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Amperometric screen-printed biosensor arrays with co-immobilised oxidoreductases and cholinesterases

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Abstract

Amperometric screen-printed biosensor arrays for detection of pesticides (organophosphates and carbamates) and phenols have been developed. Cholinesterases (AChE and BChE), tyrosinase (TYR), peroxidases (SBP, soybean and HRP, horseradish) and cellobiose dehydrogenase (CDH) were combined on the same array consisting of one Ag/AgCl reference electrode surrounded by eight radially distributed working electrodes of either carbon or platinum. Mainly cross-linking with glutaraldehyde was employed for enzyme immobilisation. The substrates for the enzymes were acetylthiocholine for cholinesterases (ChEs), cellobiose for CDH and hydrogen peroxide for peroxidases. Hydrogen peroxide was generated in the presence of glucose by co-immobilised glucose oxidase (GOx). All measurements were performed in an electrochemical steady state system specially constructed for eight channel screen-printed electrode arrays. The achieved relative standard deviation values calculated for different enzyme substrates (10 measurements) were typically below 7% and one assay was completed within less than 10 min. The detection limits for pesticides and phenols were in the nanomolar and micromolar ranges, respectively. The developed biosensor array was evaluated on wastewater samples. To simplify interpretation of results, the measured data were treated with multivariate analysis–principal component analysis (PCA).
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1. Introduction

The interest for environmental monitoring increases and represents an urgent issue because of the expanding use of chemicals and biologically active substances in industry and agriculture. In some cases, such substances can exhibit fairly high toxicity. Phenols (especially chlorinated ones), organophosphates and carbamates as insecticides are typical examples of such compounds [1,2].

Enzymatic biosensor arrays represent promising pre-screening methods for rapid and simple measurements and express analysis of many pollutants, which can function either directly as substrates or as inhibitors of enzymes selected for the sensing array. A number of sensors for determination of phenols and pesticides include a variety of transducers based on amperometric [3–10], potentiometric [11,12] and optical [13,14] detection. However, these sensors enable determination of only individual compounds or subgroups of related pollutants, while samples usually consist of a complex matrix of different compounds. This problem can be addressed by designing multibiosensors consisting of several transducer elements with different bioselective components specific for various substances. In the environmental area, with the exception of immunosensors [15,16] applied mainly in the clinical field, the application of enzyme arrays suitable

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for analysis of multiple samples in various field conditions has progressed during the last decade. The examples include multienzymatic biosensors based on immobilisation of different enzymes from the same class, e.g. oxidases [17], arrays combined with the same type of enzyme but from different natural sources, e.g. different types of cholinesterases [18]. Usually, this approach enables detection of one type of analyte in samples according to different substrate specificity: biosensors for discrimination of phenols with immobilised tyrosinase together with laccase and/or peroxidase [8,19], acetylcholinesterase and butyrylcholinesterase based biosensors for determination of organophosphate and carbamate pesticides [3,20]. Additionally, especially cholinesterases can be included in biosensor arrays for heavy metal detection, often co-immobilised with urease [21,22]. The use of enzymes from different classes in one array can provide more information about heterogeneous composition of samples. Various biosensor arrays fulfilling such requirements have been developed, mainly with enzymes belonging to oxidoreductases and hydrolases, e.g. biosensor arrays for determination of glucose, lactate, glutamine, glutamate with glucose oxidase, catalase, lactate oxidase, glutamate oxidase, glutaminase and asparaginase as biorecognition elements [23] and glucose oxidase, lactate oxidase, creatininase/creatinase, sarcosine oxidase and urease sensors for detection of glucose, lactate, urea, creatinine, chloride, ionised sodium, potassium, calcium and magnesium [24]. No principal limitations for combining several enzymes in one array seem to exist. However, the requirements of different co-substrates for functioning of the above mentioned enzyme-modified electrodes make the answer less straightforward. The compatibility between oxidoreductases and hydrolases when functioning in the same array based on four enzymes (combination of acetylcholinesterase, butyrylcholinesterase, tyrosinase and horseradish peroxidase) has already been studied [25,26].

The present work investigates the possibility to include additional enzymes (oxidoreductases cellobiose dehydrogenase and soybean peroxidase) with different substrate specificities as one of the ways to obtain more information on environmental samples with unknown composition. The developed biosensor array was tested in the amperometric steady state system using model phenolic and pesticide compounds and later it was evaluated on wastewater samples from industrial sources. The obtained results were treated with principal component analysis (PCA) to facilitate interpretation of measured signals for qualitative classification of samples.

2. Experimental

2.1. Chemicals

Acetylcholinesterase (AChE—electric eel, 244 IU/mg), butyrylcholinesterase (BChE—horse serum, 345 IU/mg), peroxidase (HRP—horseradish, 263 purpurogallin units/mg and SBP—soybean, 108 units/mg), tyrosinase (TYR—

mushroom, 2590 units (ΔA_{280})/mg), bovine serum albumin (BSA), glutaraldehyde, acetylthiocholine chloride (ATChCl), glucose, cellobiose, catechol and *p*-aminophenol were obtained from Sigma (St. Louis, MO, USA). Glucose oxidase (GOx—*Aspergillus niger*, 270 IU/mg) was from Biozyme (Biozyme Laboratories, Gwent, UK). Cellobiose dehydrogenase (CDH—*Phanerochaete chrysosporium*, 2.45 g/l, $A_{420}/A_{280} = 0.64$) was purified according to the method of Henriksson et al. [27]. Pesticides were provided by Dr. B. Šafář (Military Research Institute of Protection, Brno). All other chemicals (including phenol, *p*-chlorophenol, *p*-cresol) were obtained from Merck (Darmstadt, Germany). Water purified in a Milli Q system (Millipore, Bedford, MA, USA) was used to prepare all solutions.

