Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 62 (2004) 649–654
Talanta
www.elsevier.com/locate/talanta

Optimized multilayer oxalate biosensor

Pablo A. Fiorito, Susana I. Córdoba de Torresi*

Instituto de Química, Universidade de São Paulo, C.P. 26077, 05513-970, São Paulo SP, Brazil

Received 11 June 2003; received in revised form 15 September 2003; accepted 17 September 2003

Abstract

The optimization of a biosensor prepared by the immobilization of *oxalate oxidase* (OOX) with a cross-linking agent onto a multilayer inorganic/organic modified electrode, is presented. A very thin Prussian Blue (PB) film covered by a self-doped polyaniline (SPAN) layer acts as very sensitive amperometric sensor for the H_2O_2 formed by the enzymatic reaction. The electrode allows the very reliable and sensitive oxalate detection in the 0.08 to 0.45 mmol l^{-1} concentration range. The observed sensitivity was 131.3 μA $mmol^{-1}$ cm^{-2} at the operation potential of 0.05 V versus Ag/AgCl in a succinate buffer solution (pH = 3.8). The bilayer Prussian blue/SPAN leads to a very stable, sensitive and selective system that not only minimizes the interference caused by ascorbic and uric acids but also forms a very adherent sensing film that allows repetitive successive determinations.

Keywords: Amperometric biossensor; Oxalate detection; Conducting polymers

1. Introduction

Oxalate sensing in clinical samples such as urine, is a very important problem that requires the search for simple, low cost and reliable methods due to the fact that small oxalate excess in the organism could lead to very serious health problems. Oxalate can be ingested or produced as the final product of ascorbic acid metabolism leading to the formation of insoluble complexes with divalent cations and producing renal calculus [1].

Current oxalate determination methods such as colorimetry [2], liquid or gas chromatography [3,4], capillary electrophoresis [5], usually require complicate and expensive equipments and sample pre-treatment. Amperometric biosensors are a very attractive and suitable strategy for oxalate determination due to specificity, simple use and the possibility of analyzing complicate samples without treatment [6]. In this case, the biological recognition is made by the *oxalate oxidase* (OOX) enzyme and the transduction is made by the electrochemical detection of H_2O_2 produced by the enzymatic reaction:

$$(COOH)_2 + O_2 \xrightarrow{OOX} 2CO_2 + H_2O_2 \quad (1)$$

Specialized literature in oxalate biosensing is not as extensive as bibliography on other analytes like glucose, for example. Reddy et al. [7,8] reported OOX based biosensors where the enzyme is immobilized between two membranes, the inner one being permeable only to H_2O_2 and the outer one avoiding the passage of interferents, macromolecules or colloidal particles and limiting the amount of substrate in order to extend the linear range response. Kubota et al. [9,10] reported different alternative strategies for oxalate sensing; an enzymatic micro-reactor for the potentiometric determination, and a flow injection analysis system (FIA) with spectrophotometric detection by using a two enzyme reactor. Amperometric oxalate biosensors require the monitoring of H_2O_2 oxidation current at very high potential values; this fact leads to the problem that the interference of the oxidation of other substances that could be present in the sample, must be eliminated. A suitable and elegant strategy to overcome this problem can be the diminution of the operating potential as reported by Kubota et al. [11] by the immobilization of two enzymes, oxalate oxidase and peroxidase (HRP). In this biosensor the H_2O_2 produced by the OOX reaction is detected by HRP at −0.10 V. Other authors used metallic hexacyanide complexes as the transductor of oxygen peroxide signal [12,13].

The present work presents the design and optimization of an oxalate biosensor prepared by immobilizing oxalate oxidase enzyme onto a glassy carbon (GC) electrode modified

* Corresponding author. Tel.: +55-11-30912165; fax: +55-11-38155579.
E-mail address: storresi@iq.usp.br (S.I. Córdoba de Torresi).

0039-9140/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2003.09.010

by a bilayer inorganic–organic film that allows very stable and sensitive oxalate determinations, in a wide concentration range and minimize the interference of uric and ascorbic acids, currently present in clinical samples.

