Fişa suspiciunii de plagiat / Sheet of plagiarism's suspicion	Indexat la: 76/05

Opera suspicionată (OS) Suspicious work	Opera autentică (OA) Authentic work

OS	Ciucu, A., Ciucu, C., Organic Phase Amperometric Biosensor for Detection of Pesticides, In**:** *Roum. Biotechnol. Lett.,* Vol. 7, No. 2, 2002, pp. 667-676.
OA	Wilkins E., Carter M., Voss, J., Ivnitski D., A quantitative determination of organophosphate pesticides in organic solvents, In: *Electrochemistry Communications*, Vol.2, Issue 11, Nov. 2000, pag.786–790.

Incidenţa minimă a suspiciunii / Minimum incidence of suspicion	
p.667:8 – p.667:19	p.786:8 – p.786:16
p.667:21 – p.667:32	p.786:1s – p.786:16s
p.668:29 – p.668:39	p.786: 16d – p.787:7s
p.669: 9 – p.670: 22	p.787:19s – p.788:37
p.671: Figure 2	p.788: Fig.1
p.672: Figure 3	p.789: Fig.3
p.673: Figure 4	p.789: Fig.2
p.674: Figure 5	p.790: Fig.4

ELSEVIER

Electrochemistry Communications 2 (2000) 786–790

electrochemistry communications

www.elsevier.nl/locate/elecom

A quantitative determination of organophosphate pesticides in organic solvents

E. Wilkins [a,*], M. Carter [a], J. Voss [a], D. Ivnitski [b]

[a] *Department of Chemical & Nuclear Engineering, University of New Mexico, 209 Farris Engineering Center, Albuquerque, NM 87131, USA*
[b] *New Mexico Engineering Research Institute, University of New Mexico, University Blvd., Albuquerque, NM 87106, USA*

Received 17 July 2000; received in revised form 6 September 2000; accepted 11 September 2000

Abstract

An amperometric acetylcholinesterase (AChE) biosensor based on thiocholine-hexacyanoferrate reaction was developed for the analysis of OPCs in pure organic solvents. The enzyme (AChE) was co-immobilized with an electron mediator, Prussian Blue, on the surface of a graphite electrode. The effect of organic solvents on acetylcholinesterase activity was estimated in the presence of polar (hydrophilic) and non-polar (hydrophobic) organic solvents in the range of 0.01–100%. The ability of the AChE biosensor to detect pesticides was demonstrated by quantitative determination of dichlorvos, fenthion and diazinon in ethanol solvent. The assay allows determination of OPCs in sub-micromolar concentration ranges with an overall assay time of 10 minutes. The sensing elements of the amperometric AChE biosensor can be stored in dry state for more than 2 months. The AChE biosensor possesses distinct advantages, including monitoring of hydrophobic substrates, elimination of microbial contamination, and relative ease of enzyme immobilization. Potential application areas include food analysis and environmental monitoring.

Keywords: Amperometric biosensor; Acteylcholinesterase; Prussian Blue; Pesticides

1. Introduction

Pesticides (herbicides, fungicides, insecticides) are widely used throughout the world, and millions of tones are used each year in agriculture, medicine and industry. Many of them are highly toxic and their accumulation in living organisms can be cause of serious diseases. Because similar compounds were produced as possible nerve poisons a further area of application is in the military. The destruction of OPC-based chemical weapons mandated by international agreements or as part of routine operations also leads to problems in environmental control and protection. Pollutants of this type are found to be present in many sampled soils, ground and waste-waters streams. One of the most important preventive measures in this case is to rapidly determine the source of the pollutant and the magnitude of the threat using on-site measurements. The pesticide assay in real samples requires the stage of extraction from water samples, soils, ground and pre-concentration because of relatively low concentration of target analytes and hydrophobic nature of pesticides [1]. This is usually accomplished using organic solvents, such as methanol, acetone, acetonitrile, or hexane or combining them with solid-phase extraction [1–4]. In this respect, organic phase enzyme sensors (OPES) capable of measuring directly in the organic extract would be well suited particularly for the rapid analysis of pesticides without further sample processing [5–11]. The advantages of using organic phase enzyme sensors are summarized in some review articles [10–13].

