Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 1025–1031
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Flow-injection amperometric determination of dopamine in pharmaceuticals using a polyphenol oxidase biosensor obtained from soursop pulp

Vilma Sobral Bezerra [a], José Luiz de Lima Filho [a], Maria Conceição B.S.M. Montenegro [b], Alberto N. Araújo [b], Valdinete Lins da Silva [c,*]

[a] *Laboratory of Immunopathology Keizo Asami (LIKA), Department of Biochemistry, UFPE, 50670-901 Recife PE, Brazil*
[b] *REQUINTE, Dep. Química-Física, Fac. Farmácia U.P., 4000 Oporto, Portugal*
[c] *Laboratory of Environmental and Quality Engineering, Department of Chemical Engineering, UFPE, Cidade Universitaria, Tereza Melia Street, CEP-50740-521 Recife PE, Brazil*

Received 13 May 2003; received in revised form 21 July 2003; accepted 24 July 2003

Abstract

The amperometric determination of dopamine (Do) in pharmaceuticals formulations by flow injection analysis (FIA) is proposed. An enzymatically modified carbon paste electrode constituted by 25% (w/w) of polyphenol oxidase obtained from *Annona muricata* L. tissue, 30% (w/w) of graphite, 30% (w/w) of silicone and 15% (w/w) of 7,7,8,8 tetracyanoquinodimethane (TCNQ), was used as flow-through detector. The flow amperometric detection was carried out at a potential of 0.10 V (vs. Ag/AgCl) when an injected sample volume of 250 μl was inserted on a 0.3 M phosphate buffer carrier solution (pH 7.8) flowing at 2.5 ml/min. The developed biosensor showed good stability and reproducibility, enabling up to 500 determinations in 60 days, without considerable loss of enzymatic activity. The FIA system presented a linear response to Do concentrations in the interval from 2×10^{-2} to 2×10^{-4} M, with relative standard deviations lower than 1.5%. The kinetic parameter K_M for the soluble and immobilized enzyme was 1.45×10^{-2} and 1.91×10^{-2} M, respectively. In the analyses of different commercially pharmaceutical formulations a relative deviation lower than about 3.4% was obtained.

Keywords: Polyphenol oxidase; Dopamine; Biosensor; Amperometry; Pharmaceutical formulations

1. Introduction

Dopamine (Do) is a catecholamine (3,4 dihydroxyphenylethylamine), with adrenergic activity used for the correction of hemodynamic disorders associated with shock episodes [1]. For the Do

* Corresponding author. Tel.: +55-81-3271-8711; fax: +55-81-3274-7278.
E-mail address: vlins@ufpe.br (V.L. da Silva).

0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00412-6

determination, United States Pharmacopoeia (USP) recommends the use of a high-performance liquid chromatography with spectrophotometric detection at 280 nm.

Other alternative methods for the quantification of this drug in formulations involved techniques such as chromatography [2,3], molecular fluorescence [4], chemiluminescence [5–7] potentiometry [8–10], amperometry [11–14] and spectrophotometry [15–21].

As has been clearly demonstrated in the literature [22] Do is readily oxidized to dopaminequinone (DoQ) in a quasi-reversible electron transfer reaction. By means of a sequence of follow-up chemical reactions DoQ is transformed into the more easily oxidizable leucodopaminechrome, which in turn is oxidizable to dopaminechrome, giving rise to another redox system at lower potentials. Although a direct electrochemical quantification of Do could be performed at the oxidation peak in the first cycle [23] the electrode surface is to be renewed after each potential cycle due to the inactivation produced by side polymeric reactions.

Enzymes are catalysts with high selectivity toward a given substrate, increasing the reaction rate by many orders of magnitude. For this reason their use in the construction of biosensors has become a research field where the development has been evident in the past years. In this regard the extraction of enzymes from natural products enables the preparation of biosensors at low costs, with good selectivity characteristics for the application in the analyses of complex matrices like the pharmaceutical formulations. Biocatalytic properties of polyphenol oxidase (PPO, EC 1.14.18.1) have been reviewed [24]. This enzyme, present in microorganisms, animals, plants and fruits such as banana pulp and spinach leaves [25], fresh potato tissue [26–29], mushroom tissue [30] and apple chips [31], is responsible for browning of exposed surfaces on some fruits and tubers. PPO catalyses the *ortho*-hydroxylation of phenols and the oxidation of catechols to *ortho*-quinone in the presence of O_2 [24,32]. In this way the current obtained in the electrochemical reduction of the products can be related with the amount of the corresponding catecholamine.

