Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytica Chimica Acta 485 (2003) 263–269
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Dual amperometric biosensor device for analysis of binary mixtures of phenols by multivariate calibration using partial least squares

Renato S. Freire [a,1], Márcia M.C. Ferreira [a], Nelson Durán [a,b], Lauro T. Kubota [a,*]

[a] *Instituto de Química, Universidade Estadual de Campinas, UNICAMP, CP 6154 CEP 13083-970, Campinas, SP, Brazil*
[b] *Núcleo de Ciências Ambientais, Universidade de Mogi das Cruzes, Mogi das Cruzes, SP, Brazil*

Received 13 November 2002; received in revised form 24 March 2003; accepted 31 March 2003

Abstract

A simple and reliable method for rapid evaluation of mixtures of phenolic compounds (phenol/chlorophenol, cathecol/phenol, cresol/chlorocresol and phenol/cresol) using a dual amperometric device is described. This new approach is based on the difference between the sensitivity of laccase and tyrosinase for different phenolic compounds. A multichannel potentiostat was used to monitor simultaneously laccase- and tyrosinase-based biosensors, and the data were treated using the partial least squares (PLS) chemometric algorithm. This system showed an excellent efficiency for the resolution of the phenolic mixtures. For example, in the phenol/chlorophenol mixture it was studied the determination of individual species in a concentration range from 1.0×10^{-6} to 10.0×10^{-6} mol l^{-1} obtaining relative standard deviations of 3.5 and 3.1% for phenol and chlorophenol, respectively. The excellent correlation between the estimated and the real concentrations can also be observed by the correlation coefficients (0.9958 and 0.9981 for phenol and chlorophenol, respectively). These results show that proposed methodology can be successfully employed to the simultaneous determination of phenolic compounds in mixtures, even in more diluted solutions.

Keywords: Phenolic compounds; Amperometric biosensors; Multichannel; Multivariate calibration

1. Introduction

Nowadays the determination of phenolic compounds is of great importance, since these compounds are widely used in industrial processes, such as the manufacture of plastics, polymers, drugs and dyes [1–5]. This kind of compound also result as breakdown from some pesticides and by-products from paper pulp industry, with the types and abundances of phenolic compounds changing with the particular source or mill process [6–8]. Phenolic compounds belong to a class of chemicals polluting, easily absorbed by animals and humans through the skin and mucous membranes [9]. Their toxicity affects a great variety of organs and tissues, primarily lungs, liver, kidneys and genito-urinary system [2,9]. In addition, due to their great variety, phenolic compounds show a broad range of toxicity levels, being phenol and its chlorinated or alkylated derivatives classified as priority pollutants [8]. In the same way, the organoleptic

* Corresponding author. Tel.: +55-19-37883127; fax: +55-19-37883023.
E-mail address: kubota@iqm.unicamp.br (L.T. Kubota).
[1] Present address: Instituto de Química, Universidade de São Paulo, USP CEP 05513-970, São Paulo, SP, Brazil.

0003-2670/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0003-2670(03)00414-8

properties of many fruits, and consequently the quality of foods and beverages, are correlated with the concentration and kind of phenolic compound [10]. Furthermore, many neurotransmitters and medicines have phenolic structure with different bioactivities. Thus, the development of procedures for detection and simultaneous determination of these compounds in different matrices is highly desired.

Analytical methods for the detection and quantification of mixtures of phenols are usually based on analytical separation techniques, which allow the identification and quantification of individual constituents [11]. Many methods have been developed for the determination of phenolic compounds, such as chromatographic, fluorimetric and spectrophotometric methods [12–15]. However, these techniques do not easily allow continuous monitoring, they are expensive, time-consuming, need skilled operators, and sometimes require preconcentration and extraction steps that increase the risk of the sample loss or contamination [16–19].