The aim of our research is to develop a rapid, simple, and sensitive amperometric method for direct measurement of OPCs in organic solvent. Our strategy is based on using of the following signal-amplification systems: (1) the co-immobilization of redox mediators (Prussian Blue) and AChE on the electrode surface; (2) the accumulation of the product of enzymatic and electrochemical reactions at the membrane/ electrode interface; (3) the cyclic regeneration of the redox mediators at the electrode surface. The follow-

* Corresponding author. Tel.: (505) 277-2928; fax: (505) 277-5433.
E-mail addresses: wilkins@unm.edu (E. Wilkins), ivnitski@nmeri.unm.edu (D. Ivnitski).

1388-2481/00/$ - see front matter
PII: S1388-2481(00)00122-3

ing reactions describe the principle of amperometric detection of AChE activity:

$$(CH_3)_3N^+CH_2CH_2SCOCH_3 + H_2O \xrightarrow{AChE} (CH_3)_3N^+CH_2CH_2SH + CH_3COOH \quad (1)$$

$$2(CH_3)_3N^+CH_2CH_2S^- + 2[Fe(CN)_6]^{3-} \rightarrow (CH_3)_3N^+CH_2CH_2SSCH_2CH_2N^+(CH_3)_3 + 2[Fe(CN)_6]^{4-} \quad (2)$$

$$[Fe(CN)_6]^{4-} - e^- \xrightarrow{electrode} [Fe(CN)_6]^{3-} \quad (3)$$

Thiocholine, produced by enzymatic hydrolysis of acetylthiocholine (ATCh), reacts stoichiometrically with hexacyanoferrate (III). Then, the reduced electron mediator is reoxidized at the graphite electrode and the analytical signal is measured amperometrically.

2. Materials and methods

2.1. Materials

Purified acetylcholinesterase (AChE, EC 3.1.1.7, 950 $IUmg^{-1}$ from electric eel), acetylthioholine (ATCh) chloride, polyethylenimine (PEI) and glutaraldehyde (25% aqueous solution) were purchased from Sigma Chemical Company. The pesticides used in the tests were as follows: diazinon, fenthion, dichlorvos were purchased from Chem Service, Inc., West Chester, PA. Acetone, benzene, ethanol, propanol and butanol (US Industrial Chemicals) were used without further purification. Other chemicals of analytical grade were obtained from the standard sources.

2.2. Preparation of polyethylenimine Prussian Blue (PEI-PB) modified electrodes

Graphite rods (made from pencil lead, HB 0.9 mm) were cleaned in methanol, rinsed with double-distilled water, and dried. The cellulose acetate film (from a 45% w/v acetone solution) was used for insulating of graphite rods except for 0.1 cm (one end designed as a working electrode surface) and 1 cm (the other end left for current collecting). PEI-PB films were deposited on the exposed 0.1 cm^2 working section of the electrode surface. The working electrode was dipped for 15 min in a 0.2% methanol solution of PEI and air-dried for 8 h. The electrode surface was washed with methanol to remove excess unbound polymer. A fresh solution of 0.02 M $FeCl_3$ and 0.02 M of $K_3Fe(CN)_6$ was prepared in distilled water. The electrode was dipped in the ferric-ferricianide solution and was cathodically polarized for 15 min under galvanostatic conditions with current density of 40 $\mu A/cm^2$ [14]. Cyclic voltammetric experiments were performed in a three-electrode cell with counter (Pt) and a commercial Ag/AgCl reference electrodes and EG & G PAR 273 potentiostate interfaced to a PC computer system with PAR M270 software. The rotation of the graphite electrode was performed with a Pine Instruments rotator and with an MSRS speed controller. The electrochemistry of the electrodes covered by the thin film of PB was examined in an acidic 1 M KCl solution (pH 4.0).