Wastewater samples were received from different European industries (30 samples from a pulp and paper industry and 13 samples from a pesticide industry). The pulp and paper samples were from two subsets of wastewater marked as untreated (U) and alert (A), respectively. The latter subset was received after processing in a pilot treatment plant at ANOX, Lund, Sweden. The effluent quality was regulated to the "alert" toxicity level by the addition of nutrients N and P to the wastewater batch prior to the treatment. At DHI Water and Environment, Hørsholm, Denmark, the two subsets of pulp and paper wastewater were further fractionated by RP-HPLC (after pH adjustment and filtration) and diluted 10 times with Milli Q water. The pesticide-containing industry samples (P) were collected from an effluent of a biological wastewater treatment plant at the pesticides-producing company.

2.2. Preparation of biosensors

Screen-printed arrays were obtained from BVT Technologies (Brno, Czech Republic). Eight working electrodes $(\varnothing = 1\,\text{mm})$ were printed with a radial distribution on an alumina support (5 cm × 1.2 cm) with either graphite paste DP 7101 (Dupont, USA) or platinum. The Ag/AgCl printed layer was used as a reference electrode. The original platinum working electrodes were used for immobilisation of cholinesterases whereas screen-printed graphite electrodes were used for immobilisation of tyrosinase, peroxidase (mixed with glucose oxidase) and cellobiose dehydrogenase. About 1 μl of each enzyme solution was added on the electrode surface. The prepared sensors were left in a closed vessel overnight at +4 °C in vapours of glutaraldehyde (originating from a drop of 3% solution). Before use, the arrays were rinsed with Milli Q water. The final distribution of enzymes on the eight-electrode array was as follows (protein concentrations in the deposited drops): (1) cellobiose dehydrogenase (2.45 mg/ml); (2) horseradish peroxidase/glucose oxidase bi-layer (both in the final concentration of 5 mg/ml); (3) soybean peroxidase/glucose oxidase bi-layer (final concentrations of 5 and 10 mg/ml, respectively); (4) tyrosinase (10 mg/ml); (5 and 6) acetyl- and butyrylcholinesterase (each enzyme solution prepared as a mixture of 10 μl enzyme
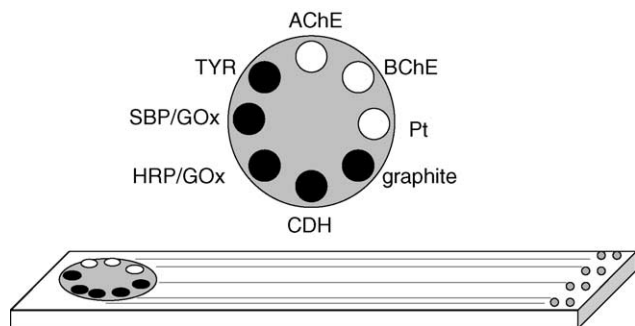
Fig. 1. The construction of the eight-electrode screen-printed array and the illustration of the final distribution of enzymes on the working electrodes, free Pt and graphite electrodes remained uncoated.

(20 nkat/μl) and 13 μl BSA (50 mg/ml) in 110 μl phosphate buffer [26]). Two electrodes (7 and 8) remained as uncoated platinum and graphite (Fig. 1).

2.3. Steady state measurements

The prepared biosensor array was fixed in the amperometric steady state cell (described elsewhere [28]) and connected to an eight-channel potentiostat (two-electrode system with working electrodes and the Ag/AgCl reference/counter electrode printed on the array) controlled by the data software program Intels 1.5 (Laboratory of Enzyme Chemistry, Institute of Biochemistry, Vilnius, Lithuania). The working potentials of 350 mV (cholinesterase modified and bare platinum electrodes) [26], 400 mV (cellobiose dehydrogenase modified and bare graphite electrodes) [10] and −100 mV (tyrosinase, horseradish and soybean peroxidase modified electrodes) [9] versus Ag/AgCl were applied. The height of 1.8 mm and speed of 15 Hz were used as cell-rotator parameters [28]. The immobilised enzymes were activated by adding substrate solutions into 10 ml of 50 mM phosphate buffer with 100 mM KCl (pH 7.0) to provide final concentrations of 0.5 mM acetylthiocholine chloride, 0.5 mM glucose and 0.5 mM cellobiose. Model compounds stock solutions of either phenols (phenol, catechol, *p*-aminophenol, *p*-chlorophenol and *p*-cresol) or pesticides (heptenophos, dichlorvos, carbaryl, fenitrothion and phosphamide) were prepared in methanol (1 M phenols and 10 g/l pesticide stock solutions).

Detection of phenols was based on a recycling mechanism between the electrode surface and the immobilised oxidoreductase (TYR, HRP, SBP and CDH). The enzymes were activated by either oxidising agents (O_2 for TYR and H_2O_2 generated from glucose by the co-immobilised glucose oxidase for HRP and SBP) or a reducing agent (cellobiose for CDH). After a stable steady state current depending on concentration of oxidoreductase substrates (glucose or cellobiose) was established due to a direct electron transfer between the enzyme and the electrode [29], a methanolic solution of phenol was added to the cell. The phenolic compounds served as

mediators shuttling electrons between the immobilised oxidoreductase and the electrode [9,10]. The second steady state current due to the concentration of phenols in the solution was recorded.

