ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 355–361

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Voltammetric determination of lactate dehydrogenase using a carbon paste electrode

Cornelia Tarmure [a], R. Săndulescu [b,*], Corina Ionescu [a]

[a] *Biochemistry and Clinical Laboratory Department, University of Medicine and Pharmacy 'Iuliu Haţieganu', Faculty of Pharmacy, Pasteur St. no. 4, 3400 Cluj-Napoca, Romania*

[b] *Analytical Chemistry Department, University of Medicine and Pharmacy 'Iuliu Haţieganu', Faculty of Pharmacy, Pasteur St. no. 4, 3400 Cluj-Napoca, Romania*

Received 5 June 1999; received in revised form 4 November 1999; accepted 27 November 1999

Abstract

A voltammetric study was performed by linear sweep voltammetry using a carbon paste electrode, in -0.1 to $+1.3$ V potential range, with the view of elaborating an assay for lactate dehydrogenase (LDH) in different enzymatic preparations and biological fluids. There have been performed studies concerning pH influence upon the enzymatic reaction, as well as the electrochemical behavior of LDH in the presence of modified carbon paste electrodes saturated with sodium pyruvate and/or NADH. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Lactate dehydrogenase; Carbon paste electrodes; Linear sweep voltammetry

1. Introduction

Electrochemical techniques are widely used in pharmaceutical and biomedical analysis from the behavior of neurotransmitters in biological media to the kinetics of polymerization and enzymatic reactions or the determination of traces of analytes in environmental and forensic analysis [1,2].

Different types of carbon paste electrodes are still the objects of a great interest as reflected by the growing number of published papers that describe the use of these electrodes in the most varied fields of electroanalysis (including drug analysis, pollutants or biologically active molecules investigations) [3–5].

Enzymes have been used combined with different electrodes for over 25 years when Clark [6] in 1962 described the first amperometric enzyme electrode and the first enzymes used in association with a carbon paste electrode have been alcohol and lactate dehydrogenase (LDH) described by Yao and Musha in 1970 [8], NAD^+ has been fixed on the CPE through the formation of a Schiff base with n-octanealdehyde. Since then a great number of enzymes have been fixed through adsorption or other techniques on the CPE surface [9].

The first investigation concerning an enzyme mixed in a carbon paste was described by Ma-

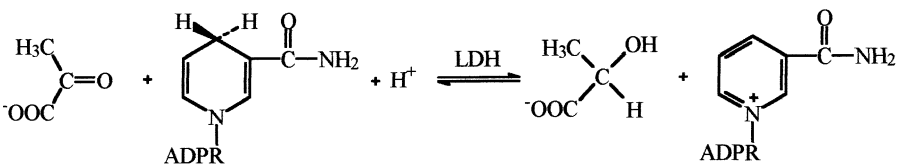
* Corresponding author. Fax: +40-64-195404.
E-mail address: rsandulescu@umfcluj.ro (R. Săndulescu)

PII: S0731-7085(99)00278-2

tuszewski and Trojanowicz in 1988 [10] and concerned glucose oxidase mixed directly in the organic phase formed of graphite powder and silicone oil. Since then a great number of works about enzyme modified CPEs, have been published [11].

LDH (EC 1.1.1.27) catalyzes the oxidation of lactate to pyruvate by direct transfer of a hydride ion from the C2 carbon of lactate to the C4 carbon of the nicotinamide ring of oxidized nicotinamide adenine dinucleotide (NAD^+). This NAD-pyruvate adduct is bound tightly to the enzyme.

LDH accelerates the oxidation of lactate by NAD^+ to pyruvate and NADH by about 10^{14}-fold according to the following scheme (ADPR, adenosine 5′-diphosphate ribose) [7].



pyruvate NADH lactate NAD^+

This reversible reaction involves the transfer of the hydride ion, H^-, from the pro-R face of NADH to the C2 carbon of pyruvate forming L-lactate and NAD^+ with a degree of fidelity approaching 100%.