2.3. AChE sensor design

AChE was immobilized covalently on PEI-PB-coated graphite electrodes [15] and stored dry in a refrigerator (4^0C) when not in use. AChE modified graphite electrodes with co-immobilized PEI and PB (AChE-PB-PEI electrodes) were used as sensing elements of the sensor. The enzyme electrodes can be stored dry for 2 months at 4°C. All electrochemical measurements were performed in the three-electrode cell ($V = 0.3$ ml) with a rotating AChE-PB-PEI modified working electrode, a graphite ink counter and Ag/AgCl ink reference electrodes. The activity of AChE on the electrode surface was estimated at the initial rate of reduction of hexacyanoferrate (III) and was determined spectrophotometrically at 420 nm [16].

2.4. Determination of pesticides in organic solvents

The AChE-PB-PEI modified electrodes have been used for measurement of fenthion, diazinon and dichlorvos in ethanol solutions. Stock pesticide solutions were prepared by withdrawing 10 μl of the pesticide and then placing its in a vial and weighting. It was then made up to a final volume of 10.0 ml with a pure ethanol solvent. The standard concentrations of pesticides were prepared daily in ethanol solvent. The kinetic approach was used for determination of pesticides. The AChE-PB-PEI modified electrode was immersed into electrochemical cell with 0.3 ml 0.1 M phosphate buffer (pH 7.5) containing 0.1M KCl, 0.01 mM $MgCl_2$ and stirred at 300 rpm. The potential of AChE-PB-PEI electrode is set at +350 mV versus Ag/AgCl. Then 0.01 ml of pesticide in pure ethanol solvent (or only ethanol) is injected and a background signal was recorded. After the current has stabilized (10 min), the 0.2 mM ATCh is added, and a second stationary current state is reached. The percent inhibition was calculated using formula (1):

$$I\% = (i_1 - i_2)/i_1 \times 100 \quad (4)$$

where $I\%$ is the degree of inhibition, i_1 is the steady-state current obtained in the presence of only ethanol and i_2 is the steady-state current obtained in the presence of pesti-

cide in the sample. A steady-state responses were obtained after 10 seconds.

3. Results and discussion

The determinant factors in the development of the fast and sensitive AChE-sensor are the method of the enzyme immobilization and microenvironment for mediated electron transfer from enzyme to the electrode surface. A variety of methods for enzyme immobilization, such as crosslinking of enzyme by bifunctional reagents, covalent binding and entrapment in a suitable metrices have been employed [17]. In this work, we have used a bifunctional reagent, glutaraldehyde, as the cross-linking agent for immobilization of AChE on the electrode surface [18]. Advantage of this technique is strong chemical binding of the biomolecules. The polymeric nature of glutaraldehyde provides attaching the enzyme to the electrode surface, which may permit greater flexibility for protein conformational changes required for enzyme activity. In order to define the optimal conditions for immobilization of AChE on the electrode surface, the relationship between the magnitude of the analytical signal, concentration of polyethylenimine (PEI) and glutaraldehyde have been studied and converted by RS-1 software into Eq. (5) [19,20]:

$$I = 288 - \{[286(A - 60)]/30 + [155(B - 10)]/5\} \quad (5)$$

where I is response of the AChE electrode (nA), A and B are the concentrations of the PEI (%) and glutaraldehyde (GA) (%), consequently. AChE (1 mg/ml) for immobilization and ATCh (1.2 mM) for measurement were used at fixed concentrations. The effect of a glutaraldehyde concentration was studied in the range from 0.1% to 5%. We found that for a glutaraldehyde (GA) concentration ranging from 0.5% to 3.0% the activity of immobilized AChE is almost constant. For a GA concentration higher then 3.0%, the activity of AChE decreases. The advantages of glutaraldehyde for AChE immobilization on the PEI-PB electrode surface are simple, rapid and this technique is suitable for manufacturing AChE electrodes with the same enzyme activities.