2. Experimental

2.1. Materials

Oxalate oxidase (E.C. 1.2.3.4), from *Barley seedings*, 0.71 units/g solid, was purchased from Sigma. Aniline from Sigma was distilled prior to use and stored under N_2 atmosphere at $-4\,^{\circ}C$. Methanilic acid, ascorbic acid, uric acid (Sigma), and glutaraldehyde (Fluka) were of analytical grade and were used without further purification. All solutions were prepared using ultra pure water (Elga System UHQ).

2.2. Instrumentation

Electrochemical measurements were performed with an Autolab PGSTAT30 (Ecochemie) potentiostat/galvanostat running over the GPES 4.7 software. All potentials are referred to a $Ag/AgCl/Cl^-$ (sat.) electrode, while platinum was employed as the auxiliary electrode.

2.3. Biosensor preparation

2.3.1. Prussian Blue deposition

Thin Prussian Blue films were prepared by polarizing a GC electrode ($\phi = 3$ mm, BAS, $A = 0.0707\,cm^2$) at 0.40 V in an aqueous $2.5\times10^{-3}\,mol\,l^{-1}$ $FeCl_3 + 2.5\times10^{-3}\,mol\,l^{-1}$ $K_3Fe(CN)_6 + 0.10\,mol\,l^{-1}$ KCl + $0.10\,mol\,l^{-1}$ HCl solution. The deposition was carried out in an electrochemical micro-cell (200 μl) using a Pt coiled wire as counter electrode and $Ag/AgCl/Cl^-$ (sat.) electrode as reference. After deposition the film was activated in a $0.10\,mol\,l^{-1}$ KCl + $0.10\,mol\,l^{-1}$ HCl solution by applying 50 triangular potential cycles in the −0.20 to 0.60 V potential range.

Electrochemical deposition leads to the formation of "insoluble" Prussian Blue ($Fe_4[Fe(CN)_6]_3$) that during cycling will be transformed in "soluble" PB ($KFe[Fe(CN)_6]$). The difference between soluble and insoluble PB results from the degree of peptization to potassium salts, this process being easier for soluble PB. This later phenomena is called lixiviation and it must be avoided in order to stabilize the electrocatalytic layer onto the substrate.

2.3.2. Self-doped polyaniline deposition

The film was deposited onto either a GC naked electrode or covered with a Prussian Blue film by applying triangular potential cycles between 0.20 and 0.90 V at $0.05\,V\,s^{-1}$ in a $0.10\,mol\,l^{-1}$ $H_2SO_4 + 0.10\,mol\,l^{-1}$ aniline $+0.10\,mol\,l^{-1}$ methanilic acid solution.

2.3.3. Enzyme immobilization

Oxalate oxidase was immobilized onto GC, GC/SPAN or onto the electrode covered with a Prussian blue/SPAN bi-layer by putting a drop (6 μl) of a solution containing $2.4\,mg\,ml^{-1}$ of enzyme and a drop (3 μl) of glutaraldehyde (2.5%) solution on the top of the electrode. The solvent was allowed to evaporate at room temperature.

2.3.4. Amperometric measurements

Tests of biosensor's response to oxalate were made by triplicate in a conventional three-electrode electrochemical cell (5 ml) containing a stirred succinate buffer solution ($0.10\,mol\,l^{-1}$, pH = 3.8) as electrolyte. Before the successive oxalate additions, the electrode was polarized at the operating potential till the stabilization of the background current.

3. Results and discussion

3.1. Sensitivity and optimization of the biosensor

The analytical curve of a biosensor prepared by the immobilization of the enzyme oxalate oxidase onto a Prussian Blue (PB) thin film is shown in Fig. 1. The current response to oxalate was recorded in a succinate buffer solution (pH = 3.8) at 0.00 V in the 0.05–0.45 $mmol\,l^{-1}$ concentration range, which covers levels of oxalate in medical analysis [8]. The amperometric response for the biosensor in these experimental conditions is also depicted in the figure. As the observed current is negative, it can be inferred that it is related to the reduction of H_2O_2 formed by the enzymatic reaction, which is catalyzed by the Prussian Blue film [14], as described in Scheme 1. The performance of this sensor was excellent with a linear response in the 0.05–0.45 $mmol\,l^{-1}$ concentration range and the sensitivity obtained was $131.3\,mA\,mol^{-1}\,cm^{-2}$. For the sake of comparison, Fig. 2 shows the analytical curve and the amperometric response of a sensor prepared by the immobilization