The position of the internal equilibrium between bound reactants and bound products for the reaction catalyzed by LDH is near unity for both the heart and muscle isoenzymes of LDH.

Different authors [7,12–18] describe a number of modified carbon paste electrodes with LDH and other enzymes used in different configurations for the detection of lactate, pyruvate and drugs.

The purpose of this paper is the development of an electrochemical method, as an alternative to the enzyme or enzyme kinetic ones, usually performed for the determination of LDH from different matrices, especially from biological fluids. For that reason we proposed to realize a new modified CPE, specific for LDH and which can avoid the interferences in clinical samples.

2. Experimental

2.1. Apparatus

The linear sweep voltammetric study was made with a Bruker E100 potentiostat and a XY Hewlett Packard 7035 B recorder, the measurements being performed in a polarographic cell with three electrodes: a CPE as working electrode, an Ag/AgCl (3 M NaCl) reference electrode and a platinum wire as auxiliary electrode.

The electroanalytical parameters of the carbon paste prepared for this study with solid paraffin are similar to the ones of the paste prepared with liquid paraffin, the mechanical features of the last one, specially under hydrodynamic conditions being clearly inferior, that is why we decided to use only the carbon paste prepared with solid paraffin.

Samples were measured with 10, 100 and 500 μl Hamilton syringes.

The pH of the solutions was measured with a Chemcadet 5986-62 pH-meter (Cole-Parmer) using a combined glass electrode. All experiments were carried out at room temperature (22–25°C).

2.2. Reagents

All chemicals were of analytical grade (Merck or Sigma) and were used as received. The stock solutions and buffers were prepared using deionized and bidistilled water.

The carbon paste electrode (CPE) was prepared using a carbon paste made by us with solid paraffin [5,19] which was packed by pressing the matrix into the Teflon body of the electrode (1 mm i.d.). Before the measurements, the electrode surface was smoothed to a mirror finish using a clean paper card.

2.3. Preparation of modified carbon pastes

Three modified carbon pastes were realized: one with sodium pyruvate, one with NADH and the third with both sodium pyruvate and NADH.

The incorporation of the above mentioned compounds was made after the melting of solid paraffin ($t = 50$°C), the addition of exactly weighed amounts of sodium pyruvate, and/or NADH, the homogenization by mixing, then addition of the graphite and homogenizing until the resolidification of the paste.

The preparation of modified CPEs and the smoothing of the surface were made with the same technique as described in Section 2.2.

3. Results and discussion

3.1. Electrochemical study of separate partners of enzymatic reaction

A first step was to study the electrochemical behavior of the main partners of the LDH reaction system separately (i.e. sodium pyruvate, NADH and LDH) in order to establish the optimal working conditions, for each substance and also for the entire system. Intensity–potential curves of the three components have been recorded in water and 0.15 M phosphate buffer solution (pH 7.5), at room temperature (20–25°C), in a potential range from -0.1 to $+1.3$ V at different sweep rates, between 2 and 20 mV s^{-1}.

The results revealed that neither the sodium pyruvate nor the LDH show oxidation peaks in anodic polarization and in the potential range used above (Fig. 1a and b).

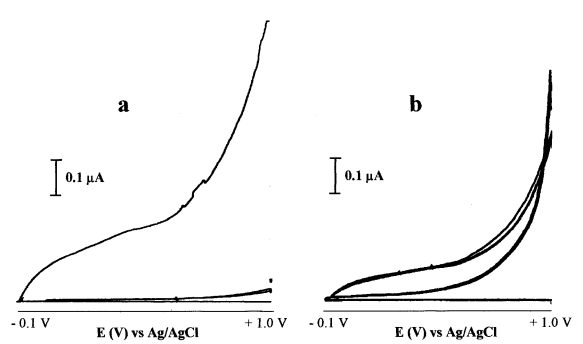


Fig. 1. Linear sweep voltammograms from -0.1 to $+1.0$ V, using CPE vs. Ag/AgCl reference electrode, 20 mV s^{-1}, 0.15 M phosphate buffer (pH 7.4). (a) 5.6×10^{-3} M pyruvate, 200 nA V^{-1}; (b) 2.35 U ml^{-1} LDH, 200 nA V^{-1}, 23°C.