Different methods have been developed in an attempt to overcome the problem of electron communication between the electrode and the enzyme membrane [21]. One possible approach involves a combination of biosensors with electron mediators that mediate electron transfer between the enzyme and the electrode. This demands a close contact between the enzyme molecules, the mediator and the electrode. In this respect, amperometric detection of the acetyl- or butyrylthiocholine hydrolysis process, catalyzed by AChE on the electrode surface is very attractive [2,22,23]. Thiocholine, the product of the enzyme reaction, can be anodically oxidized on an electrode surface. However, the large over voltage (700 mV versus Ag/AgCl) makes acetyl- or butyrylthiocholine chloride or iodide as a substrate inconvenient for this purpose [23]. This problem may be overcome by the use of chemical modified electrodes [24–27]. For example, immobilization of Prussian Blue (PB) film on the electrode surface has found considerable application as redox mediator [25–30]. The reasons for employing Prussian Blue (PB) films include their simple preparation and their potential for applications in aqueous and non-aqueous media [25,26,30]. In addition, this redox system has the advantages of a well-defined electron stoichiometry, insensitivity of the mediation reaction to changes in pH and ionic strength, and a high value of the rate constant for electron transfer between the enzyme and the electrode.

In our work the positively charged PEI was used in the biosensor design not only for immobilizing the AChE, but also for accumulating and creating a high local concentration of negative charged products of enzymatic and electrochemical reactions within an electrode surface. Fig. 1 illustrates the cyclic voltammograms of a PB film on a bare and a PEI-PB modified graphite electrodes. We found that the rate of electron transfer is significantly enhanced at the PEI-PB modified electrode that on a bare graphite electrode. The same amplification effect, the enhanced response to ATCh, was obtained also in the case of AChE-PEI-PB modified electrodes (Fig. 2). The AChE-PEI-PB modified electrode exhibits nearly ten times better sensitivity (curve 1) than the AChE electrode without PEI-PB films (curve 2). Responses of the AChE-PEI-PB electrode were measured over the concentration range 0–7 mM of ATCh chloride, at an applied potential of 350 mV versus Ag/AgCl. The relationship between the AChE-PEI-PB electrode response and the value of ATCh concentration was linear in the range 0–900 μM ATCh. The response time was one min. Several factors may contribute to this amplification: (1) The co-immobilization of PEI and the redox mediators on the electrode surface. (2) Positively charged PEI film on the electrode surface is effective not only for immobilizing the AChE, but also for accumulating

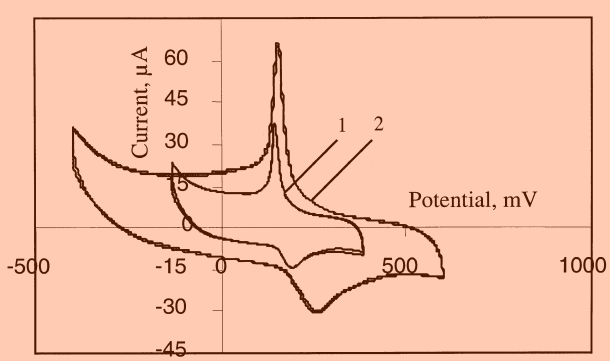

Fig. 1. Cyclic voltammograms of (1) PB and (2) PEI-PB modified graphite electrodes in 0.5 M KCl solution, 50 mV s^{-1}, 300 rpm, 20°C.

