia, S. Chiavarini, J. AOAC Int. 75 (1992) 511.
[10] M.T. Tena, P. Linares, M.D. Luque de Castro, M. Valcarcel, Chromatographia 33 (1992) 449.
[11] J.L. Besombes, S. Cosnier, P. Labbe, G. Reverdy, Anal. Lett. 28 (1995) 405.
[12] I.C. Vieira, O. Fatibello-Filho, Anal. Lett. 30 (1997) 895.
[13] M. Lutz, E. Burestedt, J. Emnéus, H. Lidén, S. Gobhadi, L. Gorton, G. Marko-Varga, Anal. Chim. Acta 305 (1995) 8.
[14] C. Nistor, J. Emnéus, L. Gorton, A. Ciucu, Anal. Chim. Acta 387 (1999) 309.
[15] E.A. Cummings, S. Linquette-Mailley, P. Mailley, S. Cosnier, B.R. Eggins, E.T. McAdams, Talanta 55 (2001) 1015.
[16] T. Mai Anh, S.V. Dzyadevych, A.P. Soldatkin, N. Duc Chien, N. Jaffrezic-Renault, J.-M. Chovelon, Talanta 56 (2002) 627.
[17] S. Campuzano, B. Serra, M. Pedrero, F.J. Manuel de Villena, J.M. Pingarrón, Anal. Chim. Acta 494 (2003) 187.
[18] S. Zhang, H. Zhao, R. John, Anal. Chim. Acta 441 (2001) 95.
[19] A.I. Yaropolov, A.N. Kharybin, J. Emnéus, G. Marko-Varga, L. Gorton, Anal. Chim. Acta 308 (1995) 137.
[20] B. Serra, S. Jiménez, M.L. Mena, A.J. Reviejo, J.M. Pingarrón, Biosens. Bioelectron. 17 (2002) 217.
[21] F. Ortega, E. Domínguez, E. Jönsson-Pettersson, L. Gorton, J. Biotechnol. 31 (1993) 289.
[22] J. Švitel, S. Miertuš, Environ. Sci. Technol. 32 (1998) 828.
[23] T. Ruzgas, J. Emnéus, L. Gorton, G. Marko-Varga, Anal. Chim. Acta 311 (1995) 245.
[24] T. Ruzgas, L. Gorton, J. Emnéus, G. Marko-Varga, J. Electroanal. Chem. 391 (1995) 41.
[25] A. Lindgren, J. Emnéus, T. Ruzgas, L. Gorton, G. Marko-Varga, Anal. Chim. Acta 347 (1997) 51.
[26] T. Ruzgas, E. Csöregi, J. Emnéus, L. Gorton, G. Marko-Varga, Anal. Chim. Acta 330 (1996) 123.
[27] S. Gaspar, K. Habermüller, E. Csöregi, W. Schuhmann, Sens. Actuators B 72 (2001) 63.
[28] S.-Q. Liu, H.-Z. Ju, Anal. Biochem. 307 (2002) 110.
[29] E. Ferapontova, E. Puganova, J. Electroanal. Chem. 518 (2002) 20.
[30] R. Huang, N. Hu, Bioelectrochemistry 54 (2001) 75.
[31] S.S. Rosatto, L.T. Kubota, G.O. Neto, Anal. Chim. Acta 390 (1999) 65.
[32] P. Skládal, G.S. Nunes, H. Yamanaka, M.L. Ribeiro, Electronalalysis 9 (1997) 1083.
[33] P. Skládal, Anal. Chim. Acta 252 (1991) 11.

[34] C. La Rosa, F. Pariente, L. Hernández, E. Lorenzo, Anal. Chim. Acta 308 (1995) 129.
[35] D. Martorell, F. Céspedes, E. Martínez-Fàbregas, S. Alegret, Anal. Chim. Acta 290 (1994) 343.
[36] N. Mionetto, J.-L. Marty, I. Karube, Biosens. Bioelectron. 9 (1994) 463.
[37] G.S. Nunes, P. Skládal, H. Yamanaka, D. Barceló, Anal. Chim. Acta 362 (1998) 59.
[38] P. Skládal, M. Fiala, J. Krejčí, Int. J. Environ. Anal. Chem. 65 (1996) 139.
[39] T. Montesinos, S. Pérez-Munguia, F. Valdez, J.-L. Marty, Anal. Chim. Acta 431 (2001) 231.
[40] P. Skládal, Food Technol. Biotechnol. 34 (1996) 43.
[41] A.A. Ciucu, C. Negulescu, R.P. Baldwin, Biosens. Bioelectron. 18 (2003) 303.
[42] F.N. Kok, V. Hasirci, Biosens. Bioelectron. 19 (2004) 661.
[43] R.W. Keay, C.J. McNeil, Biosens. Bioelectron. 13 (1998) 963.
[44] N.F. Starodub, N.I. Kanjuk, A.L. Kukla, Yu.M. Shirshov, Anal. Chim. Acta 385 (1999) 461.
[45] N.F. Starodub, Biosensors for Direct Monitoring of Environmental Pollutants in Field, in: D.P. Nikolelis, U.J. Krull, J. Wang, M. Mascini (Eds.), NATO Advanced Series, Series 2–Environment, 38, Kluwer Publishing Co., The Netherlands, 1998, pp. 209–219.
[46] S.C. Chang, K. Rawson, C.J. McNeil, Biosens. Bioelectron. 17 (2002) 1015.
[47] S.E. Stanca, I.C. Popescu, L. Oniciu, Talanta 61 (2003) 501.
[48] S. Sapelnikova, E. Dock, R. Solná, P. Skládal, T. Ruzgas, J. Emnéus, Anal. Bioanal. Chem. 376 (2003) 1098.
[49] M. Stoytcheva, Bull. Soc. Chim. Belg. 103 (1994) 147.
[50] R. Gruss, F. Scheller, M.J. Shao, C.C. Liu, Anal. Lett. 22 (1989) 1159.
[51] J. Järv, M. Speek, Biochim. Biophys. Acta 706 (1982) 174.
[52] B. Hagnigni, L. Gorton, T. Ruzgas, L.J. Jönsson, Anal. Chim. Acta 487 (2003) 3.
[53] Council Directive 80/778/EEC of 15 July 1980. OJ No L 229, 30.8.1980.