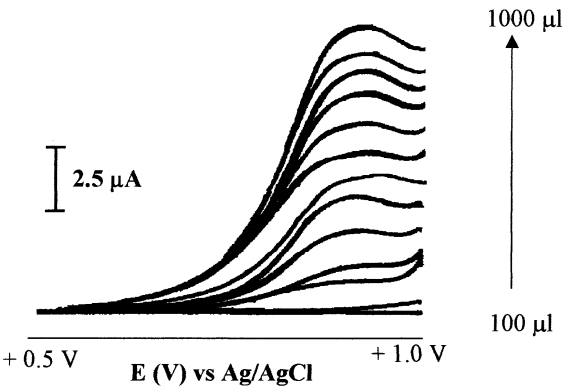


Fig. 2. Linear sweep voltammograms of 5.6×10^{-5}–5.6×10^{-4} M NADH solutions from $+0.5$ to $+1.0$ V, with CPE vs. Ag/AgCl reference electrode (20 mV s^{-1}, 5 μA V^{-1}, 0.15 M phosphate buffer).

As expected, the only oxidizable compound was NADH. The intensity–potential curves present a well defined oxidation peak at $+0.95 \pm 0.05$ V. In current response as a function of NADH concentrations, the oxidation curves have been registered using linear sweep voltammetry (same CPE surface) in 4 ml water and phosphate buffer, at room temperature 22 ± 1°C, by successively spiking samples of 100–1000 μl 2.25 mM NADH solution, stirring 30 s after each addition. The oxidation curves in the potential range from -0.1 to $+1.3$ V, registered at the rate of 5 mV s^{-1} on the scale of 5 μA at 22°C and then again in a higher potential range comprised between $+0.5$ and $+1.0$ V with a rate of 20 mV s^{-1} confirmed the linear relationship between the NADH concentration and the current intensity (Fig. 2). The equation of the regression line for the concentration range 5.6×10^{-5}–5.6×10^{-4} M is $y = 39.8x + 0.9$ where y is μA and x is μl ($r^2 = 0.992$) and allows the determination of NADH with good results in different media. The calculated repeatability of several injections ($n = 37$) corresponded to a RSD of 2.1% and the reproducibility for three series of six samples was found to be 3.6%. The accuracy of the method was good and can be compared to other analytical methods: 102 ± 2% and the detection limit is 2.75 nM.

Although in the individual cases things look simple and confirming what we proved so far,

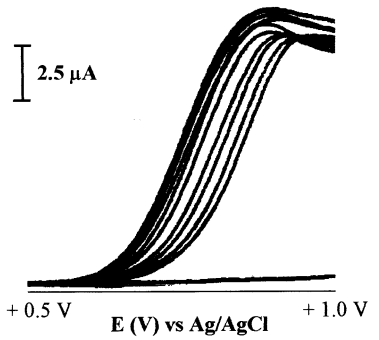


Fig. 3. Continuous drift of the oxidation potential of 5.6×10^{-4} M NADH solutions in the presence of sodium pyruvate (5.6×10^{-4}–3.4×10^{-3} M).