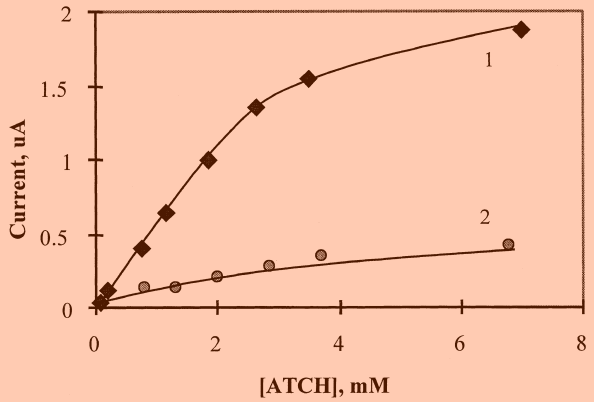

Fig. 2. Calibration plots for the ATCh in 0.1 M phosphate buffer (pH 7.5) containing 10% water–ethanol mixture, 0.1 M KCl, 0.01 mM $MgCl_2$ and stirred at 300 rpm. Potential is 0.35 V versus Ag/AgCl; 20°C. 1) AChE-PB-PEI modified electrode; 2) AChE-modified electrode.

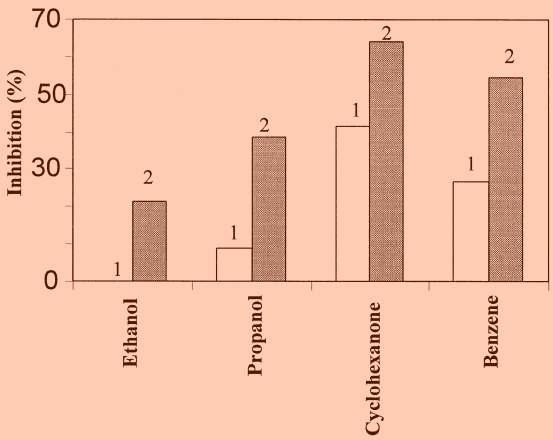

Fig. 3. Inhibition of acetilcholinesterase on the electrode surface after a 10-minute incubation in (1) 10% water–organic solvent mixture and (2) pure organic solvent.

and creating a high local concentration of products of enzymatic and electrochemical reactions within an electrode surface. (3) The permanent electrochemical regeneration of hexacyanoferrate (III) ions within the diffusion layer.

The short diffusion distance on the interface of PEI/PB/electrode permits a rapid diffusion of the electroactive mediators to the electrode surface. Thus, the co-immobilization of the positively charged PEI in the PB layer changes the behavior of the working electrode dramatically. The best results where obtained with 0.2% PEI.

The effect of organic solvents on activity of AChE immobilized on the electrode surface have been studied in the presence of polar and non-polar organic solvents in the range of 0.01–100%. The response of AChE-PEI-PB electrode was measured in 0.1M phosphate buffer, pH 7.5, and 0.1 M KCl, in the presence of a fixed concentration of ATCh, before and after the working electrode was incubated for 10 min in aqueous–solvent mixtures or pure organic solvents. The percent inhibition was calculated using the formula: $I\% = (I_1 - I_2)/I_1 \times 100$, where $I\%$ is the degree of inhibition, I_1 is the steady-state current obtained in buffer solution, and I_2 is the steady-state current obtained after the working electrode was 10 min incubated in organic solvent. Studies of AChE stability on the electrode surface in different organic solvents revealed that exposure of AChE electrodes to benzene or cyclohexanone the catalytic activity of the AChE is markedly decreased (Fig. 3). However, the AChE-PEI-PB electrode can operate in the buffer solution in the presence of 0.1%–10% polar solvents (ethanol or propanol). We found that the activity of AChE on the electrode surface in mixture of the water solution with 0.1%–10% of ethanol, was higher than in pure aqueous solution. The same effect was described also for alcohol dehydrogenase and other enzymes in the water–ethanol mixture [11,31]. With the right amount of water and polar organic solvent, lowering of the dielectric constant of the enzyme active site microenvironment can probably be manipulated to obtain an enhanced sensor response compared to responses in the aqueous phase [11]. However, in water free polar solvent the enzyme on the electrode surface is inactivated. Therefore, for the assay of pesticides in pure ethanol solvent we used a kinetic approach, which was described in the Material and methods section.