The association of the PPO enzymatic characteristics with electrochemical transduction enables to reduce the interferences of Do metabolically related compounds thus providing more accurate results when stability trials of pharmaceuticals are intended.

In this work an amperometric biosensor was constructed by using a natural source of enzyme and used in a flow-injection system for the determination of Do in pharmaceuticals in a more expeditious way. The biosensor was prepared by using soursop (*Annona muricata* L.), which constitutes a natural source of PPO on an electronic modified carbon paste doped with 7,7,8,8-tetracyanoquinodimethane (TCNQ) used as chemical mediator (electron acceptor) in order to diminish the working potential and to minimize interfering effects [33].

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade, used without further purification. Doubly deionized water was used throughout.

In order to prepare a 0.3 M phosphate buffer solution, 18.4 g sodium hydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) were weighted and dissolved in 500 ml of water. To this, 0.3 M sodium dihydrogen phosphate solution was added until a pH of 7.8 at 25 °C. The buffer solution was used in evaluation tests of enzymatic activity and also as carrier in the flow system.

A 10^{-3} M pyrocatechol stock solution used as substrate was daily prepared by dissolving 0.005 g of pyrocatechol powder (Sigma–Aldrich) in 50 ml of the buffer solution. The calibration solutions for Do spectrophotometric determination and for the enzymatic activity trials in the soursop extracts were prepared from this solution by appropriate dilution.

To carry out the assessment of the quality of results furnished by the developed procedure amounts of 10 μl of different pharmaceutical products, namely, Do (Cristalia, Brazil), Revimine (União Química, Brazil), Dopamina (Eurofarma,

Brazil) and Revivan (Zambon, Brazil) were dissolved in 10 ml of phosphate buffer.

2.2. Apparatus

The flow-injection manifold (Fig. 1) comprised a Gillson Minipuls 3 peristaltic pump (Villier-le-Bel, France) as propelling device, an home-made injector [34], and several types of other homemade perspex devices namely, a confluence and detector supports. The different devices were interconnected by PTFE tubing (0.8 mm i.d.). The analytical signals were obtained by means of a potentiostat MQPG 01, equipped with the developed biosensor, a Pt auxiliary electrode (wire 0.5 mm diameter and 10 cm long) and an Ag/AgCl home-made reference electrode. The potentiostat output was connected through an interface card to a computer where the collected data was saved and processed.

A Femto 435 spectrophotometer was employed to assess the Do levels in the pharmaceutical preparations by the conventional procedure [35]. Centrifugation of extracts was conducted in a Kubota (KR-20000T model) centrifuge.

2.3. Biosensor preparation

The biosensor was prepared from a mixture of 30% (w/w) of graphite powder 1–2 μm (Fluka), 30% (w/w) of silicone wax (Nuclear, Brazil); 15% (w/w) of TCNQ (Sigma–Aldrich) and 25% (w/w) of the vegetal tissue of soursop (pulp's chip). This material was weighed and placed on a plastic holder (pipette tip) having 3 mm of diameter and 2 mm of depth, in contact with a gold pin (Fig. 2). The surface of the sensor was polished with vegetal paper. The biosensor was stored in a 4 °C dry place between utilization.

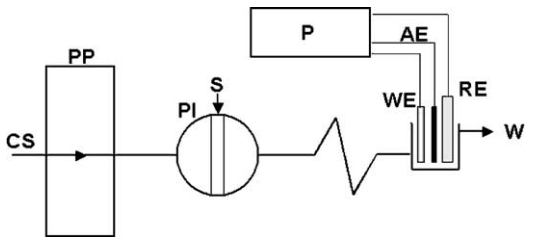

Fig. 1. Schematic representation of the implemented flow-injection system. CS, carrier solution; PP, peristaltic pump; PI, proportional injector; S, sample; RE, reference electrode; AE auxiliary electrode; WE, working electrode; W, waste and P, potentiostat.