Inhibition of pesticides was characterised by the relative inhibition (Eq. (1)), where ΔI was calculated as steady state minus background currents (Eq. (2)). After addition of the cholinesterase substrate, acetylthiocholine, a steady state current, I_{ss}, was reached within 1 min. Addition of a sample containing the cholinesterase inhibitor followed and resulted in the decrease of activity demonstrated by a decrease of signal dI/dt. The resulting dI/dt measured as the slope of the current-time dependence, was proportional to the concentration of inhibitors. About 10 μl of the pesticide methanolic solution was added into 10 ml of working buffer.

$$RI = \frac{(dI/dt)}{\Delta I} \tag{1}$$

$$\Delta I = I_{ss} - I_0 \tag{2}$$

The developed biosensor array was tested on samples received from industrial sources known to contain phenols and pesticides. In the beginning, a standard solution of substrates giving cell concentrations of 0.5 mM ATChCl, 0.5 mM glucose, 0.5 mM cellobiose and 15 μM catechol was added into the working solution. After stabilisation of a steady state current, addition of the sample followed (finally 10 times diluted). Milli Q water and additional catechol (final concentration increase of 15 μM) were used as reference samples. A typical real record of addition substrates and sample is shown in Fig. 2. To eliminate time shifts in the recorded curves (important for the multivariate analysis treatment), the sample additions during all measurements were aligned to the same recording time point.
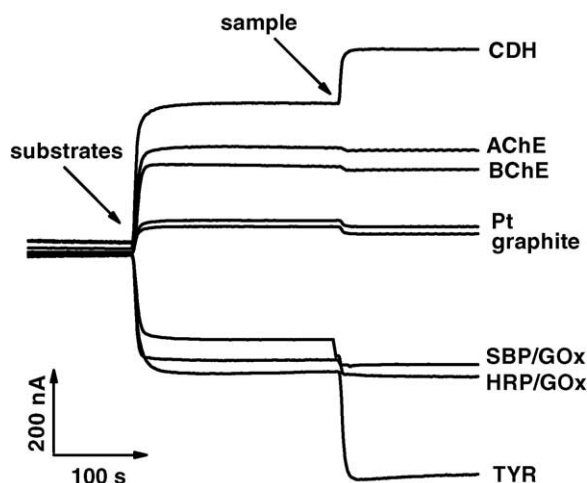

Fig. 2. A typical plot recording responses of all eight working electrodes. As substrates, 0.5 mM ATChCl, 0.5 mM glucose, 0.5 mM cellobiose and 15 μM catechol were used. As example, sample from pulp and paper industry was added.

2.4. Treatment of sample data for chemometric analysis

Chemometric treatment of signals from multienzyme biosensor arrays can transform the complex responses into formats that are easier for interpretation. Principal component analysis (PCA) as a multivariate analysis method was used for this approach. In PCA, the raw data matrix, in our case built of wastewater samples (rows) and sensor responses (columns), is decomposed into a structured part based on the systematic variance of the data and a noise part built of the random variations [30]. From the structured part, phenomena hidden in large amounts of data can be visualised. The samples can be characterised by selecting to subsets and relationships can be found among the sensor responses [31]. PCA calculations were carried out using the commercial software Unscrambler (v 8.0, Camo Process, Oslo, Norway).

Since PCA models are based on structural variations in the data set, drift caused by the sensors can have large effects on the calculated model. Thus, prior the PCA analysis, the measured data were pre-processed by correcting the responses for baseline shift. The drift caused by the loss of sensitivity and activity of the enzymes with time (stability of the sensor) was corrected by measuring a standard (0.5 mM ATChCl, 0.5 mM glucose, 0.5 mM cellobiose and 15 μM catechol) before each wastewater sample. The whole steady state response (810 equally time-distributed values) of the sample was then divided by the mean steady state value of the corresponding standard. The data were additionally normalised to Milli Q water responses obtained from all electrodes. The data were arranged into a raw data matrix with the selected response variables defining the columns and the rows referring to the sample measurements. When the raw data matrix was decomposed by PCA, new axes, called principal components (PC1 and PC2), were created, such that PC1 described the largest variance in the data and PC2 the second largest amount of data variance constructed orthogonal to PC1 and independent on PC1. In general, only few components accounted for most of the data variance and were likely to provide most of the reliable information.

3. Results and discussion

This chapter briefly summarises the results from determinations of model phenols and pesticide compounds (organophosphate and carbamate) with the amperometric eight-electrode sensor system based on immobilised acetylcholinesterase, butyrylcholinesterase, tyrosinase, cellobiose dehydrogenase, horseradish and soybean peroxidase (both peroxidases were co-immobilised with glucose oxidase), and its ability to evaluate industrial samples.

The electrocatalytic determination of phenols and pesticides using the above mentioned enzyme electrode array takes advantage of the well-known reactions shown in Scheme 1. The detection of organophosphate and carbamate insecticides using cholinesterases (ChEs) as the bio-

A) ChE

thiocholine ester $\longrightarrow$ thiocholine + acid

2 thiocholine $\xrightarrow[anode]{}$ dithiocholine + 2e$^-$ + 2 H$^+$

B) TYR

monophenol $\xrightarrow{O_2}$ o-diphenol $\underset{cathode}{\overset{O_2}{\rightleftharpoons}}$ o-quinone + 2e$^-$ + 2 H$^+$

C) HRP(SBP)/GOx

β-glucose $\xrightarrow{O_2}$ gluconolactone + H$_2$O$_2$

H$_2$O$_2$ + HRP(or SBP)$_{native}$ $\longrightarrow$ HRP(SBP)$_{ox}$ + H$_2$O

HRP(SBP)$_{ox}$ + phenols $\longrightarrow$ HRP(SBP)$_{native}$ + phenoxy radicals

phenoxy radicals $\xrightarrow[cathode]{}$ phenols

D) CDH

cellobiose $\longrightarrow$ cellobionolactone + 2e$^-$ + 2 H$^+$

quinones + 2e$^-$ + 2 H$^+$ $\longrightarrow$ phenols

phenols $\xrightarrow[anode]{}$ quinones

Scheme 1. Summary of the enzyme reactions included in biosensor arrays with immobilised AChE, BChE, TYR, CDH, HRP/GOx and SBP/GOx, using thiocholine ester (ATChCl), glucose and cellobiose as enzyme substrates present simultaneously in the solution. When positive (350 mV vs. Ag/AgCl for ChEs and 400 mV for CDH) or negative (−100 mV for TYR, SBP and HRP) electrode potentials were applied, currents were recorded on all electrodes responding to the conversion of electroactive compounds generated in the following reactions.

