Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 830 (2006) 41–46
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Rapid high-performance liquid chromatographic method for Vitamin C determination in human milk versus an enzymatic method

M. Romeu-Nadal, S. Morera-Pons, A.I. Castellote, M.C. López-Sabater*

Dept. Nutrició i Bromatologia, Centre de Referència en Tecnologia dels Aliments (CeRTA), Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII, s/n, 08028 Barcelona, Spain

Received 21 July 2005; accepted 14 October 2005
Available online 2 November 2005

Abstract

Vitamin C is an antioxidant that can be considered a possible biomarker of oxidative stability in human milk. A high-performance liquid chromatographic method was developed and validated for determining the total Vitamin C (ascorbic acid and dehydroascorbic acid) and ascorbic acid levels in human milk. This method was then compared with an enzymatic method (a Colorimetric technique) for quantifying ascorbic acid levels. Repeatability and reproducibility were acceptable for all methods. However, the high-performance liquid chromatography (HPLC) technique provided more satisfactory results than the enzymatic method due to this last method detected 37% less ascorbic acid and does not determine the total Vitamin C because of the enzymatic method cannot reduce the dehydroascorbic acid (DHA) to ascorbic acid. Furthermore, the HPLC method has the added advantages that it requires less reagents and material, and is simpler and less time consuming than the enzymatic method. In conclusion, the drawbacks of this enzymatic method would justify its substitution for a HPLC method.

Keywords: Human milk; Ascorbic acid; Vitamin C; HPLC; Enzymatic method

1. Introduction

In 1997, the American Academy of Pediatrics issued a statement on breast feeding, summarizing its benefits to the infant and the mother, and set forth guidelines for pediatricians [1]. Breast milk is considered an ideal nutrient for both term and preterm infants up to 6 months of age [2], improving host defenses, digestion and absorption of nutrients, gastrointestinal function, and neurodevelopment [3].

Preterm infants have a reduced antioxidising capacity [4–6] and are often exposed to oxidant stress caused by infection, mechanical oxygen ventilation, intravenous nutrition and blood transfusions. Many of the disorders common to preterm infants, including chronic lung disease, necrotizing enterocolitis, prematurity retinopathy and intraventricular–periventricular hemorrhage are thought to be due to this imbalance between antioxidising capacity and oxidative stress [7,8].

Vitamins A, E and C play an important role in antioxidant activity and immunomodulation [9,10]. Ascorbic acid (AA) is the principal biologically active form but dehydroascorbic acid (DHA) also exhibits biological activity since it can be easily converted into AA in the human body [11–13]. Therefore, it is important to measure both AA and DHA when reporting total Vitamin C levels. Vitamin C presents in human milk plays several biochemical roles linked to the functioning of the immune system. It helps in the maintenance of a natural barrier against infection, stimulates leukocytes for their phagocytic and antimicrobial activity, augments antibody production and complement levels [14] and also enhances synthesis of interferon [15]. For growth, development and survival, infants need an optimum supply of ascorbic acid.

A survey of infant feeding practices indicated that 40% of the mothers who breastfed their infants expressed and stored their milk frequently in their home refrigerator/freezer prior to feeding [16]. Such handling and storage of human milk can result in the loss of components sensitive to oxidation, such as the Vitamin C [17]. The concentrations of the hydrophilic antioxidants ascorbic acid and dehydroascorbic acid in some biological samples for some time have been considered possible biomarkers

* Corresponding author. Tel.: +34 93 4024512; fax: +34 93 4035931.
E-mail address: mclopez@ub.edu (M.C. López-Sabater).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.10.018

of oxidative stress [8]. Therefore, AA and DHA could be also considered biomarker of oxidation or storage time of the human milk, being it the application of the method.

Several methods have been developed for the estimation of Vitamin C levels [18]. High-performance liquid chromatography (HPLC), using a UV detector is currently the most commonly used technique for the analysis of ascorbic acid in food [12,19–26]. Some HPLC methods require electrochemical [17,27,28] o fluorimetric detection [29–31], because of the low absorptivity of DHA in the ultraviolet range of the spectrum, but the necessary equipment is not always available in hospital laboratories. To solve this problem, some authors propose the previous reduction of DHA to AA using DL-dithiothreitol [8,17,27,32]. The quantification of the latter acid allows an indirect estimation of DHA levels. Enzymatic methods using commercial test kits are also used for determining ascorbic acid levels [33–36].