Thus, the development of new methods, that allows the simultaneous determination, without previous separations of these compounds is a relevant subject of research. There are some reports about the application of spectrometric methods and chemometric tools for the resolution of phenolic compounds in mixtures [20–23]. However, very few reports have described the employment of electrochemical techniques for phenols detection [24]. One of the main goals of electrochemical detection is the improvement in the versatility and in the scope of applications. However, the electrochemical oxidation of phenols has some drawbacks, such as the poisoning of the electrode surfaces due to the accumulation of reaction by-products. In order to overcome these limitations, the aim of this work was to develop a dual amperometric biosensor device capable of making high sensitivity and reliable determination of phenolic compounds in mixtures. Although some studies using cell-based electrochemical biosensors for determination of ternary mixtures of sugars have been described [25,26], the application of amperometric enzyme-based biosensors coupled to the chemometric multivariate calibration has not been reported.

Electrochemical biosensors present some advantages that can be satisfactory employed in this kind of analysis, such as intrinsic selectivity, high sensitivity, low cost and potential for miniaturization/automation. Horseradish peroxidase (HRP) [27,28], laccase- [29–32] and tyrosinase-based electrodes [2,3,5,32] have shown good sensitivity and selective for determination of phenols. Laccase and tyrosinase, which are polyphenol oxidases, can catalyze the conversion of phenolic compounds to the corresponding quinones in the presence of oxygen, the formed quinone can be electrochemically reduced to phenolic substances at low potential without any mediator [29,30]. This kind of amperometric enzyme-based biosensors have been shown several advantages over direct electrochemical oxidation of phenolic compounds [1,33]. The first one is the selectivity, since the biosensors require a lower potential (around 0.0 V) than direct oxidation (around +1.0 V), becoming less exposed to interferences [33,34]. The increase in the sensitivity due to the signal amplification through the quinone recycling mechanism is another advantage [1,29,34], as shown in Fig. 1. The biosensors tend to be more stable and more suited to environmental monitoring, clinical testing, or food assays compared with the direct electrochemical oxidation of phenols [1,14,15].

Reagentless devices based on laccase or tyrosinase immobilization onto various transducers have been developed [1]. These two enzymes display different substrate selectivity and mechanisms of action, and thus the use of laccase- and tyrosinase-based biosensors on the analysis of mixtures of phenolic compounds can allow the screening and quantification of the

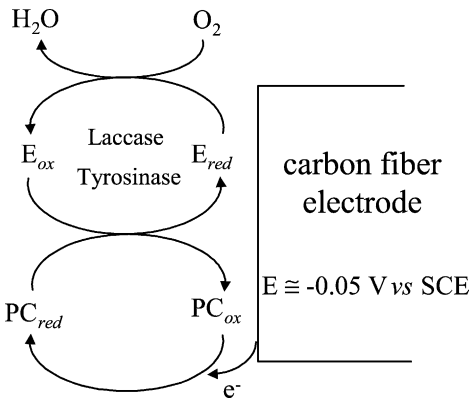

Fig. 1. Reactions mechanism on the laccase- and tyrosinase-based biosensor. PC: phenolic compound; E: enzyme; *red* and *ox* are the reduced and oxidized forms.

individual components [29,32]. Thus, for the first time, the application of a dual amperometric enzyme-based biosensor device is proposed for the resolution of binary mixtures of phenolic compounds, using multivariate calibration.

2. Experimental

2.1. Reagents

All solutions were prepared with deionized water. Laccase (from *Trametes versicolor* fungus (CCT 4521)) was produced and extracted as described by Minussi et al. [35]. Tyrosinase (2000 U mg^{-1}, EC 1.14.18.1) was purchased from ICN (USA). Bovine serum albumin, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenosulfonate, glutaraldehyde (50% (w/v), water solution, reagent grade), catechol, phenol, *p*-chlorophenol, cresol and *p*-chlorocresol were purchased from Sigma or Aldrich. All experiments were carried out using a phosphate buffer solution (pH 5.0, 10 mmol l^{-1}).