2.4. Methods

2.4.1. Extraction of PPO of soursop and determination of enzymatic activity

An amount of 25 g of *A. muricata* L. previously washed was cut into small pieces and macerated in a mortar with 50 ml of phosphate buffer (0.1 M). The macerate was filtered through gauze and centrifuged at $26\,730 \times g$ for 15 min at 4 °C to remove cellular residues and obtain the coarse extract. The protein content of the different extracts was determined in 200 μl aliquots, by the modified Bradford method [36]. This method consisted of adding 50 μl of NaOH 1 M solution to 40 μl of the sample, plus 1000 μl of the Bradford reagent (10 mg of Coomassie blue, 5 ml of 95% v/v ethanol and 10 ml of 88% v/v phosphoric acid). The absorbances of the resulting solutions were measured at 595 nm against a blank reagent and the corresponding concentrations obtained by interpolation in a calibration curve using bovine serum albumin calibrating solutions.

The determination of the specific activity of the PPO of the crude soursop extract was carried out according to Roudsari et al. [37] from aliquots of the crude extract by measuring the increase of the absorbance at 420 nm at room temperature (25.0 ± 1.0 °C) for 5 min and recording the absorbance values at intervals of 2.0 s.

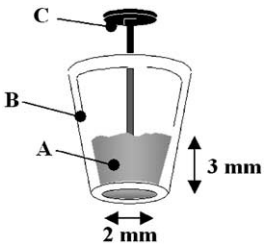

Fig. 2. Schematic drawing of the biosensor developed. (A) Carbon paste with the immobilized enzyme, (B) perspex tube, (C) gold pin.

The specific activity of the immobilized enzyme PPO was amperometrically determined using the developed biosensor. First the developed biosensor was immersed in a phosphate buffer solution containing 5.00×10^{-2} M of catechol and cycle voltammograms were registered in order to select the polarizing potential to be applied on the subsequent studies. Thereafter, the biosensor was soaked in a 3 ml phosphate buffer solution in which 100 μl of catechol solution with concentrations from 1.94×10^{-2} to 8.16×10^{-2} M were added. The enzymatic reaction was followed by 180 s being the apparent K_M assessed from the initial rate of the observed reactions.

2.5. The spectrophotometric determination of the dopamine

The Do determinations by the conventional procedure were carried out according to Dannis [35]. Calibrating solutions of catechol were prepared with amounts ranging from 0.05 to 0.2 mg, which likewise the analyzed samples were treated with 2.5 ml of ammonium hydroxide. Then, the pH was adjusted to 7.8 U with phosphate buffer. Afterwards, a solution of 1.0 ml of 4-aminoantipirine (2% w/v) and 1.0 ml of potassium ferrocyanide was added. After 15 min the absorbance was measured at 460 nm.

2.6. Flow injection analysis system

A FIA system manifold (Fig. 1) with amperometric detection for Do determination in pharmaceutical products was established. In the developed set-up an injected sample volume (250 μl) was inserted in a 0.3 M phosphate buffer carrier solution (pH 7.8) and the amperometric current was monitored electrochemically by using the incorporated biosensor (dead volume of 0.5 ml) after the application of a polarized potential of 0.10 V versus Ag/AgCl. The hydrodynamic parameters of the system were optimized by the univariate method in order to achieve a compromise between sensitivity, sample throughput and reproducibility. The variables studied: injected sample volume (100–400 μl), flow rate (1.5–8 ml/min), pH of buffer (pH 5.8–8.5) and concentration of buffer (0.1–1.0 M).

3. Results and discussion

The PPO obtained from *A. muricata* L. (soursop) in its crude extract showed a specific activity of 593.2 U/mg of protein (considering one unit of activity of polyphenol oxidase of the soursop as the amount of enzyme that catalyze the production of 1 μmol of catechol/min in the experimental conditions used). This value was in accordance with the previously reported by Oliveira [38], who found 532 U/mg of protein. A double-reciprocal plot of activity versus substrate concentration, carried out to determine the apparent Michaelis–Menton constant, yielded a K_M value of 1.45×10^{-2} M for the soluble enzyme and 1.91×10^{-2} M for the immobilized enzyme. Although the procedures used to access the K_M values were of same order of magnitude thus indicating that immobilization procedure thus not produced significant changes of the enzymatic activity. Moreover, the results obtained were similar to the reported one by Vieira and Fatibello [39] for sweet potato (1.73×10^{-2} M). It was also verified that the pH value enabling highest enzyme activity was 7.8, value that coincides with the one observed by the same authors for the enzymes obtained from the previously referred materials.

