Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 65 (2005) 710–715
Talanta
www.elsevier.com/locate/talanta

Optimisation of focused-microwave assisted digestion procedure for Kjeldahl nitrogen determination in bean samples by factorial design and Doehlert design

Maria das Graças A. Korn[a,*], Wagna P.C. dos Santos[a,b], Mauro Korn[c], Sérgio L.C. Ferreira[a]

[a] Núcleo de Excelência em Química Analítica (NQA-PRONEX), Grupo de Pesquisa em Química Analítica, Instituto de Química, Universidade Federal da Bahia, Campus Universitário de Ondina, Salvador, Bahia 40170-290, Brazil
[b] Centro Federal de Educação Tecnológica da Bahia, Salvador, Bahia, Brazil
[c] Universidade do Estado da Bahia, Campus Universitário do Cabula, Salvador, Bahia, Brazil

Received 4 May 2004; received in revised form 18 June 2004; accepted 27 July 2004
Available online 25 September 2004

Abstract

In the present paper a focused-microwave Kjeldahl digestion procedure without metal catalyst for nitrogen determination in bean samples was developed. Temperature at which the decomposition plateau occurs, mass of potassium sulphate and either volume of sulphuric acid or hydrogen peroxide were optimised. Results of the two-level full factorial design (2^4) based on an analysis of variance demonstrated that only the decomposition plateau temperature and the sulphuric acid volume were statistically significant. Optimal conditions for the digestion of bean samples were obtained by using Doehlert design. The modified digestion procedure of 0.25 g of bean samples has been performed in 27 min at optimised conditions. The accuracy of the developed procedure by the analysis of the two certified reference materials, peach leaves (NIST 1547) and apple leaves (NIST 1515). The *t*-test applied to the results revealed that they are in agreement ($p > 0.05$) with the certified values. The precision, expressed as relative standard deviation (R.S.D.) was of 0.96% for four successive Kjeldahl nitrogen determinations. In addition, interlaboratory exercises were performed with several bean samples in reference Brazilian food control laboratory.

Keywords: Kjeldahl nitrogen determination; Focused-microwave; Doehlert design

1. Introduction

Brazil is a country with continental dimensions and differences in social and economic opportunities. Different kinds of beans are daily consumed by the Brazilian population in high quantities, regardless of the region, social class, season of the year and people age. For many Brazilians, beans are the most important intake of proteins. Therefore, protein determination in different kind of beans is of paramount importance to assess people's nutrition and welfare.

Despite of spectroscopic [1,2], enzymatic [3,4] and chromatographic [5,6] methods have been proposed for protein determination in food samples, sample digestion by Kjeldahl's procedure [7] is still the most used one [8,9]. Notwithstanding, requests for environmental friendly analytical processes (e.g., characteristics of green methods) cannot be accomplished by the conventional Kjeldahl method, since, e.g., sample preparation needs a metal catalyst (Hg, Se or Cu). Kjeldahl microwave digestion is considered as a worthy alternative to overcome the drawback related to the use of a catalyst, since H_2O_2 has been used for complete decomposition of organic compounds. Some other advantages of Kjeldahl microwave digestion are the decrease of the digestion time and improved precision.

Chemometric experimental designs [10,11] and optimisation of some sample preparation procedures have been described. Factorial design was used for optimisation of a

---

[*] Corresponding author. Tel.: +55 712355166.; fax: +55 712355166.
E-mail address: korn@ufba.br (M.d.G.A. Korn).

0039-9140/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2004.07.047

dissolution procedure for food certified reference materials using microwave [12], for optimisation of microwave- and ultrasound-assisted acid leaching methods for heavy metals determination in sludges [13] and also for optimisation of digestion methods for sewage sludge [14].

Moreover, factorial and central composite designs were also employed for optimisation of food sample preparation processes, e.g., ultrasound-assisted extraction procedure for determination of tartaric and malic acids from grapes and winemaking by-products [15], ultrasound-assisted extraction procedure for the determination of trace elements in seafood products [16] and also for optimisation of a microwave pseudo-digestion procedure for determination of trace elements in seafood products [17].