However, several problems are associated with the determination of Vitamin C levels using enzymatic methods, for example, the reduction of dehydroascorbic acid to ascorbic acid cannot be measured, the minor specification of the sample and the low recovery of the ascorbic acid [36].

The aim of this study was to developed and validate two HPLC methods for the routine determination of Vitamin C content in human milk: one for determining the total Vitamin C level (AA and DHA indirectly), and a second for determining only the ascorbic acid level. The HPLC method for ascorbic acid determination was then compared with an enzymatic method (commercial test kit).

2. Experimental

2.1. Collection of breast milk

Samples of human milk were collected from both breasts by means of a Chicco manual breast pump (Chicco®, Italy), following the manufacturer's instructions, from four healthy mothers aged 20–35 years, at the Extraction Unit of the department. Informed consent was obtained from the participating mothers. All the mothers had full term pregnancy. Mature human milk was collected into sterile opaque bottles during the first expression in the morning. The milk from different mothers was immediately pooled, and aliquots of 5 ml were transferred to plastic tubes. This volume was big enough to allow the analysis for triplicate of both methods in the same day, thus, providing a direct comparison. Until the ascorbic acid analysis, aliquots were stored at −80 °C for no longer than 1 month.

2.2. Chemicals and reagents

Stock standard solutions were prepared by dissolving ascorbic acid in 0.56% (w/v) *meta*-phosphoric acid solution with Milli-Q water and stored at 4 °C. The Milli-Q water was purified by passing it through a Millipore Compact Milli-Q water system (Bedford, MA, USA). The ascorbic acid standard with a purity of 99.7% was obtained from Merck (Darmstadt, Germany). The *meta*-phosphoric acid with a purity of 33.5–36.5% was purchased from Fluka (Buchs, Switzerland).

The solvents used, such as HPLC-grade acetic acid, and HPLC-grade methanol were purchased from Panreac (Barcelona, Spain) and SDS (Peypin, France), respectively.

The commercially available test kit used was purchased from Boehringer Mannheim, R-Biopharm, Roche. It was a colorimetric method for the determination of ascorbic acid in foodstuffs and other materials. The DL-dithiothreitol with a purity of 99% and the anhydrous citric acid were obtained from Sigma (St. Louis, MO, USA).

2.3. Determination of total Vitamin C and ascorbic acid through the HPLC method

Chromatographic measurements were made using a Hewlett-Packard (Waldbrom, Germany) Model 1050 pump system, a Waters 717 plus Autosampler (Milford, MA, USA), a UV–vis detector, SPD-10 AV VP (Shimadzu, Kyoto, Japan) and an HP-3365 Series II Chemstation. The analytical column used was a Tracer Spherisorb ODS2 C_{18} (250 × 4.6 mm I.D., 5 μm particle size) protected with a guard cartridge (Tracer, C_{18}, 5 μm), both from Tracer Analitica (Tecknokroma, Barcelona, Spain).

The aliquots of human milk were thawed to around 22 °C in a water bath, protected from light, and then mixed. To analyze total Vitamin C content, dehydroascorbic acid was reduced to ascorbic acid with DL-dithiothreitol. Exactly 300 μl of this mixed human milk and 800 μl of DL-dithiothreitol 100 mM were added into a special centrifuge and filtration tube (Microsep II MF, 0.45 μm from VWR, Barcelona, Spain). The mixture was shaken mechanically for 30 s and then the tube was kept in a dark room for 15 min. Three hundred microlitres of 0.56% (w/v) *meta*-phosphoric acid solution was then added and the mixture further shaken for 30 s and centrifuged at 10 °C (10 min, 3000 × *g*).

To analyze ascorbic acid, 300 μl of mixed human milk and 300 μl of 0.56% (w/v) *meta*-phosphoric acid solution were added to the same special centrifuge and filtration tube, shaken for 30 s and centrifuged at 10 °C (10 min, 3000 × *g*).

With both HPLC techniques, 50 μl of the filtrate was directly injected into the HPLC system. Isocratic chromatographic separation was carried out using a mobile phase of Milli-Q water with acetic acid (0.1%, v/v) and methanol in a relative proportion of 95:5 (v/v). The eluent flow-rate was 0.7 ml/min and the column temperature was 25 °C.

Ascorbic acid was identified by comparing the retention time of the sample peak with that of the ascorbic standard at 254 nm. Quantification was carried out using external standardization.