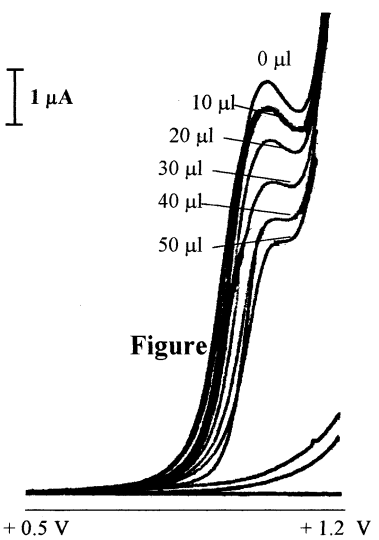


Fig. 4. Linear sweep voltammograms of LDH from + 0.5 to + 1.2 V, with CPE vs. Ag/AgCl reference electrode (20 mV s^{-1}, 2 μA V^{-1}, phosphate buffer) in the presence of 2.8×10^{-4} M NADH and 5.6×10^{-4} M pyruvate solutions (19–94 μU LDH).

problems become more complicate when studying associations.

3.2. Electrochemical study of the enzymatic reaction

To establish the concentration and optimal working conditions only the pairs NADH–sodium pyruvate and NADH–LDH have been studied, because the third combination was not very promising to give a satisfying result. In this second step of the research the working conditions and the optimal concentrations for the LDH determination were established.

As it can be seen in Fig. 3, when adding successive samples of 22.5 mM sodium pyruvate solution, between 100 and 600 μl in a solution containing 5.6×10^{-4} M NADH solution in water, a continuous shift of the oxidation potential of NADH to greater values has been observed, while the current intensity was relatively constant.

The studies have established the working conditions for the determination of LDH, by adding successive volumes of LDH to a solution containing 500 or 1000 μl 2.25 mM NADH and 100 μl 22.5 mM sodium pyruvate solution on the sensitivity scale of 2 μA V^{-1} with a sweep rate of 20 mV s^{-1} after stirring for 1 min between each replicate.

Studies done at LDH concentrations between 10 and 50 μl (120–600 μU) have revealed a linear relation between the concentration of the enzyme and the decrease of the current intensity, due to the NADH oxidation (Fig. 4). The equation of calibration curve was $y = -0.79x + 96$ ($r^2 = 0.994$) where y is in μA and x in μl.

The study has revealed the fact that the most stable and reproducible results were obtained in a 0.15 M phosphate buffer (pH 7.5) and 1 M NaCl added in order to increase the ionic strength. The working temperature was between 20 and 25°C (room temperature), because the optimal working temperature of the enzyme (37°C) complicates the analytical method without bringing any significant advantages.

3.3. Optimal parameters of LDH calibration curve

A very important factor is the electrode surface fouling due to the fact that after oxidation NADH is presumed to form polymers (dimers) that could diminish, in time, the surface of the CPE. As seen in Fig. 5a, where in the presence of 10 μl LDH the current for 100 μl 2.25 mM NADH (5.5×10^{-5} M) decreases from one replicate to another (the time between each replicate is 90 s, 60 s for stirring solution). After 20 min the

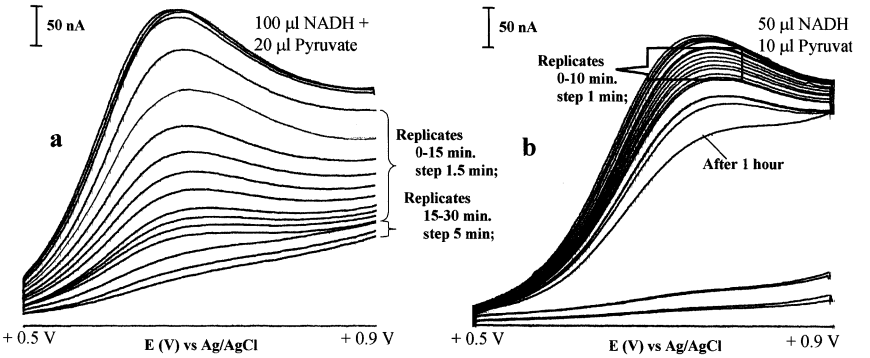


Fig. 5. Linear sweep voltammograms of LDH vs. time from $+0.5$ to $+0.9$ V, with CPE vs. Ag/AgCl reference electrode (20 mV s^{-1}, 100 nA V^{-1}, 23°C, phosphate buffer). (a) 23 µU LDH in the presence of 5.6×10^{-5} M NADH and 1.1×10^{-4} M pyruvate solutions; (b) 2.3 µU LDH in the presence of 2.8×10^{-5} M NADH and 5.5×10^{-5} M pyruvate solutions.