Doehlert design [18] is a chemometric tool, frequently used for optimisation of analytical methods [19–21]. The Doehlert design permits estimation of the parameters of the quadratic model, building of sequential designs, detection of lack of fit of the model and use of blocks, as advantages over other response surface designs, such as composite central and Box–Behnken. They need fewer experiments, which are more efficient and can move through the experimental domain [22]. For instance, this experimental design has been used for optimisation of preconcentration procedures using solid phase extraction [23–25] and cloud point extraction [26] and also for optimisation of instrumental conditions for determination of arsenic [27] and silicon [28] in naphtha using graphite furnace atomic absorption spectrometry.

In the present work, the optimisation of Kjeldahl's digestion method for the determination of total proteins in bean samples assisted by focalised microwave radiation and based on hydrogen peroxide oxidation, was studied by using factorial and Doehlert designs.

2. Experimental

2.1. Apparatus

The Star 2 Plus (CEM, Indian Trail, NC, USA) system was used for focused-microwave digestions. This microwave oven can be operated using a maximum applied power generated by a specific 650 W magnetron with two individual reaction cells. The distribution of microwave radiation is controlled using a waveguide and slots. Infrared sensors measure the temperature in each cavity and, based a feedback system, interact with the magnetron to adjust the microwave radiation incidence in each vessel. Quartz reaction cells were used in this case because of the high boiling point of sulphuric acid digests (320 °C). Quartz air condensers were used in conjunction with the standard quartz vessels. The maximum volume of the reaction system is 250 ml, allowing digestion of up to 5 g of sample or even more, depending on the adopted procedure and the sample characteristics.

A distillation unit, BUCHI Kjeldahl line, model B-324, was used for ammonia distillation.

2.2. Chemicals and standards

All reagents were of analytical grade unless otherwise stated. For mineralisations sulphuric acid (96%, Merck, Darmstadt, Germany), hydroxide peroxide (30%, Merck, Darmstadt, Germany) and potassium sulphate (Merck, Darmstadt, Germany) were used. For distillation NaOH (50%, w/v, Merck) and boric acid (4%, w/v, Merck) were used. For titration standardised sulphuric acid (0.05 mol L^{-1}, Merck) and methyl purple (Merck) were used as indicator. Ultrapure water from an Easypure RF set up (Barnstedt, Dubuque, IA, USA) was used in all experiments. Due to the lack of beans certified reference materials or more similar CRM, two commercially available reference materials – peach leaves (NIST 1547) and apple leaves (NIST 1515) – were used to evaluate the accuracy of the procedure.

2.3. Microwave digestion

Approximately 0.25 g of sample was mixed with 6 mL of sulphuric acid and 1.5 g of potassium sulphate without heating. Then, the microwave program was carried out. Afterwards, the temperature decreased up to 200 °C, and then 10 mL of hydroxide peroxide were added, in aliquots of 1 mL, during 2 min. Then, the solution was heated for more 2 min. The total digestion time was 27 min. After cooling, the mixture was alkalinised with sodium hydroxide. Nitrogen, now in the form of ammonia, was distilled into a 4% (w/v) boric acid solution. The ammonia was titrated with 0.05 mol L^{-1} standard sulphuric acid using methyl purple as indicator.

2.4. Multivariate experiments and data processing

Optimisation of the digestion conditions was carried out using a two-level full factorial experimental design and Doehlert matrix design. Temperature of the decomposition plateau, volume of concentrated sulphuric acid, volume of hydrogen peroxide and potassium sulphate mass were experimental variables evaluated. The experimental data were processed by using the Statistica computer program [29].

3. Results and discussion

3.1. Design of experiments for estimation of optimum conditions

All factorial designs were developed using a ground bean sample (32 mesh). The focused-microwave-assisted digestion procedure had some fixed experimental conditions: time to reach the decomposition temperature, 10 min; temperature for hydrogen peroxide oxidation, 200 °C; time for hydrogen peroxide oxidation, 4 min; and the sample mass, 0.25 g. Hydroxide peroxide was added in 10 aliquots of 1 mL, without heating.