logical target (A), results from their inhibition due to structural similarity with acetylcholine [32]. If a salt of acetyl- or butyrylthiocholine (e.g. ATChCl) is used as substrate for ChE, then thiocholine is produced during the enzymatic reaction and it becomes subsequently anodically oxidised at 350 mV. Tyrosinase (TYR), peroxidase (POD) and cellobiose dehydrogenase (CDH) can participate in detection of phenols through the reactions shown in Scheme 1(B–D). In the presence of oxygen, TYR catalyses a two-step reaction that includes hydroxylation of monophenols to o-diphenols and their subsequent dehydrogenation to o-quinones (B). These are further electrochemically re-reduced to o-diphenols at −100 mV, which consequently results in a bioelectrochemical amplification cycle. TYR is able to use mono-, di- and trihydroxyphenols as substrates but it exhibits higher affinity for dihydroxyphenols and it does not exhibit any activity for the oxidation of m- and p-benzenediols [33]. Phenols can also be detected through the re-reduction of PODs, which previously have been oxidised in the presence of hydrogen peroxide (C). The phenoxy radicals, formed during this reaction,

are electrochemically reduced on the electrode surface at −100 mV [34]. The direct addition of hydrogen peroxide into the working solution can be replaced by its continuous generation using co-immobilised glucose oxidase (GOx) when β-D-glucose is present in the solution. Another option for phenol detection is to use immobilised CDH (D). In this case, phenols are first anodically oxidised to quinones (at 400 mV), which are subsequently reduced by CDH back to phenols in the presence of cellobiose [35].

3.1. Evaluation of the enzyme electrode array

ChEs were immobilised according to the published procedure by mixing enzymes together with BSA and glutaraldehyde as cross-linking agents [36]. In such a way, the modified electrodes provided up to 2.5-times higher responses compared to application of glutaraldehyde cross-linking using only diluted enzyme solution. The obtained response was $\Delta I = 182/121$ nA and R.S.D. = 4.7/4.8% (10 measurements with 0.5 mM ATChCl) for AChE/BChE, respectively. The proper enzyme immobilisation concentrations of PODs and GOx were tested in the range of 5, 10 and 20 mg/ml. The highest responses were achieved for the mixtures containing 10 mg/ml HRP with 10 mg/ml GOx and 20 mg/ml SBP with 10 mg/ml GOx.

A suitable pH for measurements with this multienzyme electrode array was found at pH 7.0. It represents the lower pH of the optimal range for ChEs (e.g. at pH 6.75 BChE already lost 34% of the activity reached at pH 7.0 and AChE even 55%). On the other hand, at higher pH values, the loss of activity of the oxidoreductases increased (from 20 till 50% of the activity reached at pH 7.0).

To find suitable working concentrations of substrates, calibration curves were constructed for each component. The apparent Michaelis constants and the maximal currents were calculated by fitting the variation of current versus the concentration of substrate to the electrochemical version of the Michaelis–Menten equation (Eq. (3)), where [S] represents the substrate concentration, I_{max} the maximum current and K_m^{app} the apparent Michaelis constant.

$$I = \frac{I_{max}[S]}{([S] + K_m^{app})} \qquad (3)$$

The following concentrations were used for all following experiments:

- 0.5 mM ATChCl from the tested range of 0.1–5 mM, the calibration parameters were obtained both for AChE ($K_m^{app} = 0.17$ mM, $I_{max} = 54$ nA) and for BChE ($K_m^{app} = 0.31$ mM, $I_{max} = 152$ nA);
- 0.5 mM glucose from the range of 0.1–1 mM with following parameters for GOx in combination with either HRP ($K_m^{app} = 0.26$ mM, $I_{max} = 262$ nA) or SBP ($K_m^{app} = 0.07$ mM, $I_{max} = 181$ nA), with added 100 μM catechol;
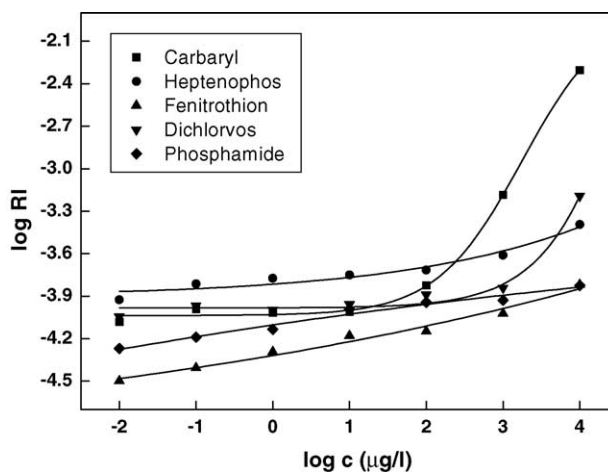

Fig. 3. Calibration curves for organophosphate and carbamate pesticides determined with the AChE sensor. AChE was activated by acetylthiocholine chloride (0.5 mM) recorded as a stable steady state current. The pesticide inhibitions were characterised as log RI, where relative inhibition is equal to $RI = (dI/dt)/\Delta I$, dI/dt characterised the time decrease in signal after addition of pesticide in the working solution containing acetylthiocholine chloride. The applied potential was +350 mV vs. Ag/AgCl. The relative standard deviations varied for both enzymes were in the range of 2.1–5.9%.

- 0.5 mM cellobiose from the range of 0.1–1 mM for CDH with $K_m^{app} = 0.30$ mM and $I_{max} = 120$ nA when 100 μM catechol was present.

Glucose can be used as CDH substrate too; however its K_m^{app} value is about 1000 times higher than for cellobiose, one of the most effective electron donors for this dehydrogenase [37].

