ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT

Journal of Pharmaceutical and Biomedical Analysis 31 (2003) 621–627

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

Chemometrics assisted spectroscopic determination of vitamin B6, vitamin B12 and dexamethasone in injectables

Alberto J. Nepote [a], Patricia C. Damiani [b], Alejandro C. Olivieri [b,*]

[a] *Cátedra de Química Analítica II, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Paraje El Pozo, Santa Fe 3000, Argentina*

[b] *Departamento de Química Analítica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario S2002LRK, Argentina*

Received 14 March 2002; received in revised form 3 August 2002; accepted 10 October 2002

Abstract

A spectrophotometric method is described and applied to resolve ternary mixtures of the corticosteroid dexamethasone sodium phosphate and the vitamins B6 and B12. It involves multivariate calibration based on partial least-squares regression. The model was built with UV–vis absorption spectra, and was evaluated by cross-validation on a number of synthetic mixtures. Satisfactory results for both artificial and commercial samples were obtained. A spectrofluorometric method was also developed for the determination of B6 in the presence of vitamin B12 and dexamethasone. The results provided by both methods for pharmaceutical formulations were compared successfully. None of the described procedures require sample pre-treatment steps.

© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Spectrophotometry; Multivariate calibration; Spectrofluorimetry; Pyridoxine hydrocloride; Hidroxocobalamin; Dexamethasone sodium phosphate

1. Introduction

Several pharmaceutical preparations contain mixtures of vitamin B6, vitamin B12 and dexamethasone. Their main clinical use is as analgesic, anti-inflammatory, myorelaxant and anti-neuritic. They are used for the symptomatic relief of acute affections of short duration, chronic rheumatic pathologies and post-traumatism of the locomotive system [1].

Vitamin B12 {Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-hydroxocobal-amide, hydroxocobalamin, B12} is used in the treatment and prevention of its deficiency, tobacco amblyopia and Leber's optic atrophy [2]. It has been determined previously in ampoules by spectrophotometry after chromatographic separation [3,4] and spectrophotometry [5].

Vitamin B6 [(5-hydroxy-6-methylpyryridine-3,4-diyl)dimethanol hydrochloride, pyridoxine hydrochloride, B6] is mainly involved in the metabolism of amino acids, carbohydrates and fats. It is also required for the formation of haemoglobin. It has

* Corresponding author. Tel./fax: +54-341-437-2704.
E-mail address: aolivier@fbioyf.unr.edu.ar (A.C. Olivieri).

0731-7085/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0731-7085(02)00677-5

been reported that pyridoxine is effective in treating pregnancy sickness, symptoms of the pre-menstrual syndrome, muscular weakness, recurrent oxalate urolithiasis and hyperkynetic syndromes in children [6,7]. B6 has been determined using spectrophotometry and HPLC [8], derivative spectrophotometry [9], colorimetry [10] and flow-injection/chemiluminescence [11].

Dexamethasone sodium phosphate (9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione disodium 21-phosphate, DEX) is a corticosteroid used for parenteral administration in intensive therapy or in emergency [12]. It has been determined previously in several pharmaceutical forms by spectrophotometry [13–15], reverse-phase HPLC [16–18], normal-phase chromatography [19,20] and capillary zone electrophoresis [21,22].

The most widely used technique for simultaneous determination of B6, B12 and DEX is HPLC [23,24]. In recent years, as part of a program devoted to the development of simple methods for pharmaceutical quality control, we have also been exploring the use of chemometrics-enhanced spectrofluorometry and spectrophotometry as suitable techniques for the analysis of active principles in different pharmaceutical forms [25–30]. This is due to the simplicity and sensitivity of spectroscopic methods, and to the speed of the determination, since preliminary separation steps are avoided. The required selectivity is provided by chemometrics. These characteristics make this methods appealing for routine quality control programs, where they may be faster and of lower cost as compared to HPLC.

In this report, commercially available injectables have been studied which contain ternary mixtures of B6, B12 and DEX, with the components in the mass ratio 125:5:1. They display strongly overlapped absorption spectra, a fact which precludes their simultaneous determination by simple spectrophotometric methods (especially in what regards the minor analytes). However, partial least-squares (PLS) combined with UV–vis absorbance measurements provides satisfactory calibration and prediction results for both artificial and unknown samples of the three components, in spite of the unfavourably low concentrations of the minor ones. The successful performance in the latter cases requires a suitable wavelength selection procedure.