Table 1
Maximum and minimum levels of selected factors

Factors	Min (−)	Central point	Max (+)
Temperature of the decomposition plateau (°C)	280	300	320
Volume of sulphuric acid (mL)	8	10	12
Volume of hydrogen peroxide (mL)	6	8	10
Mass of potassium sulphate (g)	0.5	1.0	1.5

The variables temperature of the decomposition plateau, volume of concentrated sulphuric acid, volume of hydroxide peroxide and potassium sulphate mass were studied, along the experimental ranges shown in Table 1. The levels were chosen according to previous experiments.

3.2. Factorial design

A two-level full factorial 2^4 design with two replicates of central point was carried out in order to determine the influence of these factors as well as these interactions. The factorial design was evaluated using % (w/w) of protein as analytical response. The experimental design matrix and the protein % (w/w) for each trial are shown in Table 2. An analysis of the variance (ANOVA) demonstrated that, within the experimental range, only temperature of the decomposition plateau and volume of sulphuric acid were statistically significant. Interpretation of the Pareto chart (Fig. 1) leads to conclude that the microwave digestion is more efficient for lower temperature of the decomposition plateau, as revealed by the negative sign related to this effect. This is logical, since an increase in the temperature can cause loss of nitrogen during digestion.

Table 2
Experimental design and the results obtained in % (w/w) of protein

Experiment	*T*	VA	VP	MS	%PTN
1	–	–	–	–	21.96
2	+	–	–	–	11.50
3	–	+	–	–	21.90
4	+	+	–	–	21.35
5	–	–	+	–	22.22
6	+	–	+	–	12.98
7	–	+	+	–	22.25
8	+	+	+	–	19.07
9	–	–	–	+	22.73
10	+	–	–	+	13.91
11	–	+	–	+	21.48
12	+	+	–	+	22.50
13	–	–	+	+	22.53
14	+	–	+	+	20.41
15	–	+	+	+	22.46
16	+	+	+	+	20.74
17(CP)	0	0	0	0	22.87
18(CP)	0	0	0	0	22.38

T, temperature of the decomposition plateau; VA, volume of sulphuric acid (mL); VP, volume of hydroxide peroxide (mL); MS, mass of potassium sulphate (g); %PTN, protein (%).

3.3. Doehlert design

The factorial design demonstrated that the variables in the studied levels required a final optimisation. Thus, a Doehlert design involving the two most important variables was developed with such purpose. The experiments required by the Doehlert design are described in Table 3. Temperature of the decomposition plateau varied from 270 to 310 °C and volume of sulphuric acid from 8 to 12 mL, respectively.

The Doehlert matrix yielded the relation among temperature of the decomposition plateau (*T*), volume of sulphuric