3.2. Determination of pesticides

The following model pesticides were determined with cholinesterases: carbaryl, heptenophos, fenitrothion, dichlorvos and phosphamide. The relationship between relative inhibition and concentration of the pesticide for AChE and BChE based sensing elements are shown in Figs. 3 and 4, respectively. Table 1 summarises sensitivities and limits of detection (LOD) for all pesticides; LODs were determined as the lowest reliably detectable decrease in I_{ss} of approximately 10 pA s^{-1}. AChE seemed to be more selective for carbaryl, representing the carbamate insecticides, compared to other tested compounds (organophosphates), which is illustrated by the sensitivity values. BChE did not provide so strict separation of carbaryl versus other organophosphates. On the other hand, compared to the AChE responses, BChE showed higher sensitivity for organophosphates except phosphamide (Table 1). The ChEs electrodes enabled the detection of pesticides in the nano- to micromolar range (0.8 nM–2.4 μM) depending on pesticide molecule. As might be seen, the variation between limits of detection for different pesticides was relatively high. Therefore, considering future analysis of
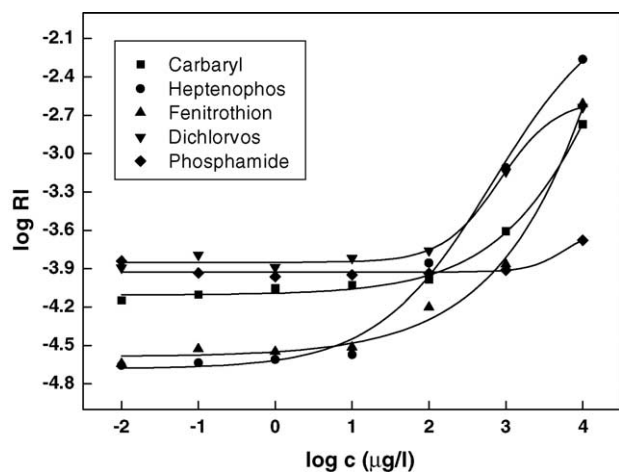
Fig. 4. Calibration curves for pesticides obtained with the BChE-immobilised sensor. The meaning of *RI* (relative inhibition) is the same as mentioned in Fig. 3, as well as the working conditions. The relative standard deviations were found to be in the range 3.4–7.8%.

heterogeneous and unknown samples; the array seems to be better suited for preliminary screening than for exact quantitative characterisation.

The low inhibition effect of phosphamide, as evident from Figs. 3 and 4 and Table 1, probably depends on its phosphorodithioate structure. In general, organothiophosphates, which are characterised by one thione moiety (P=S) and three −OR groups attached to a phosphorus atom, are only weak ChEs inhibitors. Some of them can be slowly degraded photochemically to hydrolytic and oxidation products. To speed up the conversion into the corresponding oxon forms, treatment with bromine water has been suggested as an easy and efficient method for in situ oxidation [38,39].

3.3. Determination of phenols

The selectivity for five phenolic compounds (catechol, *p*-aminophenol, *p*-chlorophenol, *p*-cresol and phenol) was studied with the TYR-, CDH-, HRP/GOx- and SOP/GOx-modified electrodes. All measurements were performed in the amperometric steady state cell. Parameters from calibra-

tion curves fitted by the Michaelis–Menten equation (K_m^{app}, I_{max}) and LODs are summarised in Table 2. The sensitivity was calculated as I_{max}/K_m^{app} and LOD according to the S/N = 3 ratio. From the parameters, the following conclusions can be made. The TYR and HRP/GOx sensors responded to all tested phenols (Table 2). The TYR sensor showed higher sensitivity for *p*-chlorophenol than for phenol and *p*-cresol and the trend was similar for the HRP/GOx sensor. On the other hand, the results are completely different for the same sensors with regards to *p*-aminophenol and *p*-cresol (Table 2). The TYR sensor showed a lower sensitivity for *p*-aminophenol compared to the other phenols. It was possible to make a similar conclusion for the HRP/GOx sensor except for the sensitivity to *p*-cresol. For the SBP/GOx sensor, no responses for phenol and chlorophenol were observed, and a lower sensitivity for catechol and *p*-cresol compared to HRP/GOx was noticed. Similar sensitivities were found only for *p*-aminophenol. As expected, CDH preferred diphenols (i.e. catechol and *p*-aminophenol), which after anodic oxidation to quinones enter the CDH enzyme reaction. Table 3 gives a brief overview of relative responses for all phenols compared to the catechol response detected with the corresponding oxidoreductase electrode. I_{max} was used as the initial value for calculation.

3.4. Precision of measurements and stability of the biosensor arrays

The relative standard deviation for 10 repeated measurements, calculated for final concentrations of 0.5 mM ATChCl, 0.5 mM glucose, 0.5 mM cellobiose and 20 μM catechol in the measuring solution, varied in the range between 1.7 and 6.7% depending on the enzyme electrode used. With regards to the storage stability, the enzyme electrodes lost up to 20% of their activity on the second day, 50% on the fifth day (except TYR which was more stable and lost only 30%), and on the seventh day only 20% of the initial enzyme activity remained (tyrosinase 50%). The time required for one analysis was equal to the sum of the times necessary to establish a stable steady state current after substrate or sample additions together with the time interval needed to receive a reliable determination of d*I*/d*t* for relative pesticide inhibition (around 5 min).

