ELSEVIER

Journal of Chromatography A, 778 (1997) 247–253

JOURNAL OF CHROMATOGRAPHY A

Short communication

Determination of water-soluble vitamins in infant milk by high-performance liquid chromatography

Soledad Albalá-Hurtado, M. Teresa Veciana-Nogués, María Izquierdo-Pulido, Abel Mariné-Font*

Unitat de Nutrició i Bromatologia, Facultat de Farmacia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain

Abstract

A rapid, simple and reliable liquid chromatographic method has been developed for the simultaneous determination of nicotinamide, thiamin, riboflavin, pyridoxine, pyridoxal, pyridoxamine, cyanocobalamine and folic acid in liquid and powdered infant milk. Ion-pair chromatography with a reversed-phase C_{18} column is used. Six vitamins were resolved in a single analysis; total analysis time never exceeded 55 min. A mobile phase of methanol–water (15:85), 5 m*M* octanesulfonic acid, with 0.5% triethylamine at pH 3.6 and a flow-rate of 1.0 ml/min gave the most satisfactory separation of these vitamins using a UV detector set at different wavelengths. Sample preparation involves acidification to precipitate proteins, and centrifugation followed by gravity filtration. Linearity, precision, recovery and sensitivity were always satisfactory. Detection limits ranged from 0.02 to 0.10 μg/ml and determination limits ranged from 0.03 to 0.25 μg/ml. © 1997 Elsevier Science B.V.

Keywords: Food analysis; Infant formulas; Vitamins

1. Introduction

Vitamins are essential for the normal health and growth of the child and hence, there is a need to measure the amounts of each type of vitamin present in infant foods [1]. Loss of vitamins can be related to the intensity of food processing and to the duration of food storage. Supplementation of infant milk formulae with vitamins is intended to compensate for the loss of these compounds due to the heat treatment to which they are subjected during manufacture.

Traditional methods for vitamin determination require the analysis of each vitamin individually by widely differing physical, chemical and biological methods [2,3], including colorimetric, fluorometric, spectrophotometric, and titrimetric techniques. The choice of method usually depends on the accuracy and sensitivity required and the interferences encountered in the sample matrix [4]. Nowadays, there is a growing need for more rapid and specific methods for vitamin analysis. High-performance liquid chromatographic (HPLC) techniques allow rapid separation and quantification of water-soluble vitamins in food using ion-exchange [4,5] or reversed-phase [6–8] columns and UV or fluorometric detection.

Early liquid chromatographic methods for the determination of water-soluble vitamins were tedious, and their utility was limited because each water-soluble vitamin presents particular chemical characteristics, such as stability, polarity and acidity. In 1979, Toma and Tabekhia [9], by including an ion-pairing reagent in the mobile phase and a reversed-

*Corresponding author.

PII S0021-9673(97)00387-7

phase column, obtained good resolution in the analysis of thiamine, riboflavin, and niacin. Since then, several ion-pairing chromatographic methods have been published for the determination of water-soluble vitamins, in a wide range of products, such as rice [9], multivitamin mineral preparations [10], milk [5,7,11], oral liquid tonics [6], and eggs [12].

Several detection methods can be applied; however, UV detection is the most common [6,9,10,12]. Although UV detection is inherently less sensitive than fluorometric detection, sample preparation for UV is simpler than for fluorometric detection, since derivatization of the vitamins is necessary to obtain the corresponding fluorescent derivatives [13–16], except for pyridoxine and riboflavin, which show a natural fluorescence response. Moreover, the advantage of measuring several compounds in a single chromatographic run offers food analysts an attractive alternative for vitamin determination.

Today a variety of different infant formulae is available on the world market. Most of these are marketed in Europe in powdered form, whereas formulae in the USA market are mostly in liquid form [17]. Recently, HPLC procedures have been used to determine thiamin and riboflavin in infant formulae [5,7]. However, no references are available for the simultaneous determination of more than two water-soluble vitamins in these products. The aim of this study was to develop a rapid and reliable method for the simultaneous determination of eight water-soluble vitamins in both liquid and powdered infant milk products. The ion-pair HPLC method proposed involved the use of a variable-wavelength UV detector which allowed the determination of a wide range of water-soluble vitamins at their optimal wavelength of absorbtion: nicotinamide, pyridoxal, pyridoxine and pyridoxamine (vitamins B_6), riboflavin (vitamin B_2), thiamine (vitamin B_1), cyanocobalamin (vitamin B_{12}), and folic acid.