2.2. Apparatus and procedure

Experiments were performed using a multichannel potentiostat PGSTAT 10 model from AUTOLAB (The Netherlands) connected to a PC microcomputer for data acquisition and potential control. Voltammetric experiments were carried out scanning the potential from 0.1 to −0.3 V for both enzyme-based working electrodes simultaneously and using a saturated calomel electrode (SCE) as reference and a platinum wire as the counter electrode. All electrodes were displayed in the same electrochemical cell, always keeping the same position. Scan rate: 25 mV s^{-1}. All experiments were carried out at room temperature.

2.3. Electrode pre-treatment and enzyme immobilization

The carbon fiber electrodes (home made [24] using about 20 fibers PAN type T-800 sized with 8 μm diameter, Toray Industries Inc., Japan) were pre-treated at a potential of +0.8 V versus SCE for 180 s in 0.1 mol l^{-1} phosphate buffer (pH 7.2). After this process, the electrodes were allowed to react for 2 h with a solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenosulfonate in 0.05 mol l^{-1} acetate buffer at pH 4.8 (with continuous stirring) [36]. Then, the electrodes were dipped, during 30 min, into a solution of glutaraldehyde (10 mg ml^{-1}), bovine serum albumin (6 mg ml^{-1}) and enzyme (65 U ml^{-1} for laccase or 140 U ml^{-1} for tyrosinase).

Table 1
Factorial design for the binary mixtures composition

P_0C_0	P_2C_0	P_4C_0	P_5C_0	P_6C_0	P_8C_0	$P_{10}C_0$
P_0C_2	P_2C_2	P_4C_2	P_5C_2	P_6C_2	P_8C_2	$P_{10}C_2$
P_0C_4	P_2C_4	P_4C_4	P_5C_4	P_6C_4	P_8C_4	$P_{10}C_4$
P_0C_5	P_2C_5	P_4C_5	P_5C_5	P_6C_5	P_8C_5	$P_{10}C_5$
P_0C_6	P_2C_6	P_4C_6	P_5C_6	P_6C_6	P_8C_6	$P_{10}C_6$
P_0C_8	P_2C_8	P_4C_8	P_5C_8	P_6C_8	P_8C_8	$P_{10}C_8$
P_0C_{10}	P_2C_{10}	P_4C_{10}	P_5C_{10}	P_6C_{10}	P_8C_{10}	$P_{10}C_{10}$

P: Phenolic compound 1; C: phenolic compound 2. The subscripts indicate the concentration, e.g. $1 = 1.0 \times 10^{-6}$; $2 = 2.0 \times 10^{-6}$, respectively until $10 = 10.0 \times 10^{-6}$ mol l^{-1}.

2.4. Dataset and samples selection

The samples utilized for simultaneous determination of binary mixtures of phenols (catechol/phenol, phenol/chlorophenol, cresol/chlorocresol and phenol/cresol) were synthetics, resulting from the factorial design, as shown in Table 1. The sample sets were prepared in the concentration range from 1.0×10^{-6} to 10.0×10^{-6} mol l^{-1} (which are inside the linear response range), resulting in a total of 49 samples for each binary mixture. Randomly, for every sample the current was monitored in three different potential: −50, −75 and −100 mV versus SCE and all of them were used in the models. Models made with less than three potentials showed a very poor fit, on the other hand, models with four or more potentials do not presented any significant improvement compared to the system with three potentials. Thus, for each sample it was recorded three different currents, resulting in a total of 147 independent variables for each binary mixture, which provided necessary information to the multivariate calibration.

The dataset was split in two subsets, one for calibration and other for external validation of the model. The calibration set had 35 samples and the validation set 15 samples. The selection of the samples to subsets was made using the principal component analysis

(PCA) [37], in order to guarantee the homogeneity in the subsets.