Table 1
Limits of detection (LOD) and sensitivities for carbaryl, heptenophos, fenitrothion, dichlorvos and phosphamide determined with the ChE based. Acetylthiocholine chloride was used as substrate for the ChEs. The relative standard deviations for limits of detection for AChE a BChE were in the ranges 2.1–5.9% and 3.4–7.8%, respectively

Pesticide	AChE		BChE	
	LOD (nM)	Sensitivity ($s^{-1} mol^{-1} l$)	LOD (nM)	Sensitivity ($s^{-1} mol^{-1} l$)
Carbaryl	0.80	100	93	34
Heptenophos	9.2	10	2.8	137
Fenitrothion	85	4.2	6.9	68
Dichlorvos	77	14	14	51
Phosphamide	130	3.4	2390	4.8

Table 2
Maximum current (I_{max}), apparent Michaelis constant (K_m^{app}) (obtained from the calibration curves of the tested model phenols), limit of detection (LOD, S/N = 3) and sensitivity (I_{max}/K_m^{app}) determined through amperometric steady state measurements with TYR, HRP/GOx, SBP/GOx and CDH immobilised in one sensor array

Enzyme	Phenols	p-Aminophenol	p-Cresol	Phenol	p-Chlorophenol	Catechol
I_{max} (nA)	TYR	104	658	1169	1378	1971
	HRP	611	131	60	29	222
	SBP	472	40	n.o.[a]	n.o.	200
	CDH	333	n.o.	n.o.	n.o.	178
K_m^{app} (μM)	TYR	80	71	121	89	284
	HRP	106	43	13	3.4	36
	SBP	93	167	n.o.	n.o.	82
	CDH	148	n.o.	n.o.	n.o.	125
Sensitivity (mA M^{-1})	TYR	1.3	9.3	9.7	16	6.9
	HRP	5.8	3.0	6.4	8.5	6.2
	SBP	5.1	0.24	n.o.	n.o.	2.4
	CDH	2.3	n.o.	n.o.	n.o.	1.4
LOD (μM)	TYR	2.4	0.33	0.41	0.19	0.43
	HRP	0.74	1.0	1.8	0.39	0.41
	SBP	0.60	14	n.o.	n.o.	1.2
	CDH	1.4	n.o.	n.o.	n.o.	2.1

Glucose and cellobiose were used as substrates for GOx and CDH. Applied potentials: -100 mV (TYR, HRP, SBP) and $+400$ mV (CDH) vs. Ag/AgCl. The values were calculated as the average of three measurements and the relative standard deviations for I_{max} were in the range 4.7–18%, for K_m^{app} within 7.3–17% and for limits of detection within 1.6–10%.

[a] Not observed.

3.5. Sample analysis

3.5.1. Steady state measurement of samples

The developed biosensor array was further tested for industrial samples potentially containing phenols and pesticides. A total number of 43 samples from a pulp and paper industry (two subsets of 15 samples each) and a pesticide industry (13 samples) were analysed. The working buffer containing 0.5 mM ATChCl, 0.5 mM glucose, 0.5 mM cellobiose and 15 μM catechol was used as a standard. After measurements, all signals were divided by the signals of the standard and corrected with the responses received from the blank sample (Milli Q water); the relative response values were calculated in this way. It was found that most of the samples responded at least on one of the sensing enzyme electrodes. Table 4 summarises the relative responses from the biosensor array obtained with industrial samples.

The CDH immobilised electrode responded only to one sample from the pulp and paper industry (A10). In general,

the pulp and paper industry samples affected mainly the POD based electrodes. U-group samples (untreated wastewater) influenced HRP and TYR sensors more compared to group A samples (alert wastewater). Some of the A-group samples provided responses on the cholinesterase-based sensors (especially with AChE). However, this effect did not appear with high probability due to the presence of pesticides in samples but may be due to other undefined compounds. According to different enzymes with variable substrate specificity, it might be possible to eliminate presence of some substrates (at least at detectable levels), e.g. monohydroxy or o-dihydroxybenzene and quinone derivatives that should appear at the CDH modified electrode. On the other hand, not only $2e^-$ acceptors as quinones play role in the CDH reactions, but as well $1e^-$ acceptors, e.g. ferricyanide, ferricitrate, Cu^{2+} ion, tetramethylbenzene and 4-aminopyridine cation radicals [35]. PODs responded to a wide range of substrates (phenols, biphenols, anilines, benzidines, polyphenols, ascorbate, iodide), which can have interference effects if present

Table 3
Relative responses for p-aminophenol, p-cresol, phenol and p-chlorophenol with respect to catechol calculated from the I_{max} values from TYR, CDH, HRP/GOx, and SBP/GOx sensors

Enzyme based electrode	Relative response (%)				
	Catechol	p-Aminophenol	p-Cresol	Phenol	p-Chlorophenol
TYR	100	5.3	33	59	70
HRP	100	276	59	27	14
SBP	100	236	20	n.o.[a]	n.o.
CDH	100	119	n.o.	n.o.	n.o.

The calculated relative standard deviations were below 10%.

[a] Not observed.

Table 4
Relative responses (%), received from division of steady state responses for samples by the corresponding response from a standard and with subtracted blank sample responses (Milli Q water), from the enzyme modified array determined for undiluted samples from pulp and paper (P&P) and pesticide industries

Samples P&P	Relative response (%)					
	CDH	HRP	SBP	TYR	AChE	BChE
A1	<1	2.2	86	<1	<1	<1
A2	<1	<1	<1	<1	<1	<1
A3	<1	<1	<1	<1	<1	<1
A4	<1	<1	<1	<1	<1	<1
A5	<1	<1	15	<1	<1	<1
A6	<1	6.9	19	<1	<1	<1
A7	<1	<1	34	<1	<1	<1
A8	<1	<1	<1	<1	<1	<1
A9	<1	<1	<1	<1	16	63
A10	15	9.6	17	<1	1.5	<1
A11	<1	4.3	9.4	<1	5.4	<1
A12	<1	<1	<1	<1	<1.	<1
A13	<1	<1	12	<1	9.6	<1
A14	<1	<1	40	<1	6.3	<1
A15	<1	<1	13	<1	48	<1
U1	<1	<1	<1	<1	<1	<1
U2	<1	40	57	6.7	<1	<1
U3	<1	44	24	<1	<1	<1
U4	<1	31	42	<1	<1	<1
U5	<1	<1	<1	250	<1	<1
U6	<1	47	36	<1	<1	<1
U7	<1	46	33	<1	<1	<1
U8	<1	377	<1	<1	<1	<1
U9	<1	25	<1	<1	<1	<1
U10	<1	32	86	<1	<1	<1
U11	<1	<1	<1	<1	<1	<1
U12	<1	<1	<1	198	<1	<1
U13	<1	<1	<1	<1	<1	<1
U14	<1	21	<1	37	<1	<1
U15	<1	10	<1	9.9	<1	<1
Pesticides						
P1	<1	<1	<1	<1	29	100
P2	<1	<1	<1	<1	19	<1
P3	<1	<1	<1	<1	46	5.2
P4	<1	<1	<1	<1	100	<1
P5	<1	<1	<1	<1	<1	<1
P6	<1	<1	<1	<1	39	20
P7	<1	<1	<1	<1	29	9.5
P8	<1	<1	<1	<1	<1	<1
P9	<1	<1	<1	<1	38	100
P10	<1	<1	<1	<1	15	<1
P11	<1	<1	38	<1	18	<1
P12	<1	<1	<1	<1	20	<1
P13	<1	<1	<1	<1	33	<1