For the determination of pesticides in our experiments ethanol was used as an extraction organic solvent. The advantage of using ethanol as organic solvent lies in a good solubility of ATCh and pesticides in the solvent and also in causing the need to avoid the inactivation effect of the solvent on biosensor response. Fig. 4 shows the result of a quantitative determination of dichlorvos, diazinon and fenthion in ethanol obtained by the kinetic mode. Data are recorded in terms of percentage inhibition as a function of the inhibitor concentration. Standard deviation is repre-

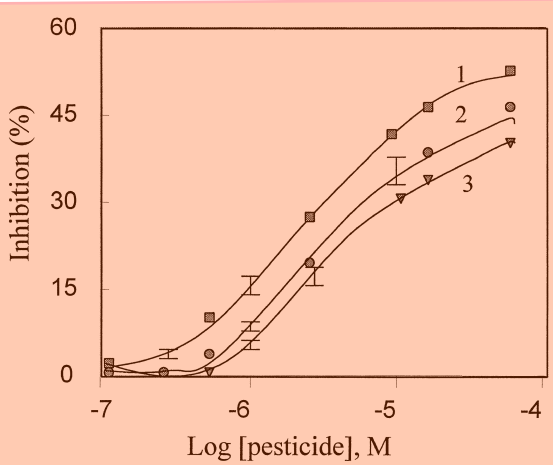

Fig. 4. Determination of pesticides with the AChE-PB-PEI modified electrode. Experimental conditions: 0.01 ml of pesticide in pure ethanol solvent was injected into electrochemical cell with 0.3 ml 0.1M phosphate buffer, pH 7.5, containing 0.1 M KCl, 0.01 mM $MgCl_2$ and 0.1 mM ATCh at 300 rpm and at 20°C. (1) Dichlorvos; (2) Diazinon; (3) Fenthion. Error bars denote deviations for $N = 5$. Technique of measurement is the kinetic approach.

sented as an error bar. The coefficient of variation of the blank measurements and inhibition measurements were found to be 5.8% and 6.3%, respectively. The lowest detection limits for dichlorvos, diazinon, and fenthion are 0.5×10^{-6} M, 0.8×10^{-6} M, and 1.0×10^{-6} M (signal-to-noise ration = 4), respectively. Unlike potentiometric AChE sensors, which based on pH measurement, the response of the amperometric AChE sensor increases with increasing of the buffer concentration. AChE electrodes are stable over at least 60 days at 4°C when stored under dry conditions. After 60 days the retained activity was 65% of the original activity.

4. Conclusion

Our results demonstrate the potentiality of the amperometric AChE biosensor based on disposable AChE-PB-PEI modified graphite electrode for the detection of organophosphate pesticides in polar organic solvent. Co-immobilization of AChE, PEI and PB on the electrode surface provides reagentless determination of pesticides with high sensitivity and fast. Unlike the pH-sensitive AChE sensors described in the literature, this solid-state AChE sensor is operated at high ionic strength and high buffer capacity. The substrate sensitivity of the AChE sensor based on the ATCh–hexacyanoferrate system in six times higher than that of the potentiometric AchE sensors [32]. The sensing elements can be mass-produced at low cost. The technique of a quantitative determination of OPCs in ethanol is simple and the complete assay is carried out in 10 min. The biosensor proposed here can be easily miniaturized and transformed to the multi-channel flow-injection monitoring instrument. Application of the AChE-PEI-PB sensor may be in clinical laboratory, food industry and for environmental monitoring where analytes of interest have poor water solubility or where existing methods lack sensitivity.

Acknowledgements

This work was supported by the US Department of Energy, through the WERC, a Consortium for Environmental Education and Technology Development. The authors thank Dr. Cecchi of the University of New Mexico for collaboration and support in the software program.