2. Experimental

2.1. Reagents and solvents.

Methanol was of HPLC grade (SDS, Barcelona, Spain). Other chemicals were of reagent grade. Trichloroacetic acid (TCA) and glacial acetic acid were obtained from Panreac (Montplet and Esteban, Barcelona, Spain); triethylamine and sodium bicarbonate was from Merck (Barcelona, Spain); and sodium octanesulfonate was from Romil Chemicals (Cambridge, U.K.). Double-distilled water was obtained from a Milli-Q System (Millipore, Bedford, MA, USA).

Standards of nicotinamide, riboflavin, pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal, folic acid, thiamine hydrochloride, and cyanocobalamin were purchased from Sigma (Madrid, Spain). (1) Stock solutions: (a) 100 mg/l of riboflavin in 2.4% (v/v) aqueous acetic acid; (b) 500 mg/l of folic acid in 5% (w/v) sodium bicarbonate; (c) 1000 mg/l of nicotinamide, pyridoxal, pyridoxine, pyridoxamine, thiamine, and cyanocobalamin in 2.4% (v/v) aqueous acetic acid;. (2) Intermediate solutions: 10 mg/l of riboflavin in aqueous acetic acid, 5 mg/l of folic acid in sodium bicarbonate and 50 mg/l of the rest of vitamins in aqueous acetic acid; (3) Working solutions: 3, 5, 7, 8, 10, 20 and 25 mg/l for nicotinamide and 0.05, 0.1, 0.5, 0.8, 1, 1.5, 2, 3 and 5 mg/l for the other analytes, all of them in 2.4% aqueous acetic acid. All standard solutions should be filtered through a 0.45 μm membrane (Millipore), protected from light, and stored at 4°C.

The mobile phase containing 5 m*M* octanesulfonic acid, 0.5% triethylamine, 2.4% glacial acetic acid, and 15% of methanol was prepared as follows: Transfer 1.10 g of octanesulfonic acid sodium salt to a large beaker and add approximately 800 ml double-distilled water. Then, add ca. 24.0 ml glacial acetic acid and 5.0 ml of triethylamine to the aqueous solution. Mix thoroughly, and adjust pH to 3.6±0.1 with acetic acid or triethylamine. Add 150 ml LC grade methanol, mix thoroughly, and transfer to 1.0 l volumetric flask. Fill to volume up to 1.0 l with double distilled water. Filter solution through 0.45 μm membrane before HPLC injection.

2.2. Equipment.

The HPLC system (Hewlett-Packard, CA, USA) consisted of an HP 1050 system controller pump, an HP 1050 Series degassing device, an HP 1100 autosampler with 20 μl fixed loop injector, and an HP 1050 Series UV detector. Data acquisition was accomplished by a Chemstation system HP 3365-II.

The separation was performed on a Tracer Spherisorb ODS 2 C_{18} column 250×4.6 mm, 5 μm (Teknokroma, Barcelona, Spain), with a matching guard cartridge. Analyses were carried out isocratically at room temperature at a flow-rate of 1 ml/min. The total run time required was less than 55 min.

2.3. Sample preparation.

(a) Liquid infant milk: 10.5 g of sample were accurately weighed into a 50 ml centrifuge tube (30 mm diameter). Then, 1 g TCA solid and a magnetic stirring bar were added. The mixture was thoroughly shaken for 10 min over a magnetic stirring plate and centrifuged for 10 min at 1250 g to separate the two phases. After, 3 ml 4% TCA were added to the solid residue obtained, mixed thoroughly for 10 min, and centrifugated. Solid-phase was discarded. The two acid extracts were combined in a 10 ml volumetric flask and the volume was filled with 4% TCA. Samples should be always protected from light by covering tubes and flasks with aluminum foil and working under subdued lighting conditions. (b) Powder infant milk: 8.0 g were accurately weighed, and 10 ml of double-distilled water were added. Then, preparation of sample was as described for liquid infant milk. Acid extracts were filtered through a 0.45 μm filter prior to HPLC analysis.