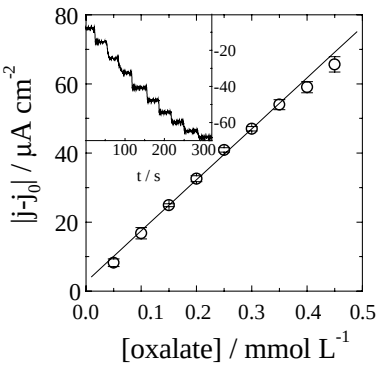
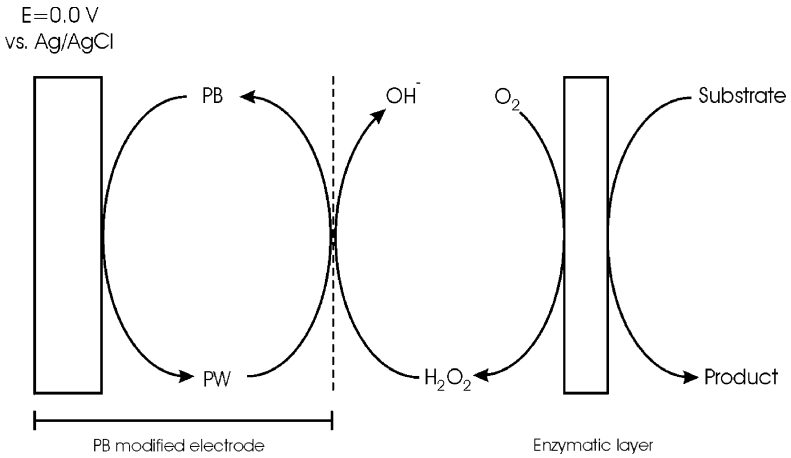


Fig. 1. Analytical curve (mean of three determinations) obtained at 0.00 V vs. Ag/AgCl with PB/OOX biosensor in succinate buffer ($0.10\,mol\,l^{-1}$, pH 3.8). Each step corresponds to an increase of $0.05\,mmol\,l^{-1}$ of oxalate concentration. Inset: typical amperometric response.



Scheme 1. General overview for an oxidase enzyme/Prussian Blue modified electrode.

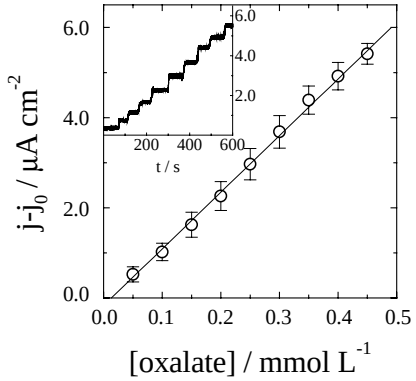
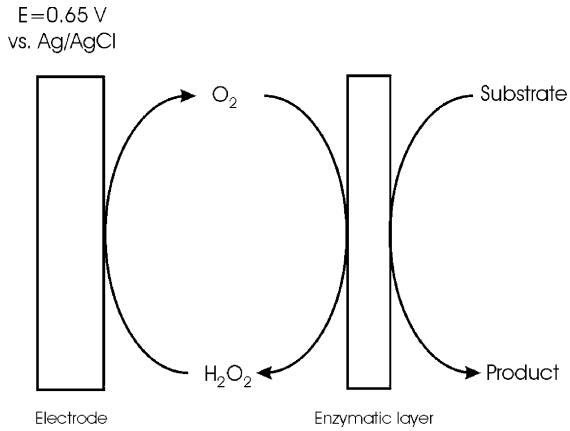


Fig. 2. Analytical curve (mean of three determinations) obtained at 0.65 V vs. Ag/AgCl with OOX biosensor in succinate buffer (0,10 mol l^{-1}, pH 3.8). Each step corresponds to an increase of 0.05 mmol l^{-1} of oxalate concentration. Inset: typical amperometric response.

of the enzyme directly onto the vitreous carbon substrate. It can be seen that, in this case, the current observed for each oxalate addition is positive. This sensor works as described in Scheme 2 where the H_2O_2 produced by the enzymatic reaction is oxidized at 0.65 V. It can be observed that, in this case, the sensitivity obtained, 12.5 mA mol^{-1} cm^{-2}, is a 10-fold lower than that obtained with the sensor containing Prussian Blue and working at 0.00 V.