2.5. Partial least squares (PLS) method

The chemometric calculations were carried out using the commercial Pirouette software (version 3.02, Infometrix Inc.). In partial least squares (PLS) analysis [37,38], a regression model is built between the $\boldsymbol{X}$ block of independent variables (where each column contains the variables measured for each sample) and the response variable $\boldsymbol{C}$. In reality, two models are built:

$$\boldsymbol{X} = \boldsymbol{T}\boldsymbol{P}^{\mathrm{T}} + \boldsymbol{E}$$
$$\boldsymbol{C} = \boldsymbol{T}\boldsymbol{q} + \boldsymbol{f}$$

where $\boldsymbol{E}$ and $\boldsymbol{f}$ are the residual matrix and vectors, respectively, $\boldsymbol{T}$ and $\boldsymbol{P}$ are the scores and loadings matrices. Note that the scores $\boldsymbol{T}$ is a link between the two models. This means that the scores are obtained in such way to model $\boldsymbol{X}$ and have maximum covariance with $\boldsymbol{C}$ simultaneously. The product $\boldsymbol{T}\boldsymbol{P}^{\mathrm{T}}$ approximates $\boldsymbol{X}$ while the product $\boldsymbol{T}\boldsymbol{q}$ to $\boldsymbol{C}$. An important feature of PLS is that it takes into account the errors in both $\boldsymbol{X}$ and $\boldsymbol{C}$. A commonly used procedure to determine the number of factors in the model is to use "leave-one-out" cross-validation to estimate prediction errors. In this procedure, one sample is excluded at time, the model is constructed and the concentration of the omitted sample predicted. Each sample is predicted once and the standard error of prediction (SEP) defined as

$$\mathrm{SEP} = \sqrt{\frac{\sum_{i=1}^{n}(c_i - \hat{c}_i)^2}{n}}$$

is calculated. In this equation, c_i is the experimental value, $\hat{c}_i$ the predicted value and n is the number of samples used for model building. The number of factors is chosen to minimize SEP [37,38].

3. Results and discussion

The biocatalytic activity of laccase and tyrosinase, immobilized over carbon fiber, was used for the biorecognition of phenolic compounds in this multichannel system. Laccase- and tyrosinase-based biosensors presented different selectivity for different phenolic compounds, as can be observed in Table 2. Both biosensors presented a wide linear response range for each phenolic compound (between 0.1 and 100 $\mu\mathrm{mol\,l^{-1}}$). Although, the biosensors allowed the convenient quantification of these compounds at levels down to $\mathrm{nmol\,l^{-1}}$ [32], the range between 1.0×10^{-6} and $10.0 \times 10^{-6}\,\mathrm{mol\,l^{-1}}$ was chosen to illustrate the favorable characteristic of the proposed method. Furthermore, the response signals were addictive for the compounds in the binary mixtures, probably due to the biosensors signals for different compounds presenting neither competition between the two substrates for the active centers of the enzyme nor overlapping of the diffusion layers. In other words, although both biosensors were set closed on a same holder and monitored simultaneously, they presented an independent response for the phenolic compounds presented in the binary mixtures. These characteristics combined with the biosensors selectivity allowed building a multivariate calibration method using PLS.

Two PLS models were separately elaborated one for each component of the binary mixture. The data sets were meancentered and the optimal number of factors

Table 2
Comparison between the relative responses for different phenolic compounds using the laccase- and tyrosinase-based biosensors

Compound	Laccase		Tyrosinase	
	Sensitivity (na, $\mu\mathrm{mol\,l^{-1}}$)	Relative response (%)	Sensitivity (na, $\mu\mathrm{mol\,l^{-1}}$)	Relative response (%)
Catechol	80.3	100.0	45.7	100.0
Phenol	0.4	2.6	14.2	25.2
Chlorophenol	0.1	1.9	20.0	41.4
Cresol	0.7	4.1	4.5	9.1
Chlorocresol	0.6	3.7	30.7	52.3

Working potential: −75 mV vs. SCE.