3. Results and discussion

First, scan analysis of standard vitamins was performed to check the optimum conditions for the detection. Wavelengths were changed according to the elution time of each vitamin, as is shown in Table 1. The mobile phase used is based on the phases described by Lam et al. [10] for determination of water-soluble vitamins in multivitamin preparations and by Maeda et al. [6] for oral liquid tonics. A study of pH and the proportion of methanol was necessary to improve resolution in the milk infant formulae. When the proportion of methanol was 20% the vitamins eluted in less than 30 min; however, resolution was poor since nicotinamide eluted within the matrix. We used a mobile phase with 15% of methanol, which increased the total time of the analysis, but provided a better resolution,

Table 1
Program of wavelength changes during elution time for water-soluble vitamin determination in infant milk formulae

Vitamin	Time (min)	λ (nm)
Nicotinamide	0.0– 6.0	261
Pyridoxal	6.1– 8.0	287
Pyridoxine	8.1–13.0	290
Pyridoxamine	8.1–13.0	290
Folic Acid	13.1–16.0	282
Riboflavin	16.1–19.5	268
Cyanocobalamin	19.6–40.0	361
Thiamine	40.1–60.0	246

since nicotinamide eluted after the matrix. The pH of the mobile phase was an extremely critical factor for the separation of vitamins, as previously has been reported [6,10]. pH values from 3.3 to 4.0 were studied and pyridoxamine and folic acid were well resolved only when pH was 3.6±0.1. Fig. 1 shows typical chromatograms of vitamin standards and an infant milk formula. No differences were found between chromatograms obtained from liquid and powder infant milk formulae.

High contents of pyridoxal in infant milk have been related with convulsive attacks in infants [18]. The method proposed allows the resolution of different forms of vitamin B_6, such as pyridoxal, pyridoxine and pyridoxamin. In infant milk formulae only the addition of pyridoxine is allowed by Spanish law regulations [19]. However, pyridoxal is the main form present in raw milk, and it could be determined by the proposed method.

3.1. Analytical characteristic of the HPLC method

3.1.1. Linearity

Linearity was verified by analysis of variance of the regression (Table 2). An r value above 0.9919 was obtained for all vitamins ($P<0.001$), except for vitamin B_{12} with an r value above 0.9765. Coefficients of determination (r^2) were greater than 95.36% for vitamin B_{12} and greater than 98.38% for the other standard curves.

3.1.2. Precision

Eight determinations of the same sample were performed using the same reagents and apparatus to