Aiming to optimize the performance of this bi-layer biosensor, amperometric tests at different pH were carried out. Previous experiments analyzing the enzyme activity have shown that the optimum pH for oxalate oxidase activity is 3.8. It is well known that catalytic properties of Prussian Blue can be modified by H^+ concentration [15]; so that, the investigation of the dependence of sensor's performance on pH was carried out. Fig. 3 shows a plot of the containing Prussian Blue sensor's sensitivity obtained from analytical curves as a function of pH of the buffer succinate solution. It can be seen that the optimum pH is still 3.8 showing that the Prussian Blue film is not affected at this pH and that it is the enzyme activity the main responsible for the sensor's pH dependence.

Another important parameter that must be optimized is the operation potential. As mentioned above, Prussian Blue acts as mediator of H_2O_2 reduction and, at the operation potential, it must be in the reduced state (Prussian White). The analyte is reduced producing the oxidation of Prussian White to Prussian Blue that leads to the amperometric signal



Scheme 2. General overview for an oxidase enzyme based amperometric biosensor.

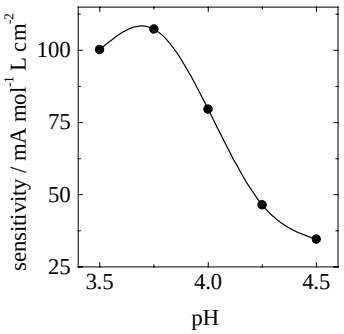


Fig. 3. Amperometric response of PB/SPAN/OOX biosensor as a function of pH. $E_{app} = 0.05$ V vs. Ag/AgCl.

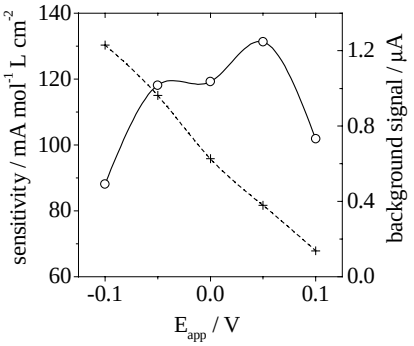


Fig. 4. Applied potential influence on (○) sensitivity and (+) background signal of PB/SPAN/OOX biosensor.

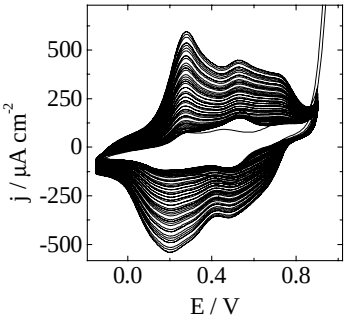


Fig. 5. Electrosynthesis of SPAN film onto a PB modified glassy carbon electrode. Electrolytic solution: 0.10 mol l^{-1} H_2SO_4 + 0.10 mol l^{-1} aniline + 0.10 mol l^{-1} methanilic acid. $v = 0.10$ V s^{-1}.