In order to provide an analytical application of the developed biosensor its was incorporated on a FIA system and the optimization of some chemical and hydrodynamic parameters was carried out. Therefore, the effect of ionic strength of the buffer solution (pH 7.8) used as carrier was evaluated by injecting catechol solution at the concentration of 0.001 M. The obtained results showed an increase of the enzymatic activity of about 40%, obtained when the buffer concentration changed from 0.3 to 0.6 M (Fig. 3). Above this value a decrease of the enzymatic activity was observed, may be related with the neutralization of the hydrogen ions involved in DoQ reduction. Besides this, increase of the ionic strength corresponded higher oxidation on the biosensor surface leading to unstable signals and reducing the lifetime of the detector.

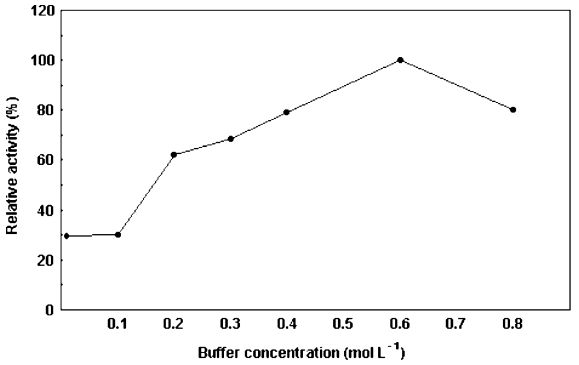

Fig. 3. Effect of the phosphate buffer concentration (pH 7.8) in the activity of the immobilized enzyme: volume of the injected sample (250 μl); flow rate (2.5 ml min^{-1}).

Thus, a buffer solution concentration of 0.3 M was selected as a compromise of enzymatic activity and biosensor stability.

The effect of pH in the enzymatic activity was studied in the range of 5.8–8.4 U (Fig. 4). The optimum pH for maximum immobilized PPO activity was 7.8, a value close to the one found by Oliveira [38] who studied the PPO activity in various stages of the maturation of the soursop and reported a pH range between 7.0 and 7.5. Previous papers with PPO of other vegetable sources like banana [29], pear [40] and mango [41] have shown that the optimum pH for the PPO activity is also close to the value determined for the immobilized enzyme.

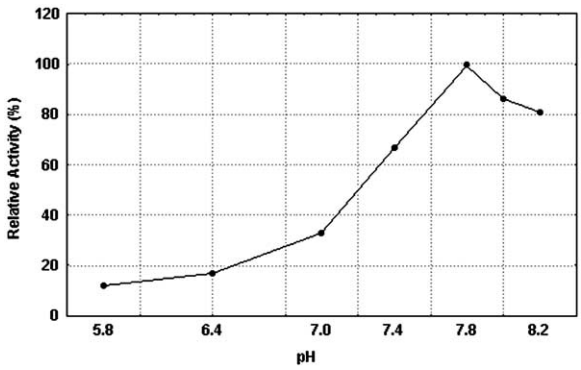

Fig. 4. Effect of the pH in the activity of the immobilized enzyme: buffer solution 0.3 M; volume of injected sample (250 μl); flow rate (2.5 ml min^{-1}).

The influence on the system performance of both, flow rate and injection volume was evaluated in the range of 1.5–8.0 ml/min and 100–400 μl, respectively. The increase in flow rate resulted in a decrease in the sensitivity particularly for flow rates higher than 3.0 ml/min, due to the lower sample residence time in the reaction chamber. However, this adverse effect was overcome by adopting a sample injection volume of 250 μl. The characteristics of the developed flow system are summarized on Table 1, in which other figures of merit are reported.