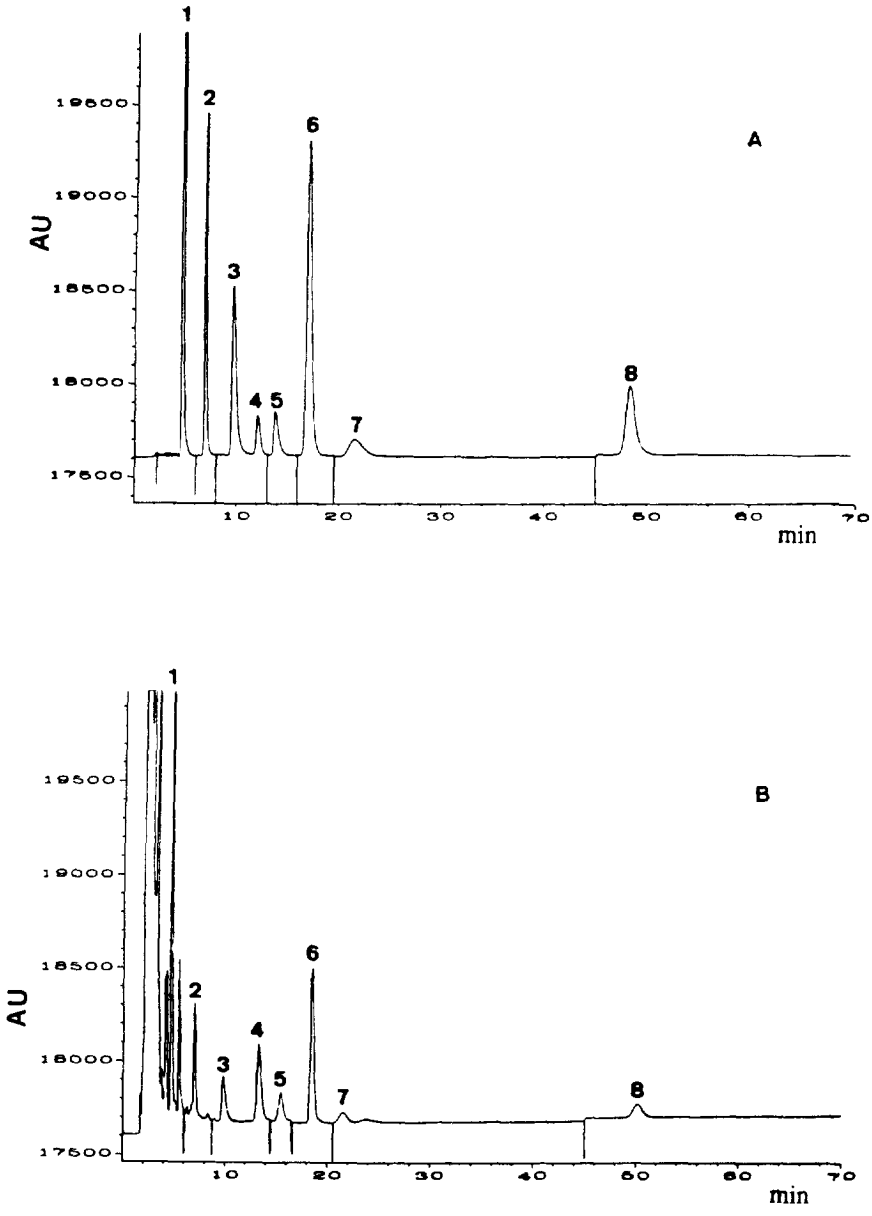

Fig. 1. Chromatograms of water-soluble vitamins. Standard solution (A), infant milk sample (B). Peak identities: nicotinamide (1), pyridoxal (2), pyridoxine (3), pyridoxamine (4), folic acid (5), riboflavin (6), cyanocobalamin (7) and thiamine (8).

evaluate the method precision in liquid and powdered infant milk. Samples were spiked with a known quantity of pyridoxal and pyridoxamine since, although both are present in raw milk, they were not detected in infant milk. Table 3 shows the precision of the method for determination of water-soluble vitamins in powder and liquid infant milk. The relative standard deviations obtained for all

Table 2
Linearity of standard curves of water-soluble vitamins

Vitamin	r	r^2	Linearity test		
			F^{a}_{exp}	DF[b]	P
Nicotinamide	0.9997	99.94	3699.7	1.39	$P<0.001$
Pyridoxal	0.9992	99.83	4257.1	1.39	$P<0.001$
Pyridoxine	0.9994	99.88	6532.5	1.39	$P<0.001$
Pyridoxamine	0.9994	99.88	5771.2	1.39	$P<0.001$
Folic Acid	0.9919	98.38	2956.2	1.39	$P<0.001$
Riboflavin	0.9997	99.95	6548.7	1.39	$P<0.001$
Cyanocobalamin	0.9765	95.36	890.3	1.39	$P<0.001$
Thiamine	0.9983	99.67	3358.4	1.39	$P<0.001$

[a] $F_{tab(1;39;\ 0.001)}=39.16$. F_{tab} and F_{exp} are tabulated and experimental Snedecor's F values, respectively, in ANOVA analysis.
[b] DF, degrees of freedom.

vitamins in both samples were less than 10%, being always satisfactory according to the Horwitz's formula for intralaboratory studies [20].

3.1.3. Recovery

Recovery was tested by the standard addition procedure. Two addition levels were used for each vitamin in powdered (Table 4) and liquid (Table 5) milk samples. Eight determinations were carried out for each addition level. By statistical analysis (Cochran's test), we verified that the accuracy did not depend on vitamin content in the sample. Mean recoveries obtained were always satisfactory, and higher than 96% for nicotinamide, pyridoxal, pyridoxine, pyridoxamine and riboflavin, higher than 88% for thiamin and higher than 76% for cyanocobalamin and folic acid.

3.1.4. Sensitivity

To determine the detection and the determination limits, the Long and Winefordner criterion was used [21], which is the most common criterion used for chromatographic procedures. The blank used in this study was 40% TCA. Determination limits were less than 0.05 μg/ml for nicotinamide, pyridoxine, pyridoxal, pyridoxamine, riboflavin and folic acid, less than 0.1 for thiamin and less than 0.3 for vitamin B_{12}.