Table 1
Influence of applied potential on the sensitivity, correlation coefficient, background signal and detection limit of the PB/SPAN/OOX biosensor

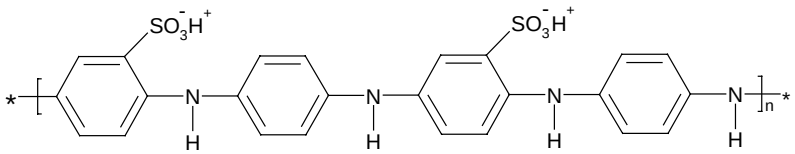
Potential (V)	Senstivity (mA mol^{-1} l cm^{-2})	*R*	Background signal (μA)	Detection limit (mmol l^{-1})
−0.10	88.1	0.998	1.23	0.29
−0.05	118	0.998	0.96	0.18
0.00	119	0.9996	0.67	0.15
0.05	131	0.996	0.38	0.08
0.10	101	0.9998	0.13	0.08

(Scheme 1), all the process depending on the applied potential [16]. Fig. 4 shows both the sensitivity value and the background current obtained at different operation potentials. It can be observed that while the sensitivity is practically not affected by the potential, the background current diminishes, as the potential is raised from −0.10 to 0.10 V. Table 1 shows the sensitivity and the detection limit obtained for each operation potential. It can be clearly seen that as the potential is raised the detection limit, calculated as the concentration of oxalate for what the signal obtained is twice the background current, diminishes till 0.08 mmol l^{-1}; consequently, the optimum operation potential was determined as being 0.05 V that corresponds to the value where the maximum sensitivity and the lowest detection limit was obtained.

Although the excellent performance obtained with the Prussian blue/enzyme bilayer biosensor, some problems related to the stability of Prussian Blue film were observed. After some oxalate determinations, the amperometric response diminishes due to the lixiviation of the electrocatalytic film. In order to avoid the dissolution process, a permeselective polymeric layer that allows the permeation of H_2O_2 was deposited onto the Prussian Blue film. A poly(sulfonate aniline) (SPAN) film was electrochemically grown by potentiodynamic sweeps onto the Prussian Blue modified electrode. Fig. 5 shows the electrochemical response recorded during SPAN electropolymerization where it can be seen the growth of the polymeric film by the continuous current increase after each cycle. The sensitivity of the biosensor GC/PB/SPAN/OOX was of the same order than that obtained for the biosensor without SPAN, but no lixiviation of the Prussian Blue film was observed.

3.2. Specificity of the biosensor

Reducting substances currently present in clinical samples, such as uric and ascorbic acids, are the most common interferents for amperometric determinations. In the present case, the Prussian Blue modified electrode allows working at ca. 0.00 V to detect the H_2O_2 produced by the enzymatic reaction; instead of 0.65 V needed for the GC/OOX interface. On the other way, as at pH 3.8 (succinate buffer) both acids are in the dissociated form, as ureate and ascorbate ions, the new configuration of the biosensor (GC/PB/SPAN/OOX) presents an additional advantage because SPAN is a self-doped polymer [17] (Scheme 3) that will allow the permeation of H_2O_2 avoiding the penetration of large anionic species. In this way, the polymeric film plays two important roles in the biosensor's design: to avoid both, Prussian Blue lixiviation and the penetration of reactive anionic species such as ureate and ascorbate. Fig. 6 shows the amperometric responses of three biosensor's configurations to 3.10^{-4} mol l^{-1} oxalate in the presence of 1.10^{-3} mol l^{-1} ureate or ascorbate solutions. As it can be seen from the Figure, the biosensor prepared



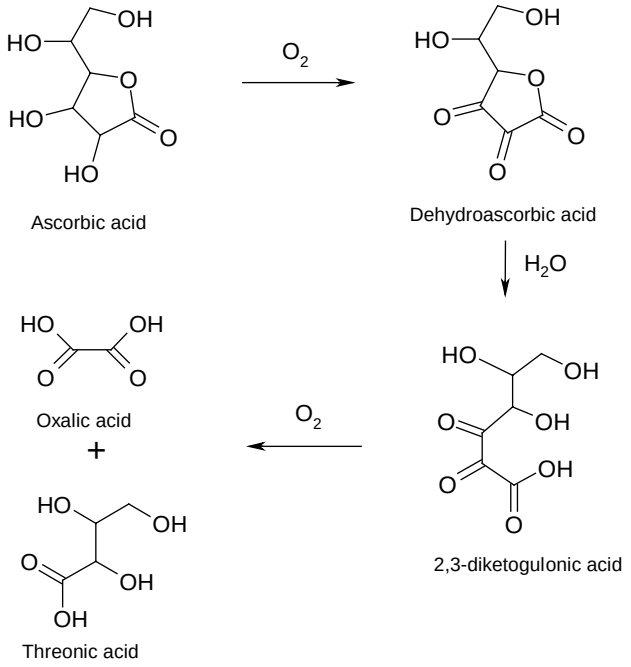
Scheme 3. Sulfonated polyaniline (SPAN) structure.