References

[1] N. Mionetto, J.-L. Marty, I. Karube, Biosensors & Bioelectronics 9 (1994) 463.
[2] A.L. Hart, W.A. Collier, D. Janssen, Biosensors & Bioelectronics 12 (1997) 645.
[3] L. Campanella, G. Favero, M.P. Sammarintino, M. Tomassetti, Anal. Chim. Acta 393 (1999) 109.
[4] L. Campanella, S. De Luca, M.P. Sammarintino, M. Tomassetti, Anal. Chim. Acta (1999) 59.
[5] L. Campanella, M.P. Sammarintino, M. Tomassetti, Sensors & Actuators B 7 (1992) 383.
[6] L. Campanella, G. Favero, M.P. Sammarintino, M. Tomassetti, Talanta 46 (1998) 595.
[7] A.M. Klibanov, Chemtech 16 (1986) 354.
[8] G.F. Hall, D.J. Best, A.P.F. Turner, Anal. Chim. Acta 213 (1988) 113.
[9] J. Wang, Y. Lin, Anal. Chim. Acta 271 (1993) 53.
[10] S. Saini, G.F. Hall, A.P.F. Turner, Anal. Chim. Acta 249 (1991) 1.
[11] E.I. Iwuoha, M.R. Smyth, M.E.G. Lyons, Biosensors and Bioelectronics 12 (1997) 53.
[12] J. Wang, Talanta 40 (1993) 1905.
[13] M.P. Connor, J. Sanchez, J. Wang, M.R. Smyth, S. Mannino, Analyst 114 (1989) 1427.
[14] K. Itaya, H. Akahoshi, S. Toshima, J. Electrochem. Soc. 129 (1982) 1498.
[15] D.M. Ivnitski, J. Rishpon, Biosensors & Bioelectronics 9 (1994) 569.
[16] H.J. Cohen, I. Fridovich, J. Biol. Chem. 246 (1971) 359.
[17] K. Mosbach (Ed.), Immobilized enzymes and cells, Methods in Enzymology, vol. 137, Part D, 1988 p. 767, Acad. Press, NY.
[18] Espinosa M, Atanasov P, E. Wilkins, Electroanalysis 11 (1999) 1055.
[19] P.C. Kurs, Computer Software Applications in Chemistry, Chp. 9, Wiley, NY, 1986, p. 125.
[20] J.L. Glajch, L.R. Snyder (eds), Computer-Assisted Method Development for High-Performance Liquid Chromatography, 1990, Elsevier, Amsterdam.
[21] M. Aizawa, Anal. Chim. Acta 250 (1991) 249.
[22] H.C. Budnikov, G.A. Evtugyn, Electroanalysis 8 (1996) 817.
[23] Q. Deng, S. Dong, Analyst 121 (1996) 1123.
[24] P. Skladal, Anal. Chim. Acta 255 (1991) 11.
[25] F. Schubert, S. Saini, A.P.F. Turner, Anal. Chim. Acta 245 (1991) 133.
[26] S.A. Jaffari, A.P. F Turner, Biosensors & Bioelectronics 12 (1997) 1.
[27] D.W. Harak, H.A. Mottola, Biosensors & Bioelectronics 6 (1991) 589.
[28] Q. Chi, S. Dong, Anal. Chim. Acta 310 (1995) 429.
[29] A.A. Karyakin, E.E. Karyakina, L. Gorton, Talanta 43 (1996) 1597.
[30] T. McCormac, J. Cassidy, D. Cameron, Electroanalysis 8 (1996) 195.
[31] A.A. Karyakin, E.E. Karyakina, L. Gorton, O.A. Bobrova, L.V. Lukachova, A.K. Gladilin, A.V. Levashov, Anal. Chem. 68 (1996) 4335.
[32] M. Trojanowicz, M.L. Hitchman, Trends Anal. Chem. 15 (1996) 38.