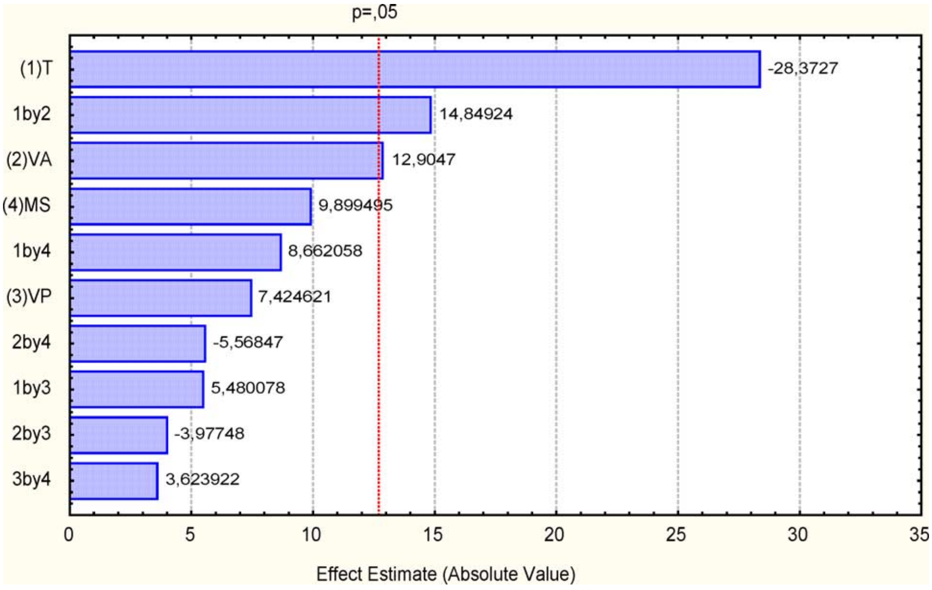

Fig. 1. Pareto chart of main effects obtained from 2^4 full factorial design. The vertical line defines the 95% confidence interval.

Table 3
Doehlert experimental design and levels of variables fixed

Experiment	Temperature (°C)	H_2SO_4 volume (mL)	%PTN (w/w)	
			Experimental	Predict
1	290	10	22.64	22.69
2	270	10	22.56	22.38
3	280	8	22.64	22.79
4	310	10	21.09	21.24
5	300	12	22.31	22.13
6	280	12	22.56	22.71
7	300	8	22.40	22.22
8 (CP)	290	10	22.74	22.69
9 (CP)	290	10	22.73	22.69

acid (VA) and % of protein (%PTN).

$$\begin{aligned}\%\text{PTN} = {} & -154 + 1.25(T) + 0.0462(\text{VA}) \\ & -2.20 \times 10^{-3}(\text{VA})^2 - 1.56 \times 10^{-3}(T)^2 \\ & -1.25 \times 10^{-4}(\text{VA})(T)\end{aligned}$$

The response surface considering the equation is shown in Fig. 2. The derivatisation of this equation as (T) and also as (VA) results in the equation system below [22]:

$$\frac{\partial\%\text{PTN}}{\partial(T)} = 1.25 - 0.00312(T) - 0.000125(\text{VA}) = 0$$

$$\frac{\partial\%\text{PTN}}{\partial(\text{VA})} = 0.0462 - 0.000125(T) - 0.00440(\text{VA}) = 0$$

From the obtained equation, after applying the Langrange's criterions [22] to verify the presence of a critical point, it was possible to deduce the coordinates of the maximum point of the surface, which corresponds to the point where the % (w/w) of protein achieves a more elevated value. So that, solving the equation, a critical point located at temperature of the decomposition plateau = 283.7 °C and volume of sulphuric acid = 3.5 mL was verified.

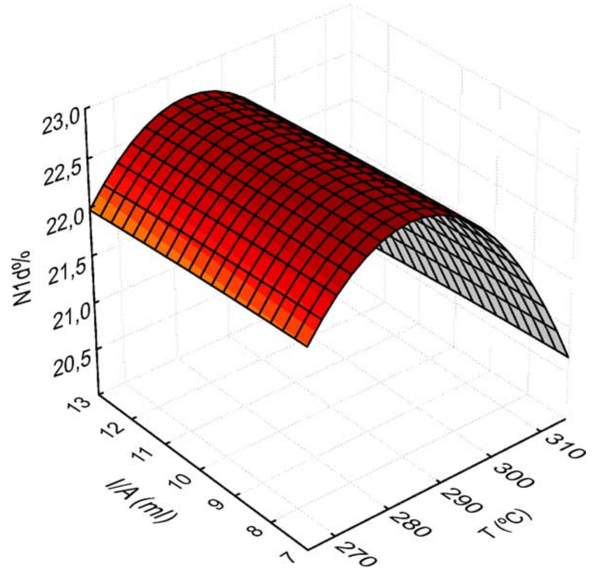

Fig. 2. Response surface estimated from factorial design by plotting temperature of the decomposition plateau (T) vs. volume of sulphuric acid (VA).