intensity–potential curves can no longer be used. Although, we obtained a linear variation of the current along with the LDH concentration variations (1–10 µl), in rigorous respected time conditions, the equation of the regression line being $y = -1.52x + 112.3$ ($r^2 = 0.997$) where y is in nA and x in µl, we 'redesigned' all the operational parameters of the system, decreasing even more the concentration of the reaction partners. The same experiment was repeated for 1 µl LDH (2.3 µU) in the presence of 50 µl 2.25 mM NADH solution (2.75×10^{-5} M) and 10 ml sodium pyruvate (5.5×10^{-5} M) (Fig. 5b). It is to be mentioned also that the oxidation potential for NADH was decreased to $+0.70 \pm 0.05$ V.

The intensity–potential curves have been registered for samples between 1 and 10 µl LDH at 100 nA V^{-1} in a potential range from $+0.5$ to $+0.9$ V, with a sweep rate of 20 mV s^{-1} and 60 s between replicates (30 s for stirring solutions). The other two components of the reaction system were in saturating concentrations 50 µl 2.25 mM NADH (2.75×10^{-5} M) and 10 µl 22.5 mM sodium pyruvate (5.5×10^{-5} M). The total time of the determinations is between 5 min (for five equal samples of 2 µl) and 10 min (for 10 equal samples of 1 µl), the linear relation between the current intensity and enzyme concentration being obvious (Fig. 6). The equation of the regression line is $y = -3.76x + 80.8$ ($r^2 = 0.997$) where y is expressed in nA and x in µl (1 µl solution contains 2.3 µU LDH). The calculated repeatability of several samples ($n = 25$) correspond to a RSD of 10.2%, the reproducibility of three series of six samples (three different days with different electrode surface) to a RSD of 10.5% and the accuracy of the method was found to be $98.5 \pm 4.5\%$.

3.4. The enzymatic reaction study using modified carbon paste electrodes

The experiments have been repeated under the same conditions, using modified carbon paste, that contains 5% sodium pyruvate and comparing the intensity–potential curves obtained when

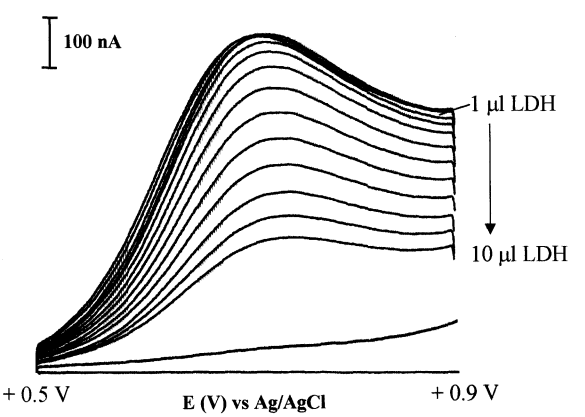


Fig. 6. Linear sweep voltammograms of LDH from $+0.5$ to $+0.9$ V, with CPE vs. Ag/AgCl reference electrode (20 mV s^{-1}, 23°C, phosphate buffer, 2.3–23 µU LDH in the presence of 5.6×10^{-5} M NADH and 1.1×10^{-4} M pyruvate solutions on 200 nA V^{-1} scale).