2.4. Determination of ascorbic acid using enzymatic method

Colorimetric measurements were performed on a Spectrophotometer UV–vis Model DU520 (Beckman, Fullerton CA, USA).

Ascorbic acid was determined following the commercially available test kit instructions for milk samples. It uses the capa-

bility of ascorbate to reduce 3-(4,5-dimethylthiazoyl-2)-2,5-diphenyltetrazolium bromide (MTT) in the presence of the electron carrier 5-methylphenazinium methosulfate (PMS) at pH 3.5 to the corresponding formazan molecule, which can be spectrophotometrically quantified by means of its light absorbance in the visible range at 578 nm. For the specific determination of ascorbic acid in a blank sample assay, ascorbic acid is the only fraction of the reducing substances present in the sample to be oxidatively removed by ascorbate oxidase in the presence of oxygen from the air. Dehydroascorbic acid does not react with MTT/PMS.

In this method, the absorbance differences for both the sample and blank sample were determined. Then, the absorbance differences for the blank sample were subtracted from the absorbance differences for the sample ($(A_2 - A_1)$ sample $- (A_2 - A_1)$ blank sample). Finally, ascorbic acid concentrations were calculated with the aid of the extinction coefficient of MTT-formazan, according to the general equation:

$$C = \frac{V \times \mathrm{MW}}{\varepsilon \times d \times v \times 1000}(\times \Delta A)$$

where C is the concentration g ascorbic acid/l human milk; V, the final volume (2700 ml); MW, the molecular weight of the substance to be assayed (176.13 g/mol); ε, the extinction coefficient for MTT-formazan at 578 nm (16.9 l mmol^{-1} cm^{-1}); d, the light path (1.00 cm); v, the sample volume (0.100 ml); and ΔA, the absorbance differences $(A_2 - A_1)$ sample $- (A_2 - A_1)$ blank sample.

The pH of human milk was adjusted to 3.5–4.0 by the addition of citric acid. All pH measurements were made with a MicropH 2001 pH meter (Crison, Alella, Barcelona, Spain). The aliquots of human milk were thawed to around 22 °C in a water bath protected from light, and then they were mixed. Specifically, 1 ml of mixed human milk and approximately 0.01 g citric acid were added into a special centrifuge and filtration tube (Microsep II). The tube was then shaken mechanically for 10 s and centrifuged for 10 min at 3000 × g at 10 °C. Finally, 100 μl of the filtrate was used in the test kit.

2.5. Statistical analysis

The values for ascorbic acid levels reported by the different techniques for each aliquot were compared using paired Student's t-tests. The level of statistical significance was set at 5%. The results were processed using the statistical package SPSS 10.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Validation of the HPLC method for total Vitamin C and ascorbic acid determination

The determination of the total Vitamin C using the HPLC method was developed following the method described by Furusawa, 2001. We optimized the reaction time and the reductor concentration to apply to the human milk.

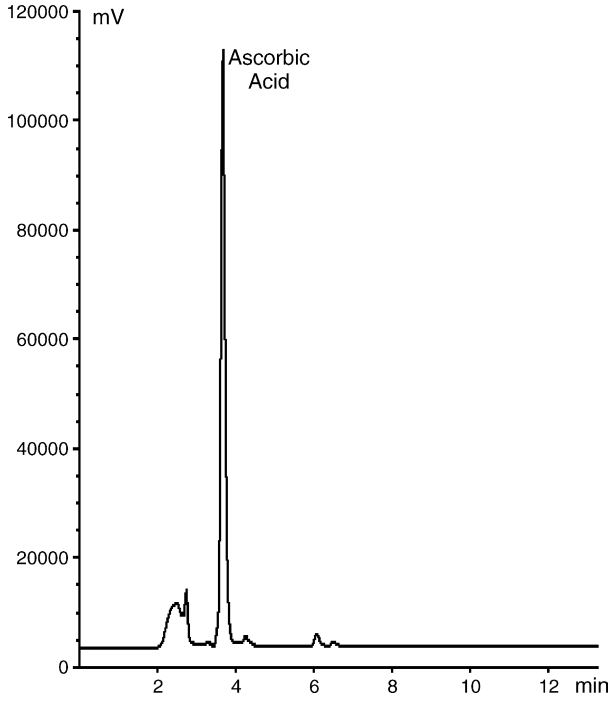

Fig. 1. Chromatogram of ascorbic acid in human milk. Refer to text for the HPLC conditions.