It should be noticed that the absorbances of B6, B12 and DEX are a function of the pH of the solution. Therefore, a previous study of the influence of the aqueous acid-base equilibria on their absorption spectra was undertaken in order to develop a successful procedure for the quantification of the analytes.

Since vitamin B6 is fluorescent, a method was also developed for its determination in the presence of both B12 and DEX, by measuring the fluorescence emission of B6 at a suitable excitation wavelength. The results were favourably compared with those provided by spectrophotometry coupled to PLS.

2. Experimental

2.1. Reagents

Vitamin B6 (Parafarm-Saporiti Laboratories, Buenos Aires, Argentina), vitamin B12 (Saporiti Laboratories, Buenos Aires, Argentina) and DEX (Saporiti Laboratories, Buenos Aires, Argentina) were of analytical grade quality. Aqueous stock solutions of B6 (1000 mg l^{-1}), B12 (1000 mg l^{-1}) and DEX (4000 mg l^{-1}) were prepared. Commercial injectables were obtained from the following laboratories: Flexicamin B12, Sidus SA (Buenos Aires, Argentina) and Sindrolen, Temis-Lostalo (Santa Fe, Argentina). Artificial samples were also prepared with the analytes in the same ratio as in the commercial samples. Doubly distilled water was used in all cases.

2.2. Apparatus and software

Absorption measurements were made on a Beckman DU 640 spectrophotometer, using 1.00 cm quartz cells. Spectra for subsequent manipulation by the PLS-1 program were saved as ASCII text files and transferred to a microcomputer.

Fluorescence spectra were collected on a Shimadzu RF 5300 PC spectrofluorometer, equipped

with a 150 W xenon lamp, and using 1.00 cm quartz cells.

PLS-1 was applied with the multivariate calibration program MULTIVAR, an in-house program written in Visual Basic 5.0 [31].

Capillary electrophoretic experiments were run on a SpectraPhoresis 100 instrument, equipped with a UV detector (Thermo Separation Products). Silica capillars of 0.75 μm (internal diameter), 70 cm (total length) and 42 cm (effective length) were employed.

2.3. Calibration samples for PLS-1

A calibration set of fifteen samples was prepared, using a central composite design in which five levels of concentrations of B6, B12 and DEX were introduced. The levels were in the range 53.00–87.00 mg l^{-1} for B6, 2.20–3.80 for B12 and 0.40–0.80 mg l^{-1} for DEX, spanning a range of B6:B12:DEX ratios from 45:2.5:1 to 160.5:1. The electronic absorption spectra for these samples were collected each 1 nm in the range 200–600 nm.

2.4. Unknown samples for PLS-1

In order to apply the multivariate PLS-1 method to commercial samples, the latter were treated in the following way. The volumes of ten ampoules of each pharmaceutical were measured in order to obtain the average volume. They were then mixed to obtain a pool. An appropriate aliquot of the latter solution was placed in a 10.00 ml volumetric flask, and completion to the mark was achieved by adding distilled water. The final concentrations were within the corresponding calibration ranges. Artificial samples (in triplicate) were prepared by mixing appropriate volumes of the three stock solutions and diluting with water, with final concentrations of B6, B12 and DEX lying in the corresponding calibration ranges and maintaining the concentration ratio present in the commercial pharmaceuticals. The electronic absorption spectra were then read and subjected to PLS-1 analysis.

2.5. Fluorescence emission method

In order to apply the fluorescence emission method to commercial samples for the determination of B6 (the only strongly fluorescent analyte), they were prepared in a similar way to that described above. B6 was determined by reading the emission intensity at 388 nm using $\lambda_{exc} = 290$ nm. The calibration plot was linear from 0 to 20 mg l^{-1}. Triplicate samples were prepared to final concentrations of ca. 10 mg l^{-1}.

2.6. Capillary electrophoretic method

All runs were performed in the presence of a borate buffer (5.0×10^{-2} mol l^{-1}, pH 8.5), using a voltage of 15 kV. Detection proceeded by UV–vis absorption at $\lambda = 240$ nm. The linear ranges of the three analytes were determined to be 0–300 mg l^{-1} (B6), 0–150 mg l^{-1} (B12) and 0–100 mg l^{-1} (DEX). Quantitation in the unknown samples proceeded by comparison of the corresponding peak areas with those of standard solutions of the following concentrations: 250.0 mg l^{-1} (B6), 10.0 mg l^{-1} (B12) and 10.0 mg l^{-1} (DEX).