The method proposed to determine water-soluble vitamins in infant milk yields satisfactory results for

Table 3
Precision of method for water-soluble vitamin determination in liquid and powdered infant milk

Vitamin	Powdered milk (μg/g)			Liquid milk (μg/ml)		
	Mean±S.D.[a]	R.S.D. (%)	R.S.D.H.[b]	Mean±S.D.[a]	R.S.D. (%)	R.S.D.H.[b]
Nicotinamide	56.32±0.99	1.76	5.81	10.3±0.35	3.39	7.50
Pyridoxal	5.38±0.26	4.83	8.28	0.99±0.05	5.05	10.68
Pyridoxine	6.23±0.14	2.22	8.10	1.20±0.09	7.50	10.38
Pyridoxamine	4.96±0.33	6.66	8.38	0.85±0.06	7.05	10.93
Folic Acid	1.32±0.09	6.81	10.23	0.54±0.03	5.55	11.70
Riboflavin	11.0±0.36	3.27	7.43	1.42±0.05	3.52	10.11
Cyanocobalamin	2.26±0.13	5.75	9.43	0.39±0.02	5.12	12.29
Thiamine	3.99±0.18	4.51	4.33	0.55±0.04	7.27	11.67

[a] Mean±standard deviation.
[b] Maximum relative standard deviation allowed for intralaboratory studies according to Horwitz's formula.

Table 4
Recovery of method for determination of vitamins in powdered infant milk

Compound	Initial content (μg/g)	Content after addition(μg/g)		Cochran's test G^c_{exp}	Recovery (%)	
		Level I[a]	Level II[b]		Mean±S.D.	R.S.D. (%)
Nicotinamide	56.32	79.36±1.15	104.28±1.15	0.6298	97.94±1.29	1.32
Pyridoxal	5.38	7.54±0.29	10.04±0.26	0.7801	96.33±3.66	3.80
Pyridoxine	6.23	8.41±0.20	10.88±0.23	0.5139	96.95±1.96	2.02
Pyridoxamine	4.96	6.98±0.36	9.67±0.31	0.1439	97.09±3.21	3.31
Folic Acid	1.32	1.50±0.09	1.98±0.09	0.6017	78.96±3.53	4.47
Riboflavin	11.0	15.57±0.32	20.56±0.27	0.7212	97.80±1.23	1.26
Cyanocobalamin	2.26	2.68±0.13	3.70±0.14	0.5618	77.23±3.31	4.28
Thiamine	3.99	5.62±0.19	7.95±0.19	0.6526	88.92±2.63	2.96

[a] 200 μg for nicotinamide, 40 μg for riboflavin, 20 μg for pyridoxal, pyridoxine, pyridoxamine and thiamine, 10 μg for vitamin B_{12} and 5 μg for folic acid.
[b] 400 μg for nicotinamide, 80μg for riboflavin, 40 μg for pyridoxal, pyridoxine, pyridoxamine and thiamine, 20 μg for cyanocobalamine and 10 μg for folic acid.
[c] Cochran's test $G_{tab\ (7,\ 2,\ 0.05)}$=0.8332, C_{tab} and C_{exp} are tabulated and experimental Cochran's C values, respectively.

both liquid and powdered samples. In the literature, many methods have been reported for water-soluble vitamins in foods but the present study is believed to be the first reported on the simultaneous determination of six vitamins in infant milk. The method proposed is fast and offers satisfactory specificity, precision and accuracy. In addition, it is also capable of separating pyridoxal, pyridoxine and pyridoxamine.

Acknowledgments

The study was supported by the Comisión Interministerial de Ciencia y Tecnología (ALI 91-0252, ALI 94-0400) of the Ministerio de Educación y Ciencia and the Comissió Interdepartamental de Recerca i Innovació Tecnològica of the Generalitat de Catalunya. The authors thank Dr. Montserrat Rivero (ORDESA S.A.) for helpful discussions, Ms.