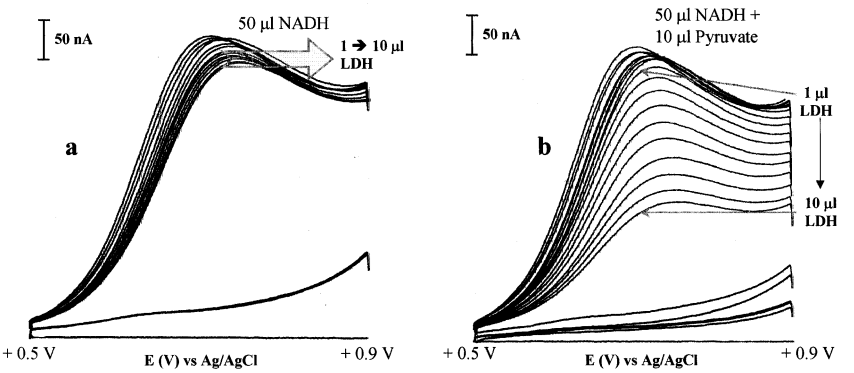


Fig. 7. Linear sweep voltammograms of LDH from +0.5 to +0.9 V, with 5% pyruvate modified CPE vs. Ag/AgCl reference electrode (20 mV s^{-1}, 100 nA V^{-1}, 23°C, phosphate buffer). (a) 2.3–34.5 μU LDH in the presence of 2.8×10^{-5} M NADH solution; (b) 2.3–23 μU LDH in the presence of 2.8×10^{-5} M NADH and 5.5×10^{-5} M pyruvate solutions.

adding volumes of LDH between 1 and 10 μl to a solution of 2.75 M NADH. The current decreases very slowly but obvious (Fig. 7a), so we came to the conclusion that the absence of the pyruvate ion in solution is the limiting parameter, fact that was proved doing the same experiments after adding 10 μl of 22.5 mM sodium pyruvate (5.5×10^{-5} M) (Fig. 7b) in the measuring cell and we obtained curves that were similar to those made with the unmodified carbon paste (calibration curve equation is $y = -6.84x + 130.7$; $r^2 = 0.993$).

Similar results were also obtained when using a modified carbon paste with 0.1% NADH, in this case the NADH concentration being the limiting factor. An interesting fact was that in this case the intensity of the initial registered current was very high and this determined us to decrease sensitivity.

Finally, the experiments have been done with a carbon paste modified with both 0.1% NADH and 2.5% sodium pyruvate. The electrode stabilizes after repeated polarizations for 20–30 min, when a reproducible baseline is obtained. The shape of the oxidation curve of NADH is present, but poorly defined, which makes the procedure too long and useless.

The addition of increasing concentrations of LDH solution (1–10 μl) showed oxidation curves a little higher (until 5–6 μl) that than the response decreased progressively. That can probably be explained by the fact that the presence of the enzyme favored, in the initial phase, the displacement and the solubilisation of the NADH from the carbon paste. The same LDH concentrations have no influence if the measuring cell contained also 5.5×10^{-5} M sodium pyruvate. The intensity of the currents registered increased linearly with the LDH volumes added until 56 μU, then remained constant. If the measuring cell contained both 1.4×10^{-5} M NADH solution and 2.75×10^{-5} M sodium pyruvate solution, similar results can be observed. The intensity of the signal increased linearly with the LDH concentration from 2.3–23 μU and then decreased. In all the above-mentioned cases, discrete differences between oxidation curves for different LDH concentrations and the curve shapes do not allow their analytical use.

4. Conclusions

For the assay of LDH from an enzyme preparation and in biological media, the electroanalytical study of the LDH reaction system by linear sweep voltammetry has been initiated using a CPE versus an Ag/AgCl reference electrode and a platinum wire as auxiliary electrode.

The sodium pyruvate and LDH are not electroactive in positive polarization in the potential range from −0.10 to +1.30 V. NADH has a well defined peak at $+0.95 \pm 0.05$ V, within concentration values of 5.6×10^{-5}–5.6×10^{-4} M in 0.15 M phosphate buffer (pH 7.5) at 22°C with a sweep rate of 20 mV s^{-1}.