Table 2
Quantitative information about ascorbic and uric acid effect on biosensor response in 0.10 mol l^{-1} succinate buffer, pH 3.8, E_{app}: 0.65 V for OOX and SPAN/OOX configurations and 0.05 V for PB/SPAN/OOX configuration

Electrode	Response ($\mu A\,cm^{-2}$)				
	Oxalate[a]	Oxalate + ascorbate[b]	Difference (%)[a–b]	Oxalate + ureate[c]	Difference (%)[a–c]
OOX	12.8	15.6	21.6	14.0	9.37
OOX/SPAN	12.0	14.1	17.5	12.1	0.83
AP/OOX/SPAN	−29.8	−30.4	2.00	−29.8	0.00

[a] 3.10^{-4} mol l^{-1} oxalate. [b] 3.10^{-4} mol l^{-1} oxalate + 1.10^{-3} mol l^{-1} ascorbate.
[c] 3.10^{-4} mol l^{-1} oxalate + 1.10^{-3} mol l^{-1} ureate.

by immobilizing the enzyme onto the GC substrate an operating at 0.65 V, presents the interference of both ureate and ascorbate. The second sensor (SPAN/OOX) prepared by immobilizing the enzyme onto a glassy carbon electrode covered by a SPAN film and operating at 0.65 V, is shown with the aim of demonstrating the efficiency of the self-doped poly(aniline) layer for stopping anionic interferents. Table 2 shows quantitative information obtained from these experiments. As it can be inferred, ureate interference is almost completely eliminated (0.9% versus 9.3%) while the ascorbate response is still observed even that in a lower extent than for the first biosensor (17.5% versus 21.6%). Finally, the response obtained with the third configuration using Prussian blue, SPAN and the enzyme and operating at 0.05 V for reducing H_2O_2 is also depicted in the figure. It can be seen that, while the ureate interference is eliminated (see column 6, line 3, in Table 2), the ascorbate response is still present. It must be paid attention to the fact that, in this case, the observed current is negative; so that, it means that the interference is caused by the reduction of some species in a different way than when the sensor operates at 0.65 V and the interference is caused by the oxidation of the ascorbate. The ascorbate interference is related to the oxidative degradation process of ascorbic acid leading to oxalate (Scheme 4) already described by Arendse et al [18]. On the other hand, it must be emphasized that the relative interference of ascorbate is lower (2.0% versus 21.6%) for this sensor because the oxalate response is much higher due to Prussian Blue electrocatalytic character.



Scheme 4. Oxidation and hydrolysis of ascorbic acid.

3.3. Operational stability

The operational stability of the biosensor was evaluated by performing repetitive determinations in an oxalate solution

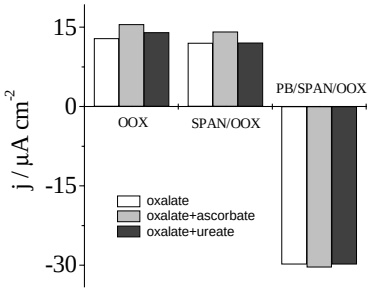


Fig. 6. Ascorbic and uric acid effect on biosensor response in 0.10 mol l^{-1} succinate buffer, pH 3.8, E_{app}: 0.65 V for OOX and SPAN/OOX configurations and 0.05 V for PB/SPAN/OOX configuration.

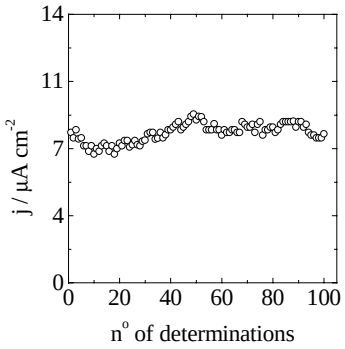


Fig. 7. Operational stability of the PB/SPAN/OOX biosensor. Amperometric response of the biosensor for 0.15 mmol l^{-1} of oxalate. E_{app}: 0.05 V vs. Ag/AgCl.