2.7. Theory of PLS-1

Multivariate methods such as PLS involve a calibration step in which the relation between spectra and component concentrations is estimated from a set of reference samples, and a prediction step in which the results of the calibration are used to estimate the component concentrations in an unknown sample spectrum [32]. The PLS-1 version is optimised for the determination of a single analyte of interest, setting the optimum number of loading vectors A is in order to avoid overfitting. This is done by applying the cross-validation method described by Haaland [33].

2.8. Analytical figures of merit

Selectivity, sensitivity and limit of detection can be calculated and used for method comparison or to study the quality of a given analytical technique. We have calculated the sensitivity from:

$$\mathrm{SEN}_k = 1/\ \|\ \mathbf{b}_k\ \| \qquad (1)$$

where $\|\cdot\|$ indicates the Euclidian norm, and the selectivity from:

$$\mathrm{SEL}_k = \|\ \mathbf{s}_k^*\ \|\ /\ \|\ \mathbf{s}_k\ \| \qquad (2)$$

where $\mathbf{s}_k$ is the vector of spectral sensitivities of component k in pure form and $\mathbf{s}_k^*$ is the corresponding projection onto the net analyte signal space. Details concerning the latter calculations can be found in previous papers [34]. The limit of detection is given by:

$$\mathrm{LOD}_k = 3\ \|\ \boldsymbol{\varepsilon}\ \|\ \|\ \mathbf{b}_k\ \| \qquad (3)$$

The analytical sensitivity is defined as the ratio between the sensitivity and the noise level:

$$\gamma_k = \mathrm{SEN}_k /\ \|\ \boldsymbol{\varepsilon}\ \| \qquad (4)$$

It allows to compare analytical methods regardless of the specific technique, equipment and scaled employed, and establishes the minimum concentration difference (γ_k^{-1}) which is statistically discernible by the method along the dynamic range.

2.9. Wavelength selection

Although PLS-1 is usually considered as a full spectrum method, literature shows a growing tendency to perform variable selection before multivariate regression, in order to improve its predicting ability. This is especially so when highly diluted analytes occur within a matrix of more concentrated components with strongly overlapped spectra. Many different procedures have been published for wavelength selection [35]. We have employed a moving window strategy, previously found to be successful in chemical systems of similar complexity to the one under study [31]. Briefly, the cross-validation variance is computed using a variable number of latent factors in spectral ranges characterized by two parameters: the first sensor and the window width. A three-dimensional plot of the variance for the optimum number of factors in each window is produced, as a function of the latter two parameters. Location of the minimum variance allows to select an appropriate working spectral region. However, the technique should not be blindly applied: after selecting an adequate region, care should be taken in checking whether the results are consistent with the spectroscopic properties of the analytes at hand.

3. Results and discussion

3.1. Spectrophotometry-multivariate calibration

UV–vis spectra of solutions of the three pure components were recorded at three different pH (acid, neutral and alkaline), with the corresponding results shown in Fig. 1. A significant spectral overlap is apparent between the spectra of all three analytes in the region 215–300 nm at the three selected pH values. Furthermore, B6 and DEX display a strong collinearity in alkaline solutions (see Fig. 1C), which is not present in either strongly acid or neutral solutions. Since the mutual relationship among the three spectra is relatively unaltered in the pH range from acid to neutral, it was decided to work at a pH of ca. 6, which results on dissolving and diluting the commercial samples with distilled water.

The next step consisted in the study of Beer's law for the three analytes at pH 6. The estimated linearity ranges were as follows: 0–80 mg l^{-1} for B6 at the absorption maximum of 290 nm, 0–90 mg l^{-1} for B12 at 350 nm, and 0–15 mg l^{-1} for DEX at 235nm.

Spectral analysis suggested that the combination of derivation and zero-crossing could not be used for the simultaneous determination of all three analytes. Indeed, no optimum wavelength in the analyzed spectral range was found at which spectral derivatives could be useful. It was thus decided to apply the multivariate method PLS-1.