The stability of the biosensor was examined over a 60 days period by successive injection of a 0.001 M catechol sample. In this condition it was possible to carry out about 500 determinations using the same biosensor, with a loss on the initial activity of about 30%. These results compete favorably with the ones obtained by Uchyama et al. [41], Vieira and Fatibello [39] and Navaratne et al. [42], that obtained a lifetime of 8 days for units done from spinach leaves, 15 days for others from crude sweet potato extract, and 23 days for a biosensor from eggplant vegetable tissue. Using a biosensor based on mushroom extract Lindgren et al. [43] does not disclose the lifetime of the detector. Throughout the referred evaluation the developed biosensor showed a within batch relative standard deviation of 1.3% for ten repeated injections.

The Do content of real samples was evaluated using the previously defined optimal conditions for the proposed FIA system. The pharmaceutical

Table 1
General working characteristics for the Do readings with FIA system

Optimized parameters	Optimum values
Sample rate (h^{-1})	40
Flow rate (ml/min)	2.5
Sample injection volume (μl)	250
Polarizing voltage (mV)	100
Buffer concentration (M)	0.3
PH	7.8
Linear dynamic range (M)	0.0002–0.02
Reproductibility (μA/day)	±0.2
Detection limit (M)	0.00015

Table 2
Results obtained after triplicate analysis of pharmaceutical samples by the conventional procedure and by the proposed procedure

Sample	Conventional procedure ($\times 10^{-4}$ M)	Developed procedure ($\times 10^{-4}$ M)	Relative error (%)
DOPAMIN®	3.26±0.04	3.22±0.03	1.34
DOPAMINA®	3.06±0.03	3.23±0.04	5.55
REVIVAN®	3.04±0.03	3.60±0.04	0.65
REVIVAN®	3.07±0.04	3.25±0.03	5.86

preparations commercially available containing 5 mg/ml of Do per injection was analyzed in triplicate. The results obtained by the proposed method were comparatively evaluated with those provided by the conventional spectrophotometric procedure (Table 2). Relative errors of 1.34% (Dopamin), 5.55% (Dopamina), 5.86% (Revivan) and 0.65% (Revimine) were obtained. An evaluation of the precision evidenced similar results for both the developed and the reference procedures (Table 2).

4. Conclusions

Soursop constitutes a rich source of polyphenol oxidase, which allows the implementation of a stable and high activity enzymatic biosensor. The implemented FIA system advantageously uses the natural enzyme as the basis of a process for selective enzymatic determination of Do in pharmaceutical products. The used biosensor permitted to carry out up to 500 determinations contributing in this way towards an effective reduction in the cost of determination. The use of amperometric detection facilitated the analysis of real samples, without any previous treatment, at a highly satisfactory sampling rate of about 40 samples per hour.

Acknowledgements

The authors would like to thank CNPq, CAPES/GRICES and FACEPE for their financial support.

References

[1] L.S.G. Goldman, A. Gilman, As bases Farmacológicas e terapêutica, vol. 1, 9ª Ed., McGraw-Hill, Rio de Janeiro, 1997.
[2] W.P. King, K.T. Joseph, P.T. Kissinger, JAOC 63 (1980) 137–142.
[3] W.V. Caufield, J.T. Stewart, Chromatographia 51 (2000) 308–314.
[4] D. Djozan, M. Farajzadeh, J. Pharm. Biomed. Anal. 10 (1992) 1063–1067.
[5] N.T. Deftereos, A. Calokerinos, C.E. Efstathiou, Analyst 118 (1993) 627–632.
[6] J. Michalowski, A. Kojlo, O.A. Estrela, Chem. Anal. Warsaw 47 (2002) 267–274.
[7] J.C. Huang, C.X. Zhang, Z.J. Zhang, Chin. Chem. Lett. 9 (1998) 843–845.
[8] L.R. Junior, J.C.B. Fernandes, G.D. Neto, L.T. Kubota, J. Electroanal. Chem. 481 (2000) 34–41.
[9] J.L.F.C. Lima, M.C.B.S.M. Montenegro, Mikrochem. Acta 131 (1999) 187–190.
[10] M.C.B.S.M. Montenegro, M.G.F. Sales, J. Pharm. Sci. 89 (2000) 876–884.
[11] W.F. Peng, E.K. Wang, Anal. Chim. Acta 281 (1993) 663–671.
[12] E. Wolyniec, A. Kojlo, J. Trace. Microprobe T 21 (2003) 217–227.
[13] E. Montane, A. Vallano, J.M. Castel, Electroanalysis 14 (2002) 783–789.
[14] E.M. Garrido, J.L.F.C. Lima, C.D. Matos, J. Pharm. Biomed. Anal. 15 (1997) 845–849.
[15] A.G. Saijan, M.B. Melwanki, J. Seetharamappa, J. Anal. Chem. 56 (2001) 827–829.
[16] P. Nagajara, R.A. Vasantha, K.C.S. Murthy, K.S. Rangappa, Chem. Anal. Warsaw 46 (2001) 569–577.
[17] B.G. Gowda, M.B. Melwanki, J. Seetharamappa, Anal. Sci. 17 (2001) 533–534.
[18] A. Afkhami, D. Nematollahi, H.A. Khatami, Asian J. Chem. 14 (2002) 333–338.
[19] P. Nagaraja, R.A. Vasantha, K.R. Sunitha, Talanta 55 (2001) 1039–1046.
[20] A. Afkhami, H.A. Khatami, J. Anal. Chem. 58 (2003) 135–138.
[21] P. Nagaraja, K.C.S. Murthy, K.S. Rangappa, M.M.M. Gowda, Talanta 46 (1998) 39–44.