Table 5
Recovery of method for determination of vitamins in liquid infant milk

Compound	Initial content (μg/ml)	Content after addition (μg/ml)		Cochran's test G^c_{exp}	Recovery (%)	
		Level I[a]	Level II[b]	Mean±S.D.	R.S.D. (%)	
Nicotinamide	10.3	15.15±0.17	19.96±0.15	0.6781	98.10±0.91	0.92
Pyridoxal	0.99	1.45±0.05	1.96±0.07	0.5605	97.70±3.19	3.26
Pyridoxine	1.20	1.63±0.11	2.11±0.10	0.6539	96.95±3.82	3.94
Pyridoxamine	0.85	1.31±0.06	1.79±0.05	0.7209	98.06±2.24	2.29
Folic Acid	0.54	0.82±0.06	1.21±0.05	0.2836	78.74±2.27	2.88
Riboflavin	1.42	1.90±0.06	2.39±0.05	0.5734	98.14±1.26	1.28
Cyanocobalamin	0.39	0.69±0.04	1.07±0.04	0.7764	76.81±2.98	3.87
Thiamine	0.55	0.92±0.05	1.36±0.03	0.6395	89.46±2.14	2.39

[a] 50 μg for nicotinamide and 5 μg for riboflavin, pyridoxal, pyridoxine, pyridoxamine, vit B_{12}, folic acid and thiamine.
[b] 100 μg for nicotinamide, 10 μg for riboflavin, pyridoxal, pyridoxine, pyridoxamine, cyanocobalamin, folic acid and thiamine.
[c] Cochran's test $G_{tab\ (7,\ 2,\ 0.05)}$=0.8332, C_{tab} and C_{exp} are tabulated and experimental Cochran's C values, respectively.

Laura Gea for her technical assistance, and Mr. Robin Rycroft for English revision of the manuscript.

References

[1] R.B.H. Wills, C.G. Shaw, W.R. Day, J. Chromatogr. Sci. 15 (1977) 262.
[2] S. Adorisio, E. de Siena, G. Ingrao, G.P. Santaroni, Ital. J. Food Sci. 1 (1992) 33.
[3] P.M. Finglas, M.R.A. Morgan, Food Chem. 49 (1994) 191.
[4] R.C. Williams, D.R. Baker, J.A. Schmit, J. Chromatogr. Sci. 11 (1973) 618.
[5] B.K. Ayi, D.A. Yuhas, K.S. Moffett, D.M. Joyce, N.J. Deangelis, J. Assoc. Off. Anal. Chem. 69 (1986) 56.
[6] Y. Maeda, M. Yamamoto, K. Owada, S. Sato, T. Masui, J. Assoc. Off. Anal. Chem. 72 (1989) 244.
[7] E.C. Nicolas, K.A. Pfender, J. Assoc. Off. Anal. Chem. 73 (1990) 792.
[8] A. Sims, D. Shoemaker, J. AOAC Int. 76 (1993) 1156.
[9] R.B. Toma, M.M. Tabekhia, J. Food Sci. 44 (1979) 263.
[10] F.L. Lam, I.J. Holcomb, S.A. Fusari, J. Assoc. Off. Anal. Chem. 67 (1984) 1007.
[11] C. Lavigne, J.A. Zee, R.E. Simard, B. Béliveau, J. Food Sci. 54 (1989) 30.
[12] S. Ötles, Y. Hisil, Ital. J. Food Sci. 1 (1993) 69.
[13] J.K. Fellman, W.E. Artz, P.D. Tassinari, C.L. Cole, J. Augustin, J. Food Sci. 47 (1982) 2048.
[14] J. Augustin, J. Assoc. Off. Anal. Chem. 67 (1984) 1012.
[15] P. Wimalasiri, B.H. Wills, J. Chromatogr. 318 (1985) 412.
[16] F. Arella, S. Lahély, J.B. Bourguignon, C. Hasselman, Food Chem. 1 (1996) 81.
[17] G. Sawatzki, G. Georgi, G. Kohn, in: Ergon S.A. (Ed.), New Perspectives in Infant Nutrition, 11th International Symposium, Murcia, Spain, 1993, Ch. 5, p. 59.
[18] S.R. Tannenbaum, V.R. Young, M.C. Archer, in: S.A. Acribias (Ed.), Química de los Alimentos, Zaragoza, Spain, 1993, Ch. 7, p. 537.
[19] Ministerio de Relaciones con las Cortes y de la Secretaria del Gobierno, R.D. 1408/92, B.O.E. (Boletín Oficial del Estado), 11 (1993) 793.
[20] W. Horwitz, Anal. Chem. 54 (1982) 67.
[21] G.L. Long, J.D. Winefordner, Anal. Chem. 55 (1983) 712.