According to the results, the best temperature on the carbonisation plateau was within the studied range and close to the lower level, which could indicate losses by nitrogen volatilisation at higher temperatures. Therefore, for the following digestions using the optimised program, the temperature of 284 °C was established. The volume of concentrated sulphuric acid employed on the optimisation procedure was 6 mL. This volume was higher than the optimum indicated value of 3.5 mL. However, the response surface shows that a wide range of concentrated volume of sulphuric acid could also be used without compromising the analytical results. The choice of a higher volume was justified in order to avoid sample dehydration during digestion, as well as to keep the acid volume in the studied range. This care was necessary since loses of sulphuric acid was observed in the preliminary experiments.

4. Validation

Validation was performed considering the parameters: precision, robustness and accuracy by analysis of certified reference materials.

The precision of the proposed procedure, evaluated through the analysis of four replicates bean samples and calculated as the relative standard deviation (R.S.D.), was 0.96%.

The robustness of an analytical method is a measure of its capacity to reproduce results when the procedure is performed under small changes in the nominal values established in the optimisation step. It was studied using univariate [30] and multivariate [30–34] methodologies. Among the multivariate techniques: factorial designs [31], saturated fractional factorial design [32,33], central composite design [34] and other designs have been used for it. In the present paper, the robustness was evaluated developing a full factorial design (2^2), with four replicates of central point, involving the variables temperature of the decomposition plateau and sulphuric acid volume. The experiments were performed centred on the experimental conditions described in Table 4. The results considering the ANOVA demonstrated that these factors are not significant in the levels studied. It can also be seen in the Pareto chart (Fig. 3). Then, it can be concluded that this procedure is robust for variation of ±10 °C of temperature of

Table 4
Maximum and minimum levels of selected factors for robustness test

Factors	Min (−)	Central point	Max (+)
Temperature of the decomposition plateau (°C)	274	284	294
Volume of sulphuric acid (mL)	5	6	7

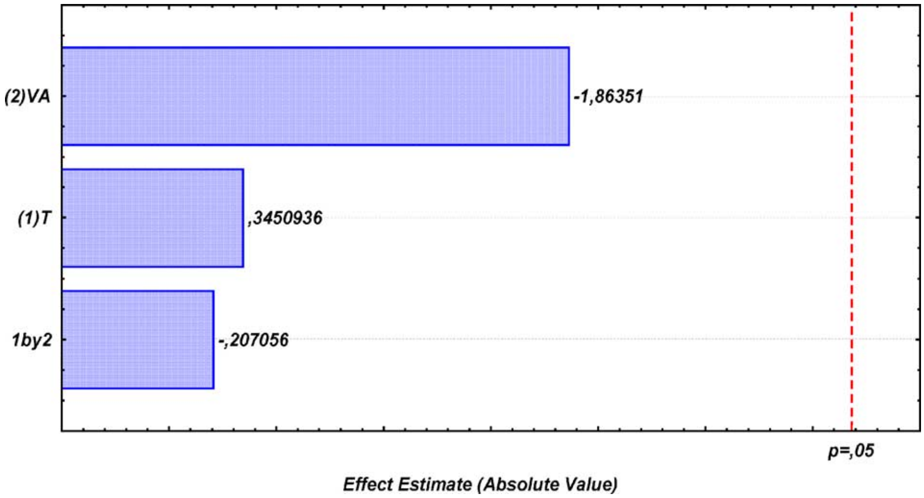

Fig. 3. Pareto chart of standardised effects for robustness study on the digestion procedure.

the decomposition plateau and sulphuric acid volume ±1 mL as recommended conditions by the procedure of 284 °C and 6 mL.

Accuracy of the developed procedure was evaluated by the analysis of two reference materials – peach leaves (NIST 1547) and apple leaves (NIST 1515). The results are shown in Table 5. Statistical comparison by the *t*-test showed no significant difference between the nitrogen values obtained from the proposed procedure and the certified values (95% confidence level).

Moreover, the proposed procedure was evaluated through microwave digestion of different amino acids bean samples constituents. Recovery achieved for determination of N in Lysine·HCl, refractory amino acid and present in the sample, was of 96.9% with R.S.D. of 1.82% (n = 3). Others amino acids were analysed and recoveries were around 100% (Table 6).