3.2. Calibration and prediction PLS-1 results

The cross-validation optimum number of factors and statistical parameters obtained when applying PLS-1 are shown in Table 1. These results were obtained in the wavelength ranges found to be optimal by the procedure described above. The use of full spectral ranges for all analytes led to very poor statistical indicators for the calibration. It should be noticed that the selected working

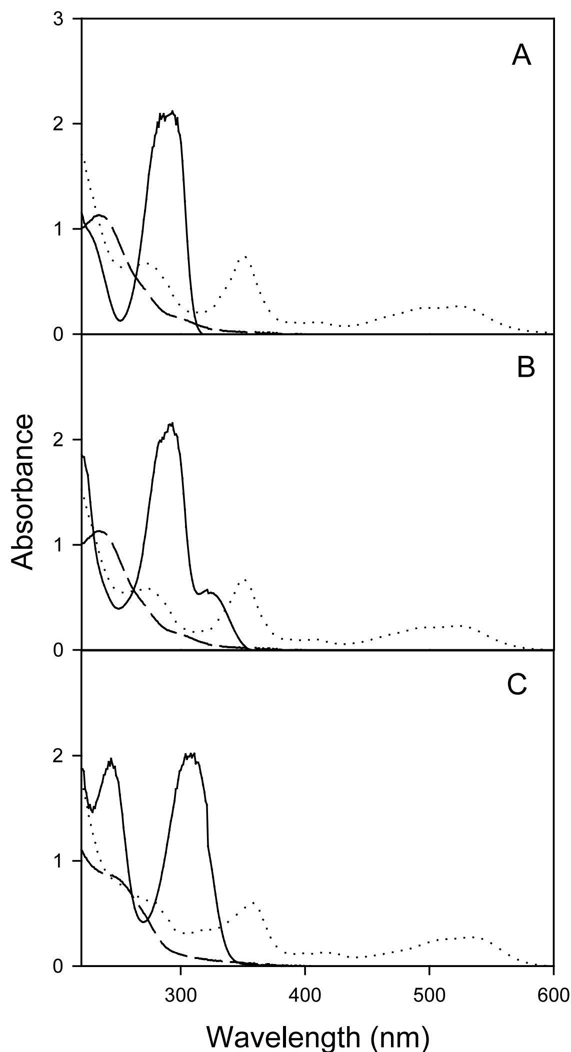

Fig. 1. UV–vis absorption spectra at different pH values of aqueous solutions of: (—) B6, (· · ·) B12 and (– – –) DEX. (A) pH 2, (B) pH 6, (C) pH 12. The concentrations are B6, 50.0 mg l^{-1}, B12 50.0 mg l^{-1} and DEX, 13.0 mg l^{-1}.

regions which are automatically found by computer calculations should always be checked against real spectra for consistency. In our case, the selected ranges coincide with the location of component spectral peaks with minimum overlapping, a fact which strongly supports their use for multivariate regression.

Both B6 and B12 show comparably good statistical indicators (Table 1). In the case of DEX these indicators are somewhat poorer (although still reasonable) due to its very low concentration in the mixtures. The figures of merit calculated by applying Eqs. (5)–(8) are summarized in Table 2. As can be seen, the sensitivity and selectivity is higher for B6, and its limit of detection is correspondingly lower as compared to the minor components.

Aqueous samples artificially spiked with vitamin B6, B12 and DEX and pharmaceutical formulations (see Section 2) were analyzed by this procedure and the results are shown in Table 3. As can be seen, the recoveries are satisfactory, even for the minor components B12 and DEX. All values reported in Table 3 are within the 90–110% limit recommended by Pharmacopeias.

Table 1
Calibration and validation results for the determination of vitamin B6, vitamin B12 and dexamethasone by spectrophotometry and PLS-1 analysis[a]

	B6	B12	DEX
Calibration results			
Spectral range	266–275	436–545	236–255
Number of factors	1	2	3
RMSE CV (mg l^{-1})	1.4	0.1	0.05
REP%	2.0	3.6	7.6
Validation results			
RMSEP (mg l^{-1})	2.2	0.1	0.04
REP%	3.0	3.3	6.4

[a]

$$\text{RMSE (CV or P)} = \left[\frac{1}{I-1}\sum_{1}^{I}(c_{\text{act}} - c_{\text{pred}})^2\right]^{1/2}$$

$$\text{REP\%} = \frac{100}{\bar{c}}\left[\frac{1}{I-1}\sum_{1}^{I}(c_{\text{act}} - c_{\text{pred}})^2\right]^{1/2}$$

Table 2
Analytical figures of merit of the spectrophotometric/PLS-1 method

	B6	B12	DEX
LOD_k (mg l^{-1})	0.04	0.09	0.08
SEL_k	0.99	0.44	0.09
SEN_k[a] (AU l mg^{-1})	0.036	0.017	0.020
γ_k (l mg^{-1})	72	34	40

[a] AU = absorbance units.