Fig. 1 shows the chromatogram of ascorbic acid standard. The resolution of the peak is good, the relative retention time is 3.7 min, and the baseline is stable.

The following parameters were determined: linearity, limits of detection and quantification, precision and recovery. In order to check the linearity of response to ascorbic acid, two linear regressions were calculated, one for the total Vitamin C determination, and the other for ascorbic acid determination (Table 1). Standard amounts ranged from 0.5 to 100 μg/ml for both determinations.

Table 1
Linearity, precision and recovery of the HPLC method for the total Vitamin C (expressed as ascorbic acid) and ascorbic acid determination

	Total Vitamin C	Ascorbic acid
Linearity[a] ($y = ax + b$)		
a: intercept	8663	12149
b: slope	−3422	−4789
r^2: determination coefficient	0.999	0.999
Precision		
Repeatability		
Mean (mg/100 ml) ± S.D.[b]	4.989 ± 0.13	4.499 ± 0.14
R.S.D. (%)	2.54	3.09
Reproducibility		
Mean (mg/100 ml) ± S.D.[b]	4.956 ± 0.18	4.528 ± 0.18
R.S.D. (%)	3.63	4.03
Recovery[c] (%)	95.06 ± 1.12	95.55 ± 1.18

Pooled human milk from $n = 4$.
[a] (y) area of ascorbic acid; (x) concentration of ascorbic acid (μg/ml).
[b] Standard deviation.
[c] Recovery of standard added in human milk expressed as mean (%) ± deviation standard.

Table 2
Linearity of the HPLC method and enzymatic method

		Linearity ($y = ax + b$)		
		a	b	r^2
HPLC method	Standard linearity in human milk[a]	13.071	−13523	0.999
Enzymatic method	Standard linearity in water[b]	0.0972	−0.0142	0.998
	Standard linearity in human milk[c]	0.0671	−0.0004	0.998

a, intercept; b, slope; r^2, determination coefficient.
[a] Linear regression from adding ascorbic acid to human milk. (y) area of ascorbic acid; (x) concentration of ascorbic acid (μg/ml).
[b] Linear regression from adding ascorbic acid to water. (y) the absorbance differences of ascorbic acid, ΔA: $(A_2 - A_1)$ sample $-$ $(A_2 - A_1)$ blank sample; (x) concentration of ascorbic acid (μg/ml).
[c] Linear regression from adding ascorbic acid to human milk. (y), the absorbance differences of ascorbic acid, ΔA: $(A_2 - A_1)$ sample $-$ $(A_2 - A_1)$ blank sample; (x) concentration of ascorbic acid (μg/ml).

Detection and quantification limits were calculated according to the USP criteria [37] by analyzing a number of blank samples and calculating the standard deviation of the background response. Multiplying the standard deviation by 3 and 10 provides estimations of the limits of detection and quantification, respectively. For ascorbic acid determination, these were 3 and 9 ng, respectively.

Precision was expressed as the relative standard deviation (R.S.D.) of replicate measurements. The repeatability was established by injecting the human milk six times. The reproducibility was determined by analyzing each sample of human milk on six different days. Table 1 shows precision results calculated for both HPLC analyses. These results met the acceptable precision standards proposed by Horwitz [38] for analyte concentrations with a range of 10–50 ppm.

Analyzing a sample five times and comparing the analytical result to the known added value showed the recovery of the method. For estimating the ascorbic acid levels, human milk samples were spiked with ascorbic acid at two fortification levels, 50 and 100% of the estimated initial ascorbic acid amount.

The recovery percentages were satisfactory for ascorbic acid measurement, using the HPLC techniques. The results obtained were acceptable because of these were of 95 and 96% (Table 1).

Table 4
Comparison of ascorbic acid percentages between different methods

Method	Ascorbic acid (%)
HPLC method using the linear regression from adding ascorbic acid to water[a]	100
HPLC method using the linear regression from adding ascorbic acid to human milk[a]	97
Enzymatic method using the linear regression from adding ascorbic acid to water[b]	83
Enzymatic method using the linear regression from adding ascorbic acid to human milk[c]	91
Enzymatic method using the Kit equation[d]	63

Percentage with different letters differ significantly ($P < 0.05$).

The conversion of dehydroascorbic to ascorbic acid was efficient because we realized different proofs: we reduced, with DL-dithiothreitol, a standard solution of DHA:AA (50:50) and we proved the conversion was 98%.