Table 5
Results of digestion used recommended procedure in certified reference material

Sample	*n*	Proposed method, %N (w/w)	Certified, %N (w/w)
Apple leaves[a]	4	2.25 ± 0.01	2.25 ± 0.19
Peach leaves[b]	4	2.84 ± 0.05	2.94 ± 0.12

[a] CRM apple leaves (NIST 1515).
[b] CRM peach leaves (NIST 1547).

Table 6
Results of focused-microwave-assisted Kjeldahl digestion of amino acids

Amino acids	%N (w/w)	*n*	Recovery (%)	R.S.D. (%)
L-Lysine.HCl	14.86 ± 0.67	3	96.93	1.82
L-Phenylalanin	8.58 ± 0.57	3	101.7	0.74
Tryptophan	13.16 ± 0.40	4	96.98	1.93
D-Leucine	10.91 ± 0.27	3	103.3	0.99
L-Asparagine	18.93 ± 0.30	4	102.5	1.00

Table 7
Protein (%, w/w) in different species beans (n = 4)

Bean type	Class	Species	%PTN (w/w)	
			Proposed method	Reference laboratory
1	White	*Vigna unguiculata*	22.7 ± 0.4	23.1 ± 0.8
2	Cores	*Phaseolus vulgaris* L.	21.3 ± 0.4	19.8 ± 0.4
3	Back	*P. vulgaris* L.	20.9 ± 0.1	21 ± 2
4	White	*P. vulgaris* L.	22.0 ± 0.2	22.3 ± 0.3

4.1. Analytical application

The proposed procedure was applied to protein determination in different samples of beans. The results, that varied from 19.8 to 23.1% (w/w) of protein (Table 7), were compared with those obtained by the official method (AOAC method 991.20, 33.2.11) and good agreement was observed when the Student’s *t*-test was applied at a probability level of 95%.

5. Conclusions

Application of factorial design and Doehlert matrix allowed the optimisation of a procedure for the rapid and safe digestion of beans assisted by focused microwave digestion. The main goals obtained with the method proposed are a reduction in sample contamination and analyte losses. It can also be considered that by applying the proposed method the sample manipulation and the sample preparation were diminished, as well as the amounts of sample and reagent. The accuracy of the proposed procedure was statistically evaluated by comparing the results obtained for certified materials (peach leaves, NIST 1547 and apple leaves, NIST 1515) with their certified values, resulted to be very good. Robustness test of the model was performed. Finally, the Kjeldahl’s analysis of nitrogen using microwave digestion was applied to several bean samples. Thus, the proposed procedure is more attractive due to a minimum reagent cost and reduced time (time less than 35 min compared with the reference method, about 4 h). Moreover, the used hydrogen peroxide greatly simplifies this method, whereas official methods require the addition of metal catalyst.

Acknowledgements

The authors are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and PRONEX/CNPq/FAPESB (Proc. No. 40/03) by the financial support. M.G.A.K., S.L.C.F. and M.K. would also like to thank CNPq for research scholarships provided.