[22] R.N. Adams, Anal. Chem. 68 (1996) 359–363.
[23] E.S. Forzani, G.A. Rivas, V.M. Solís, J. Electroanal. Chem. 382 (1995) 33–40.
[24] S.G. Burthon, Catal. Today 22 (1994) 459–487.
[25] G.A. Rechnitz, M.A. Arnold, in: A.P.F. Turner, I. Karube, G.S. Wilson (Eds.), Biosensors Fundamentals and Applications, Science Publications, London, Oxford, 1987, pp. 30–37.
[26] G.S. Forzani, G.A. Rivas, V.M. Solis, J. Electroanal. Chem. 461 (1999) 174–183.
[27] F. Brotrè, F. Mazzei, M. Lanzi, G. Lorenti, C. Brotrè, Anal. Chim. Acta 255 (1991) 59–62.
[28] J. Wang, T. Golden, P. Tuzhi, Anal. Chem. 59 (1987) 740–744.
[29] J. Besombes, S. Cosnier, P. Labbém, Talanta 43 (1996) 1615–1916.
[30] F. Mazzei, M. Lanzi, G. Lorenzi, C. Botré, Anal. Chim. Acta 255 (1992) 107–109.
[31] Y. Chen, T.C. Tan, Talanta 42 (1995) 1181–1188.
[32] K. Lerch, in: H. Sigel (Ed.), Copper Monoxygenases: Tyrosinase, Dopamine β-Monoxygenase, Copper Proteins, vol. 13 (Chapter 5), Marcel Dekker, New York, 1981 (Chapter 5).
[33] W.J. Albery, J. Electroanal. Chem. 194 (1992) 211–222.
[34] S.S. Rosatto, R.S. Freire, N. Duran, L.T. Kubota, Quim. Nova 24 (2001) 77–86.
[35] M. Dannis, Sewage Ind. Wastes 23 (1951) 1516–1522.
[36] C.M. Stoscheck, Methods Enzymol. 182 (1990) 50–68.
[37] M.H. Roudsari, A. Signoret, J. Crouzet, Food Chemi, Barking 7 (1981) 227–235.
[38] S.L. Oliveira, Polifenoloxidases nos Diversos Estádios de Maturação da graviola (*A. muricata* L.) Dissertação de Mestrado em Nutrição, UFPE, Recife, 1991, p. 85.
[39] O. Fatibello-Filho, I.C. Vieira, Anal. Chim. Acta 3 (1997) 51–57.
[40] M. Montegomery, D.H. Halim, J. Food Sci. 43 (1978) 603–608.
[41] S. Uchiyama, M. Tamata, Y. Tofuku, S. Suzuki, Anal. Chim. Acta 208 (1988) 287–290.
[42] A. Navaratne, M.S.L. Lin, G.A. Rechnitz, Anal. Chim. Acta 237 (1990) 107–113.
[43] A. Lindgren, T. Ruzgas, J. Emnéus, E. Csoregi, L. Gorton, Anal. Lett. 29 (1996) 1055–1068.