($0.15\,\mathrm{mmol\,l^{-1}}$) at 0.05 V versus Ag/AgCl, the biosensor being washed with distilled water after each assay. Fig. 7 shows the current obtained for each determination, where can be seen that the biosensor allowed to perform up to 100 determinations without any loss of signal.

4. Conclusions

A multilayer inorganic–organic oxalate biosensor that combines the high catalytic activity of Prussian Blue and a self-doped polyaniline film, that plays the double role of protecting PB film from lixiviation and of stopping anionic interferents, was presented. Oxalate oxidase was immobilized onto the PB/SPAN bi-layer by using a crosslinking agent. Optimization of the biosensor's parameters allows the very reliable and sensitive oxalate detection in the $0.08–0.45\,\mathrm{mmol\,l^{-1}}$ concentration range. The observed sensitivity was $131.3\,\mathrm{mA\,mol^{-1}\,l\,cm^{-2}}$ at the operation potential of 0.05 V versus Ag/AgCl in a succinate buffer solution (pH = 3.8). The very low detection potential combined with the permeselective SPAN layer improves selectivity and specificity of the sensor. Stability tests have shown that the biosensor allows to perform up to 100 determinations without degradation or loss of enzyme activity.

Acknowledgements

Brazilian Agencies, CNPq and FAPESP and CAPES are gratefully acknowledged. P.A.F. Thanks FAPESP (Proc. N° 01/04957-0) for the fellowship granted.

References

[1] S.R. Sharma, R. Nath, S.K. Thind, Scan. Micro. 7 (1993) 431.
[2] A. Hodgkinson, A. Williams, Clin. Chem. Acta 36 (1972) 127.
[3] N.C. France, P.T. Holland, T.K. McGhie, M.R. Wallace, J. Cromatogr. 433 (1985) 1.
[4] D.R. Fry Ian, B.J. Starkey, Ann. Clin. Biochem. 28 (1991) 581.
[5] B.C. Nelson, P.C. Uden, G.F. Rockwell, K.M. Gorsky, Z.B. Aguilera, J. Chromatogr. A 771 (1997) 285.
[6] S.M. Reddy, S.P. Higson, P.M. Vagdama, Anal. Chim. Acta 343 (1997) 59.
[7] S.M. Reddy, S.P.J. Higson, I.M. Christie, P.M. Vadgama, Analyst 119 (1994) 949.
[8] S.M. Reddy, P.M. Vadgama, Biosens. Bioelect. 12 (1997) 1003.
[9] J.R. Fernandes, G. de Oliveira Neto, L.T. Kubota, M. Tubino, Anal. Commun. 33 (1996) 397.
[10] J.R. Fernandes, G. de Oliveira Neto, M. Tubino, L.T. Kubota, Anal. Sci. 12 (1996) 443.
[11] E.F. Perez, G. de Oliveira Neto, L.T. Kubota, Sens. Actuators B 72 (2001) 80.
[12] S. Milardovic, Z. Grabaric, V. Rumenjak, N. Blau, D. Milosevic, J. AOAC Int. 84 (2001) 1927.
[13] S. Milardovic, Z. Grabaric, M. Tkalcec, V. Rumenjak, J. AOAC Int. 83 (2000) 1212.
[14] I.L. Mattos, L. Gorton, Quim. Nova 24 (2001) 200.
[15] R. Grajonyte, A. Malinauskas, Sens. Actuators B 46 (1998) 236.
[16] D. Moscone, D. D'Ottavi, D. Compagnone, G. Palleschi, Anal. Chem. 73 (2001) 2529.
[17] R.M.Q. Mello, R.M. Torresi, S.I. Córdoba de Torresi, E. Ticianelli, Langmuir 16 (2000) 7835.
[18] M.J. Arendse, G.K. Anderson, N.P. Rath, Polyhedron 20 (2001) 2495.