The studies about the couples NADH–sodium pyruvate and NADH–LDH have shown that the current intensity remained constant, but the oxidation curves are influenced by the ionic strength, pH and temperature.

The intensity–potential curves for the samples between 1 and 10 μl LDH in 0.15 M phosphate buffer and 1 M NaCl at 25°C, in the potential range from $+0.50$ to $+0.90$ V with a sweep rate of 20 mV s^{-1} have revealed the linear relation between the current and the LDH concentration. The other two components of the reaction system have been added in saturating concentrations, 2.75×10^{-5} M NADH and 5.5×10^{-5} M sodium pyruvate, respectively.

Experiments done with three new modified carbon pastes: one with 5% sodium pyruvate, the second with 0.1% NADH and the third with 0.1% NADH and 2.5% sodium pyruvate, have brought inferior results, compared to the cases in which the three reaction components are in solution. The presence of one or two reaction partners in the carbon paste does not assure the optimal concentration balance for the voltammetric dosage of LDH.

Acknowledgements

The authors thank Professor Jean-Michel Kauffmann from the Université Libre de Bruxelles for his generous help with instruments and references for all the comments and advices during the investigations.

References

[1] A.J. Bard, L.R. Faulkner, Electrochemical Methods, Wiley, New York, 1980.
[2] P.T. Kissinger, W.R. Heineman, Laboratory Techniques in Electroanalytical Chemistry, second ed., Marcel Dekker, New York, 1996.
[3] K. Kalcher, J.-M. Kauffmann, J. Wang, I. Svancara, K. Vytras, K. Neuhold, Z. Yang, Electroanalysis 7 (1) (1995) 5–22.
[4] L. Gorton, Electroanalysis 7 (1) (1995) 23–45.
[5] C. Petit, A. Gonzalez-Cortes, J.-M. Kauffmann, Talanta 42 (1995) 1783–1789.
[6] W.M. Clark, Oxidation–Reduction Potentials of Organic Systems, R.E.Krieger Publishers, Huntington, 1972.
[7] H. Deng, J. Zheng, J. Burgner, R. Callender, Proc. Natl. Acad. Sci. USA, Biophysics 86 (1989) 4484–4488.
[8] T. Yao, S. Musha, Anal. Chim. Acta 110 (1979) 203.
[9] W.J. Aston, in: A.P.F. Turner, I. Karube, G.S. Wilson (Eds.), Biosensors, Fundamentals and Applications, Oxford University Press, Oxford, 1987, pp. 276–290.
[10] W. Matuszewski, M. Trojanowicz, Analyst 113 (1988) 735.
[11] L. Gorton, E. Csoregi, E. Dominguez, J. Emneus, G. Jonsson-Petterson, G. Marko-Varga, B. Persson, Anal. Chim. Acta 203 (1991) 250.
[12] D. Weigelt, F. Schubert, F. Scheller, Fresenius Z. Anal. Chem. 259 (1987) 328.
[13] H. Durliat, C. Causserand, M. Comtat, Anal. Chim. Acta 231 (1990) 309.
[14] J.N. Valenta, S.C. Weber, R.C. Elser, Anal. Chim. Acta 62 (1990) 1947.
[15] J. Racek, Anal. Chim. Acta 197 (1987) 187.
[16] J. Wangsa, M.A. Arnold, Anal. Chem. 60 (1988) 1080.
[17] J. Wang, J. Liu, Anal. Chim. Acta 284 (1993) 385.
[18] L. Gorton, G. Marko-Varga, B. Persson, Z. Huan, H. Jarskog, E. Johansson, S. Ghobadi, M. Smolander, S. Sahni, T. Skotheim, in: L. Bulow, B. Danielson (Eds.), Mosbach Symposium on Biochemical Technology, JAI, London, 1995.
[19] C. Petit, J.-M. Kauffmann, Anal. Proc. Anal. Commun. 32 (1995) 11–12.