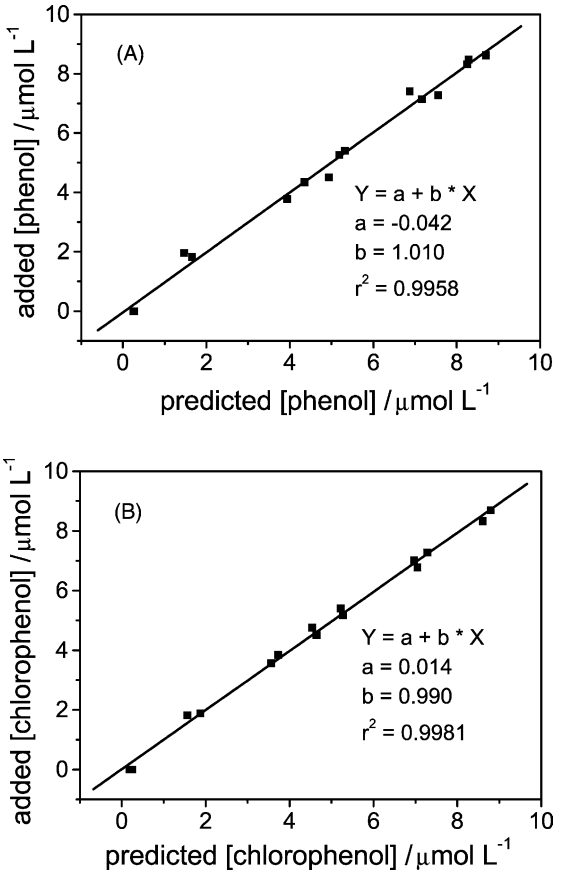

Fig. 2. Results obtained for prediction set modeled with PLS to (A) phenol and (B) chlorophenol, at a concentration range of (1.0–10.0) $\times 10^{-6}$ μmol l^{-1} in phosphate buffer 0.01 mol l^{-1} at pH 5.0.

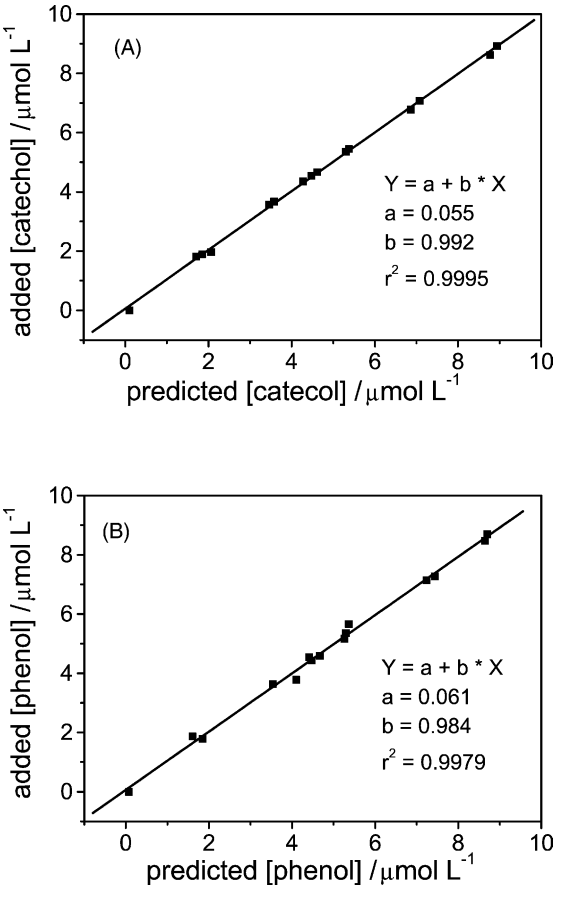

Fig. 3. Results obtained for prediction set modeled with PLS to (A) catechol and (B) phenol, at a concentration range of (1.0–10.0) $\times 10^{-6}$ μmol l^{-1} in phosphate buffer 0.01 mol l^{-1} at pH 5.0.