Table 3
Prediction results on artificial and commercial samples and comparison with capillary electrophoresis

		B6	B12	DEX
Actual content (mg)		250.0	10.00	2.00
Predictions				
Artificial sample	PLS-1	252 ± 5 (101%)	10.0 ± 0.3 (99%)	2.02 ± 0.08 (101%)
	CE	251 ± 1 (100%)	10.2 ± 0.2 (102%)	2.05 ± 0.01 (102%)
	t [a]	3.08	2.60	2.76
Flexicamin B12	PLS-1	253 ± 10 (101%)	10.1 ± 0.4 (101%)	1.93 ± 0.05 (97%)
	CE	250 ± 1 (100%)	10.0 ± 0.1 (100%)	2.01 ± 0.04 (100%)
	t [a]	2.99	3.33	2.76
Sindrolen	PLS-1	255 ± 8 (102%)	10.1 ± 0.3 (101%)	1.90 ± 0.06 (95%)
	CE	251 ± 1 (100%)	10.0 ± 0.1 (100%)	2.00 ± 0.02 (100%)
	t [a]	3.11	3.59	2.74

The results are averages of three replicates and are given in mg per sample. The sample volumes are: artificial sample, 3.00 ml, Flexicamin B12, 3.00 ml and Sindrolen, 5.00 ml. The recoveries for the commercial samples (between parenthesis) were calculated from the contents declared by the manufacturing laboratories.

[a] Calculated value of the statistical t coefficient for the comparison of means. Critical value at 99% confidence level and 4 degrees of freedom: 4.60.

3.3. Spectrofluorometric method

Vitamin B6 was determined in pharmaceutical injectables by applying conventional spectrofluorometry. Standard solutions of B6, B12 and DEX were prepared and excited at 290 nm, leading only B6 to emit fluorescence at 388 nm. In the case of B12 and DEX, no significant emission was registered in the spectral range examined for B6 (300–550 nm). Furthermore, inner filter from the minor components is not expected to seriously interfere.

Information about the maximum concentration at which linearity may be expected (i.e. absorbance of B6 < ca. 0.02 U) was obtained from its electronic absorption spectrum. According to linearity tests, the dynamic linear range was 0.07–18 mg l^{-1}, and the results obtained for the calibration graph were intercept = 1.0(5), slope = 23.9(5), r^2 = 0.9999 (fluorescence intensity units are arbitrary), RSD of a sample containing 10 mg l^{-1} = 0.4%.

The prediction results for B6 in commercial injectables by applying this procedure were excellent: 256 ± 5 mg per ampoule for Flexicamin B12 and 256 ± 7 mg per ampoule for Sindrolen (averages of ten ampoules), i.e. the recoveries calculated from the contents declared by the manufacturing laboratories are 102% in both cases. Both results are within the 90–110% limit recommended by Pharmacopeias.

3.4. Comparison with capillary electrophoresis

In order to compare the presently obtained results with a reference technique, capillary electrophoresis was applied to the same samples studied by PLS-1. The results, collected in Table 3, show comparably good recoveries using either method. Furthermore, a statistical t-test was applied in order to compare the pairs of means obtained, with the corresponding values also quoted in Table 3. In comparison with the critical t value (4.60 at 99% confidence level with 4 degrees of freedom), it can be concluded that the differences are not statistically significant.

4. Conclusions

A method has been developed for the simultaneous determination of vitamin B6, vitamin B12 and dexamethasone phosphate in injectables,

based on UV–vis spectral measurements combined with PLS-1 multivariate calibration. The recoveries on artificial and commercial samples are satisfactory. Vitamin B12 was determined in the same samples by fluorescence emission in the presence of both vitamin B6 and dexamethasone phosphate. The results compare favorably well with those obtained by the former method. PLS results were successfully validated against capillary electrophoresis.

Acknowledgements

Financial support from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Universidad Nacional de Rosario and Agencia Nacional de Promoción Científica y Tecnológica (project PICT99, no. 06-06078) is gratefully acknowledged. A. C. O. is a fellow of the John Simon Guggenheim Memorial Foundation (2001–2002).