References

[1] K. Kemsley, H.S. Tapp, A.J. Scarlett, S.J. Miles, R. Hammond, R.H. Wilson, J. Agric. Food Chem. 49 (2001) 603.
[2] F. Vianello, S. Bortoluzzi, L. Zennaro, A. Rigo, J. Biochem. Biophys. Methods 51 (2002) 263.
[3] H. Rohm, E. Tschager, D. Jaros, Lebensm. – Wiss. U. – Technol. 29 (1996) 191.
[4] S.K.C. Chang, E. Holm, J.G. Schwarz, P. Rayas-Duarte, Food Anal. Chem. 67 (1995) 127R.
[5] P.E. Jackson, J. Krol, A.L. Heckenberg, M. Mientijes, W. Staal, J. Chromatogr. 546 (1991) 405.
[6] R.D. Etheridge, G.M. Pesti, E.H. Foster, Anim. Feed Sci. Technol. 73 (1998) 21.
[7] R.B. Bradstreet, The Kjeldahl Method for Organic Nitrogen, Academic Press, London, 1965.
[8] X.T. He, R.L. Mulvaney, W.L. Banwart, Soil Sci. Am. J. 54 (1990) 1625.
[9] J. Alvarado, M. Márquez, L.E. León, Anal. Lett. 21 (1988) 357.
[10] C. Pasquini, J. Braz. Chem. Soc. 14 (2003) 198.
[11] R.M. Garcia-Rey, R. Quiles-Zafra, M.D. Luque de Castro, Anal. Bioanal. Chem. 377 (2003) 316.
[12] A.A. Mohd, R. Dean, W.R. Tomlinson, Analyst 117 (1992) 1743.
[13] S.R. Segade, M.C.D. Albor, E.F. Gomez, E.F. Lopez, Int. J. Environ. Anal. Chem. 83 (2003) 343.
[14] I. Lavilla, B. Perez-Cid, C. Bendicho, Fresenius J. Anal. Chem. 361 (1998) 164.
[15] M. Palma, C.G. Barroso, Anal. Chim. Acta 458 (2002) 119.
[16] P. Bermejo-Barrera, O. Muniz-Naveiro, A. Moreda-Pineiro, A. Bermejo-Barrera, Anal. Chim. Acta 439 (2001) 211.
[17] P. Bermejo-Barrera, A. Moreda-Pineiro, O. Muniz-Naveiro, A.M.J. Gomez-Fernandez, A. Bermejo-Barrera, Spectrochim. Acta Part B 55 (2000) 1351.
[18] D.H. Doehlert, Appl. Stat. 19 (1970) 231.
[19] M. Nechar, M.F. Molina, J.M. Bosque-Sendra, Anal. Chim. Acta 382 (1999) 117.
[20] M. Zougagh, A. García de Torres, J.M. Cano Pavón, Anal. Lett. 36 (2003) 1115.
[21] M. Zougagh, P. Cañada Rudner, A. García de Torres, J.M. Cano Pavon, J. Anal. At. Spectrom. 15 (2000) 1589.
[22] S.L.C. Ferreira, W.N.L. Santos, C.M. Quintella, J.M. Bosque-Sendra, Talanta 63 (2004) 1061.
[23] S.L.C. Ferreira, H.C. Santos, M.S. Fernandes, M.S. Carvalho, J. Anal. At. Spectrom. 17 (2002) 115.
[24] S.L.C. Ferreira, W.N.L. Santos, M.A. Bezerra, J.M. Bosque-Sendra, Anal. Bioanal. Chem. 375 (2003) 443.
[25] W.N.L. Santos, C.M.C. Santos, S.L.C. Ferreira, Microchem. J. 75 (2003) 211.
[26] M.A. Bezerra, A.B. Conceição, S.L.C. Ferreira, Anal. Bioanal. Chem. 375 (2003) 443.
[27] M.V. Rebouças, S.L.C. Ferreira, B.B. Neto, J. Anal. At. Spectrom. 18 (2003) 1267.
[28] A.A. Amaro, S.L.C. Ferreira, J. Anal. At. Spectrom. 19 (2004) 246.
[29] Statistica for Windows, Statsoft, Inc., Tulsa, USA, 1999.
[30] J. Aybar-Muñoz, A.M. García-Campaña, L. Cuadros-Rodríguez, Talanta 56 (2002) 123.
[31] K. Jorgensen, L. Jacobsen, Int. J. Pharm. 88 (1992) 23.
[32] L. Cuadros-Rodríguez, R. Blanc-García, A.M. García-Campaña, J.M. Bosque-Sendra, Chemometr. Intell. Lab. Syst. 41 (1998) 57.
[33] M.F. Molina, M. Nechar, J.M. Bosque-Sendra, Anal. Sci. 14 (1998) 791.
[34] E. Varesio, J.Y. Gauvrit, R. Longeray, P. Lanteri, J.L. Veuthey, Electrophoresis 18 (1997) 931.