(latent variables) utilized in PLS models was obtained by cross-validation, leaving one sample out at a time. Although the complexity caused by currents overlaps and the competition between the components by the electrodes surface, all the models showed a good prediction employing just two factors. The consideration of an additional factor slightly increases the percentage of variance for the components but involves an overfitting of the model.

The results obtained for the prediction sets are shown in Figs. 2–5 and in Table 3. This system showed an excellent accuracy and precision for the resolution of the binary phenolic mixtures as can be observed by the low standard error of prediction and relative standard deviation (Table 3), and also by the correlation between the estimated and real concentration (inset Figs. 2–5). Furthermore, these results show that the proposed method was extremely efficient, since the system allows a convenient screening and quantification of the phenolic compounds in binary mixtures with a low concentration level (μmol l^{-1} range). In addition, due to the high sensitivity of both biosensors the concentration range can be easily reduced to meet the practical applications needs. Moreover, both biosensors showed an excellent long-term stability maintaining their bioelectroactivity over a long period, with a variation of just 12% in the concentration prediction after 200 successive determinations of 5.0×10^{-6} mol l^{-1} phenol/chlorophenol mixture.

The results obtained show that the use of a dual electrochemical biosensor system with multivariate

Table 3
Standard error of prediction and relative standard deviations between the concentrations predicted and added

Mixture compound 1/compound 2	Compound 1		Compound 2	
	SEP (μmol l^{-1})	R.S.D. (%)	SEP (μmol l^{-1})	R.S.D. (%)
Phenol/chlorophenol	0.2344	3.5	0.1728	3.1
Catechol/phenol	0.0852	2.1	0.1603	3.5
Cresol/chlorocresol	0.5029	6.5	0.1553	2.6
Phenol/cresol	0.0931	1.8	0.2116	3.5

calibration analysis can be a promising approach for simple, fast, reproducible, selective and sensitive determination of phenolic compounds in mixtures.

Such conjugation of favorable characteristics qualifies the proposed system for monitoring phenols mixtures in industrial, clinical or environmental samples. Furthermore, the number of components analyzed in the mixtures can be improved, since more biosensors can be easily added to the system with no operational inconvenience. Thus, a HRP-based biosensor may expand the scope to additional phenolic substances.

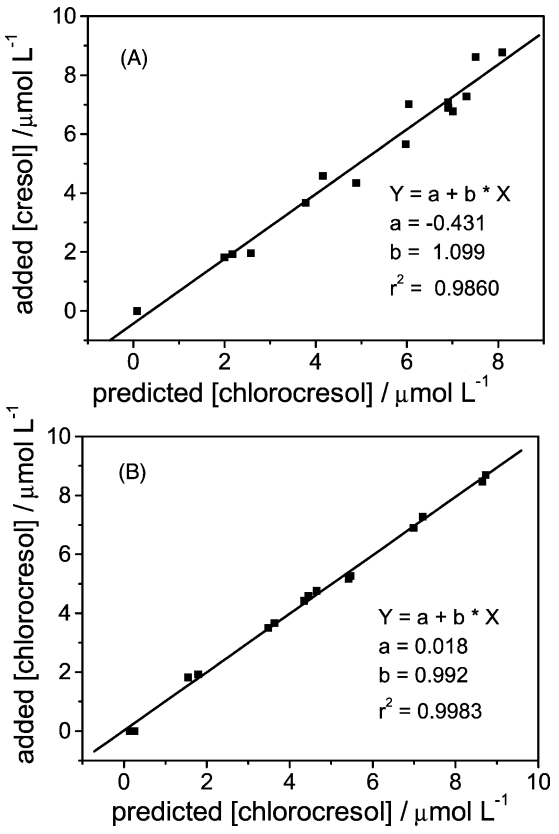

Fig. 4. Results obtained for prediction set modeled with PLS to (A) cresol and (B) chlorocresol, at a concentration range of $(1.0–10.0) \times 10^{-6}$ μmol l^{-1} in phosphate buffer 0.01 mol l^{-1} at pH 5.0.