Catechol (15 μM) and ATChCl (0.5 mM) served as initial standards.

in the samples. However, considering origin of the samples (pulp and paper industry), the most reliable group seems to be heteroaromatic compounds resulting from lignin degradation (e.g. coniferyl alcohol, *p*-coumaryl alcohol). One must also take into account that the true sample composition was unknown and thus influence from the matrix as well as other undefined components can affect the total response remarkably.

The pesticide wastewater samples affected the ChE electrodes, however, without any visible effect on the oxidoreductase-immobilised electrodes (except sample P11 providing responses even on the TYR electrode). Thus, the inhibition can be partially due to the presence of some heavy metal ions resulting in the decrease of cholinesterase activity. Even if considering only the relative responses of the enzyme sensors to samples, the pulp and paper industry samples (U-group) were clearly separated from the pesticide samples (P). This clearly appears when one focuses on ChEs versus POD and TYR responses. Due to the complexity of the obtained responses, chemometric multivariate analysis was used as a tool providing additional useful information, i.e. better qualitative resolution of different sample groups, as will be discussed below.

3.5.2. Chemometric analysis of samples data

Principal component analysis (PCA) can provide additional possibilities for investigation the measured data sets. The result of raw data matrix processing is shown in Fig. 5 as a PC1 versus PC2 plot (score plot). This two-dimensional projection accounts for 90% of the data variance and indicates three separate object groups. PC1 describes differences between the origin of the samples with pesticide-based ones (P) placed along the negative part and P&P wastewaters (A and U) situated on the positive side of the first principal component. Variation within each of the three different types of water seems to be described by PC2. The U samples are situated in the more positive parts of PC2 compared to the A-group. According to the presumption that U-samples probably have higher toxicity levels than samples from the group A, the PC2 component might reflect the toxicity content in the samples. The high discrimination of the samples according to their origin (described by PC1 in Fig. 5) would be hard or even impossible to conclude based only on the knowledge of the responses from the enzyme sensors summarised in Table 4. Thus, the score plot clearly facilitates to visualise the differences between the samples.

Another aspect of the information derived from PCA is how important particular response variables (i.e., the sensors) are for each PC in the model. These properties can be conveniently displayed in the loading plot. The relationship between objects (samples) and variables (responses) and how much they influence the system are often best illustrated in a score-loading bi-plot. This plot includes the score coordinates as well as the corresponding loading values. To more easily visualise the relationship between measured samples and the variables derived from each enzyme sensor in the array, the raw data matrix was simplified. Instead of using 810 variables to characterise response on the enzyme sensor, as was done when creating the model in Fig. 5, only one value was taken from the signal steady state plateau after sample addition and it was corrected to the mean value of the standard response. The score-loading bi-plot (PC1 and PC2 describe 74% of the data variance) in Fig. 6 shows that the scores for the three different groups of samples (P, A and U) are still
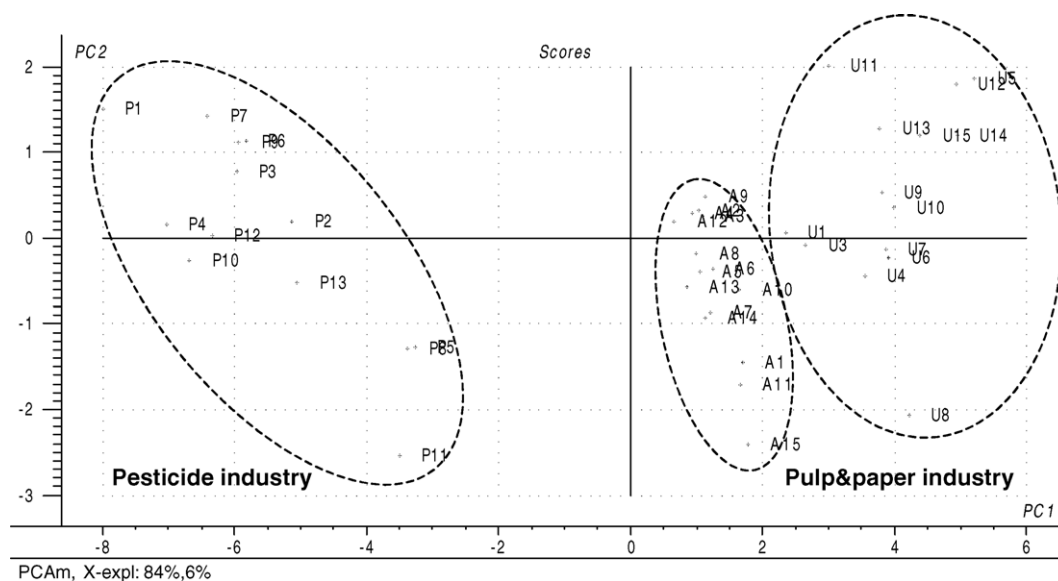
Fig. 5. PCA score plot for the first two principal components, which explain 90% of the total variation in data, obtained from environmental samples. Each sample corresponds to 6830 response variables detected by six enzyme-based sensors (each sensor responses are built of 810 variables). P represents pesticide samples whereas A and U samples represent two subsets of P&P wastewater varying in toxicity content.

clearly separated from each other, but compared to the previous score plot based on whole sample responses (Fig. 5) the groups are more tightly situated around the origin and it may be possible that important information hidden under other variables has been excluded. Three pesticide samples (P5, P8 and P11) were after PCA analysis placed into group A of pulp and paper samples (marked with arrows in Fig. 6). This deviation can be explained according to the results summarised in Table 4 (based only on one steady state response

variable for each sensor in the array) where these samples seem to be outliers of the pesticide sample group. Samples P5 and P8 did not distinguish themselves with any inhibition effect on the ChE sensors and for sample P11; some response was found on the TYR sensor.