References

[1] A. Goodman-Hillman, T. Rall, A. Nier, P. Taylor, The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, 1996.
[2] J.E.F. Reynolds (Ed.), Martindale. The Extra Pharmacopeia, The Pharmaceutical Press, London, 1993, p. 1056.
[3] W. Winkler, U. Hachula, J. Planar Chromatogr. Mod. TLC 10 (1997) 386.
[4] N. Erk, M. Kartal, Farmaco 53 (1998) 617.
[5] B. Morelli, J. Pharm. Sci. 84 (1995) 34.
[6] J.E.F. Reynolds (Ed.), Martindale. The Extra Pharmacopeia, The Pharmaceutical Press, London, 1993, p. 1054.
[7] I. Oversen, Drugs 27 (1984) 148.
[8] M. Kimura, K. Kanehira, K. Yokoi, J. Chromatgr. A 26 (1996) 722.
[9] S.F. Zhang, H.N. Li, D.M. Zhang, Q.Z. Zhou, Guangpuxue Yu Guangpu Fenxi 18 (1998) 488.
[10] R.T. Sane, V.J. Doschi, S.K. Joshi, J. Assoc. Anal. Chem. 66 (1983) 158.
[11] L.Q. Li, M.L. Yang, M.L. Feng, J.R. Lu, Fenxi Shiyanski 17 (1998) 5.
[12] J.E.F. Reynolds (Ed.), Martindale. The Extra Pharmacopeia, The Pharmaceutical Press, London, 1993, p. 728.
[13] M. Xi, Y. Yang, Z. Shao, Y. Peng, Yaowu Fenxi Zazhi 11 (1991) 291.
[14] M. Chen, X. Zhang, Q. Shao, L. Gan, Yaowu Fenxi Zazhi 11 (1991) 103.
[15] F. Song, Yiyao-Gongye 19 (1988) 177.
[16] X. Yao, Z. Qiao, Yiyao-Gongye 19 (1988) 172.
[17] P.N. Schil, B.C. Charles, J. Chromatogr. B Biomed. Appl. 658 (1994) 189.
[18] A.I. Santos-Montes, R. Gasco-Lopez, H. Izquierdo, Chromatographia 39 (1994) 539.
[19] D. Gagne, B.A. Lodge, J. Chromatogr. 193 (1980) 160.
[20] K.R. Liu, S. Hwie Chem, H.L. Wu, J Chromatogr. A 20 (1994) 455.
[21] V. Baeyens, E. Varerio, J.L. Veuthey, R. Gurny, J. Chromatogr. 13 (1997) 222.
[22] K. Raith, E. Althof, J. Bause, H. Neidhart, R.H.H. Neubert, Electrophoresis 19 (1998) 1907.
[23] W. Zhu, Yaowu-fenxi-Zashi 12 (1992) 311.
[24] M. Amin, J. Reusch, Analyst 112 (1987) 989.
[25] P.C. Damiani, M.A. Cabezon, M. Bearzotti, A.C. Olivieri, J. Pharm. Biomed. Anal. 17 (1998) 233.
[26] P.C. Damiani, M.A. Cabezon, M. Bearzotti, A.C. Olivieri, J. Pharm. Biomed. Anal. 20 (1999) 587.
[27] G.M. Escandar, Analyst 124 (1999) 587.
[28] J.A. Nepote, A.C. Olivieri, Anal. Chim. Acta 439 (2001) 87.
[29] H.C. Goicoechea, A.C. Olivieri, Talanta 49 (1999) 793.
[30] H.C. Goicoechea, A.C. Olivieri, Talanta 47 (1998) 103.
[31] H.C. Goicoechea, A.C. Olivieri, Trends Anal. Chem. 19 (2000) 599.
[32] H. Martens, T. Naes, Multivariate Calibration, Wiley, Chichester, 1989.
[33] D.M. Haaland, E.V. Thomas, Anal. Chem. 60 (1988) 1193.
[34] H.C. Goicoechea, A.C. Olivieri, Chemom. Intell. Lab. Syst. 56 (2001) 73.
[35] M.C. Ugulino Araújo, T.C. Bezerra Saldanha, R.K. Harrop Galvão, T. Yoneyama, H. Caldas Chame, V. Visani, Chemom. Intell. Lab. Syst. 57 (2001) 65.