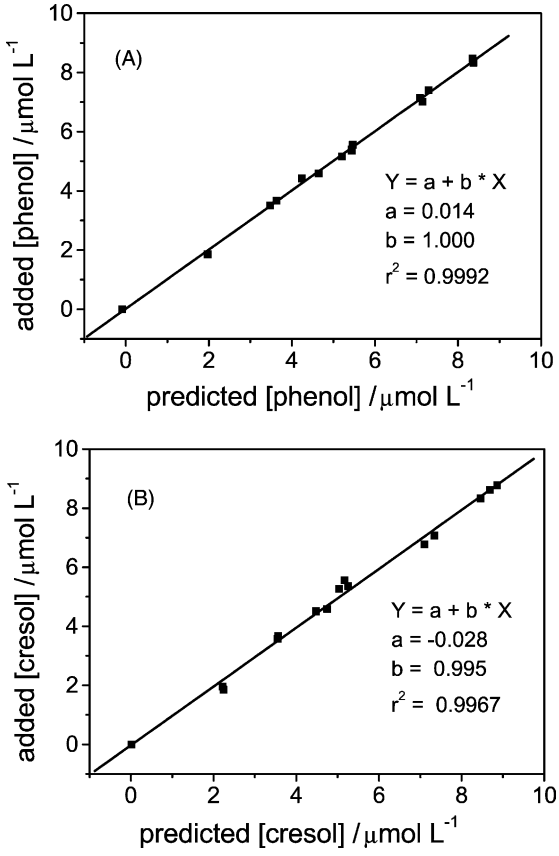

Fig. 5. Results obtained for prediction set modeled with PLS to (A) phenol and (B) cresol, at a concentration range of $(1.0–10.0) \times 10^{-6}$ μmol l^{-1} in phosphate buffer 0.01 mol l^{-1} at pH 5.0.

Moreover, this method can also easily be modified (e.g. using other biological elements) to include new kind of compounds, and promote a fast and efficient determination of a wide range of compounds in different types of matrices.

Acknowledgements

Financial support from FAPESP.