Considering Table 4, the POD sensors (HRP/GOx and SBP/GOx) responded strongly to P&P samples. The sample group A was more influenced by SBP/GOx whereas HRP/GOx responded slightly better to the sample group U.
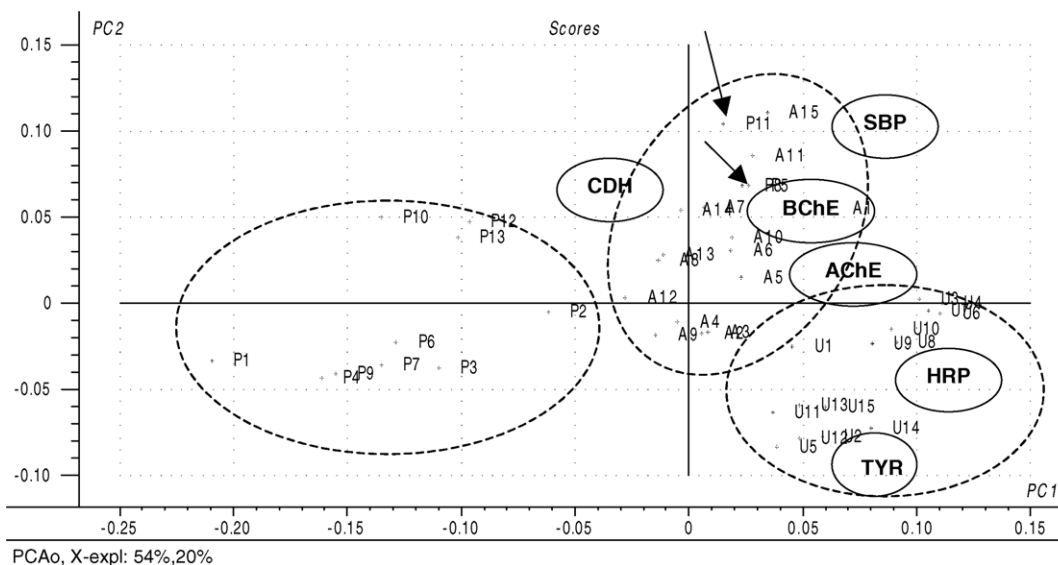

Fig. 6. PC1 vs. PC2 score-loading bi-plot that characterises the relationship between tested samples and response variables. The raw matrix was built from six variables; each represents the steady state response of one sensor corrected according to a standard response. P represents pesticide samples whereas A and U samples represent two subsets of P&P wastewater. The pesticide samples included in the cluster of pulp and paper industry subset A samples are marked with arrows.

These relations are clearly shown in the score-loading bi-plot (Fig. 6). Furthermore, as shown in Fig. 6 and Table 4, the influence of the AChE and BChE sensors on sample group A was evident, and the relation between the TYR sensor and the U-marked samples was visible, too. The CDH sensor only responded to one sample (sample A10), which resulted in a high influence on the A-marked samples indicated in the score-loading bi-plot (Fig. 6). However, it is difficult to evaluate from these experiments whether this response was an outlier, which can be excluded from the analysis, or significant. Furthermore, Table 4 shows that the pesticide samples more or less provided responses only on the pesticide-sensitive cholinesterase sensors. In Fig. 6, this effect can be seen as a negative correlation between the P-scores and the AChE and BChE loadings along PC1.

4. Conclusions

The first part of the present work proved the high potential of developing multienzyme array systems that can be used for detection of phenolic and pesticides compounds. The lowest limits of detection were determined as 0.80 nM carbaryl (AChE immobilised sensor), 2.8 nM heptenophos (BChE sensor), 0.41 μM catechol with the HRP/GOx electrode, 0.6 μM p-aminophenol with the SBP/GOx electrode and finally 0.19 μM p-chlorophenol, 0.41 μM phenol and 0.33 μM p-cresol with the TYR immobilised electrode. The achieved relative standard deviations were below 7% for 10 repeated measurements.

The second part of this contribution demonstrated successfully sensor array applications for characterisation of samples with the possibility to reach additional information using chemometric techniques. This approach represents a useful improvement of the performance of biosensor arrays and provides additional helpful information about complex samples. The obtained results show that the use of such multienzyme arrays with chemometric analysis can be a promising approach for simple, fast, reproducible, selective and sensitive detection of phenolic and pesticide substances in environmental samples and provides an initial qualitative overview about sample compositions. Finally it should be stressed that sensor arrays together with pattern recognition is often used to predict the quality of a sample without providing exact data in terms of composition and concentrations. In a later stage, classification of new objects is planned, e.g. results from other samples using these enzyme-modified electrodes, and quantitative determinations using reference values received from conventional toxicity tests.

From the practical point of view, the multienzyme arrays currently provide rather short operational lifetime and therefore application as disposable sensing devices might simplify the required recalibration procedures. The large-scale production providing affordable screen-printed sensors (around €5 per 1 eight-electrode strip) with sufficient reproducibility will justify the single-use of such biosensors. Further-

more, some portable device incorporating the multivariate data analysis for quick sample characterisation will be highly useful for field measurements.

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