References

[1] S.S. Rosatto, R.S. Freire, N. Duran, L.T. Kubota, Quim. Nova 24 (2001) 77.
[2] C. Nistor, J. Emnéus, L. Gorton, A. Ciucu, Anal. Chim. Acta 387 (1999) 309.
[3] J. Svitel, S. Miertus, Environ. Sci. Technol. 32 (1998) 828.
[4] F. Ortega, E. Dominguez, E. Burestedt, J. Emnéus, L. Gorton, G. Marko-Varga, J. Chromatogr. A 675 (1994) 65.
[5] I.M. Russell, S.G. Burton, Anal. Chim. Acta 389 (1999) 161.
[6] J.L. Gundersen, W.G. MacIntyre, Environ. Toxicol. Chem. 15 (1996) 809.
[7] E.R. Hall, J. Fraser, S. Garden, L.A. Cornacchio, Pulp Pap. Can. 90 (1989) T421.
[8] M. Olmo, C. Diez, A. Molina, I. Orbe, J.L. Vilchez, Anal. Chim. Acta 335 (1996) 23.
[9] S. Canofeni, S. DiSario, J. Mela, R. Pilloton, Anal. Lett. 27 (1994) 1659.
[10] L. Campanella, T. Beone, M.P. Sammartino, M. Tomassetti, Analyst 118 (1993) 979.
[11] A. Cladera, M. Miro, J.M. Estela, V. Cerda, Anal. Chim. Acta 421 (2000) 155.
[12] D. Puig, D. Barcelo, Trends Anal. Chem. 15 (1996) 362.
[13] J. Parellada, A. Narváez, M.A. López, E. Domínguez, J.J. Fernándes, V. Pavlov, I. Katakis, Anal. Chim. Acta 362 (1998) 47.
[14] J. Norberg, J. Emnéus, J.A. Jönson, L. Mathiasson, E. Burestedt, M. Knutsson, G. Marko-Varga, J. Chromatogr. B 701 (1997) 39.
[15] J. Wang, Trends Anal. Chem. 16 (1997) 84.
[16] R.S. Freire, N. Duran, L.T. Kubota, Anal. Chim. Acta 463 (2002) 229.
[17] J. Wang, J. Lu, S.Y. Ly, B. Tian, W.K. Adeniyi, R.A. Armendariz, Anal. Chem. 72 (2000) 2659.
[18] J. Wang, Q. Chen, Anal. Chim. Acta 312 (1995) 39.
[19] Z.L. Zhi, A. Rios, M. Valcarcel, Analyst 121 (1996) 1.
[20] A. Cladera, E. Gómez, J.M. Estela, V. Cerda, Anal. Chim. Acta 267 (1992) 95.
[21] A. Cladera, E. Gómez, J.M. Estela, V. Cerda, Anal. Chim. Acta 267 (1992) 103.
[22] M. Guardia, K.D. Khalaf, B.A. Hasan, A. Morales-Rubio, J.J. Arias, J.M. Guarcia-Fraga, A.I. Jimenez, J. Jiménez, Analyst 121 (1996) 1321.
[23] J.C.G.E. Silva, M.C.P.O. Marques, Anal. Lett. 31 (1998) 2549.
[24] R.M. Carvalho, C. Mello, L.T. Kubota, Anal. Chim. Acta 420 (2000) 109.
[25] M. Slama, C. Zaborosch, D. Wienke, F. Spener, Sens. Actuators B 44 (1997) 286.
[26] V. Plegge, M. Slama, B. Suselbeck, D. Wienke, F. Spener, M. Knoll, C. Zaborosch, Anal. Chem. 72 (2000) 2937.
[27] T. Ruzgas, J. Emnéus, L. Gorton, G. Marko-Varga, Anal. Chim. Acta 311 (1995) 245.
[28] S.S. Rosatto, L.T. Kubota, G. Oliveira-Neto, Anal. Chim. Acta 390 (1999) 65.
[29] A.J. Yaropolov, A.N. Kharybin, J. Emnéus, G. Marko-Varga, L. Gorton, Anal. Chim. Acta 308 (1995) 137.
[30] G. Marko-Varga, J. Emneus, L. Gorton, T. Ruzgas, Trends Anal. Chem. 14 (1995) 319.
[31] R.S. Freire, N. Duran, J. Wang, L.T. Kubota, Anal. Lett. 35 (2002) 28.
[32] R.S. Freire, S. Thongngamdee, N. Duran, J. Wang, L.T. Kubota, Analyst 127 (2002) 258.
[33] E. Burestedt, A. Narvaez, T. Ruzgas, L. Gorton, J. Emneus, E. Dominguez, G. Marko-Varga, Anal. Chem. 68 (1996) 1605.
[34] K.R. Rogers, J.Y. Becker, J. Cembrano, Electrochim. Acta 45 (2000) 4373.
[35] R.C. Minussi, M.A. Miranda, J.A. Silva, C.V. Ferreira, H. Aoyama, S. Marangoni, D. Rotilio, G.M. Pastore, N. Duran, FEMS Microbiol. Lett., submitted for publication.
[36] R.S. Freire, N. Duran, L.T. Kubota, Talanta 54 (2001) 681.
[37] K.R. Beebe, R.J. Pell, M.B. Seasholtz, Chemometrics: A Practical Guide, Wiley, New York, 1998.
[38] H. Martens, T. Naes, Multivariate Calibration, Wiley, New York, 1989.