3.2. Comparison between the HPLC method and enzymatic method for ascorbic acid determination

The HPLC and enzymatic methods for ascorbic acid determination were compared. Ascorbic acid levels determined using the enzymatic method used the general equation and linear regression. Specifically, two linear regressions were prepared using the enzymatic technique. One plotted the addition of increasing amounts of ascorbic acid to Milli-Q water, while the other plotted the addition of the same amounts of ascorbic acid to human milk. The two linear regressions were linear over the range 0.5–5 μg/ml. For the ascorbic acid determination using the HPLC method, a linear regression was also plotted with human milk. This was linear over the range 0.5–100 μg/ml (Table 2).

The precision of the enzymatic method and HPLC method (using the linear regression from adding ascorbic acid to human milk) was expressed as the relative standard deviation of replicate measurements. The repeatability and reproducibility were determined the same way as for the HPLC method for total Vitamin C determination. Table 3 shows precision results. These results also met the acceptable precision standards proposed by Horwitz [38].

The HPLC method gave comparable results for linearity in water and human milk (Table 4), and we did not find any signifi-

Table 3
Precision of the HPLC and enzymatic methods for ascorbic acid determination

	HPLC method		Enzimatic method	
	Standard linearity in human milk	Standard linearity in water	Standard linearity in human milk	Test Kit equation
Repeatability				
Mean (mg/100 ml) ± S.D.[a]	4.321 ± 0.13	3.700 ± 0.10	4.148 ± 0.15	2.877 ± 0.10
R.S.D. (%)	3.12	2.76	3.52	3.55
Reproducibility				
Mean (mg/100 ml) ± S.D.[a]	4.346 ± 0.18	3.630 ± 0.15	4.106 ± 0.19	2.848 ± 0.13
R.S.D. (%)	4.07	4.03	4.53	4.56

Pooled human milk from $n = 4$.
[a] Standard deviation.

cant difference ($P > 0.05$) between the concentrations of ascorbic acid.

On the contrary, the enzymatic method using the general equation and the two linear regressions reported significantly differing concentrations ($P < 0.05$) of ascorbic acid (Table 4). Concretely, the concentration of ascorbic acid in human milk samples, according to the enzymatic method, ranged from 2.88 to 4.15 mg/100 ml, the lower value resulting from the use of the enzymatic equation (Table 3).

For each sample, the comparison between the two methods was performed on the same day and only was determined the L-ascorbic content, not the total Vitamin C. We observed that the amounts of ascorbic acid reported by the two methods were not identical. The HPLC and enzymatic methods reported significantly differing concentrations ($P < 0.05$) of ascorbic acid, with the latter method giving lower values (Table 4). Thus, whereas the HPLC method nearly completely recovered a given ascorbate concentration, the commercial test kit underestimated it by about 63% when applying the enzymatic kit equation.

In order to analyze the total Vitamin C concentration using the enzymatic method, we added dithiothreitol. However, this reductor interfered with the lecture in the visible range at 578 nm being the determination of the total Vitamin C impossible because of the enzymatic method is a colorimetric technique.

4. Conclusions

The presented results suggest that the proposed HPLC methods are reliable, reproducible, and sensitive techniques for detecting total Vitamin C and ascorbic acid in human milk. In addition, the enzymatic method for ascorbic acid determination in human milk is also a precise method.

However, we believe that the HPLC technique for ascorbic acid determination in human milk provides more satisfactory results than the enzymatic method due to this last method detected only 63% of the amount which was verified using the HPLC method. Furthermore, the enzymatic method does not determine the total Vitamin C because of the reduction of the dehydroascorbic acid to ascorbic acid cannot be measured. It is demonstrated that the HPLC method is an improvement compared with a commercially available test kit for the determination of both the total Vitamin C and ascorbic acid in human milk.

The low recovery of the ascorbic acid in human milk using the enzymatic method suggests that the matrix of human milk could interfere because of the amount of ascorbic acid reported using the linear regression with water was less than that using the linear regression with human milk.

The HPLC method requires fewer reagents and material, and it is simpler and less time-consuming (approximately 20 min instead of 45 min). Further, not being a colorimetric method, it is possible to analyze more human milk samples concurrently than the enzymatic method. The near-absence of sample preparation and the easy of use of these HPLC techniques make them an ideal quality control tool for the food industry.

In conclusion, the drawbacks of this enzymatic method would justify its substitution for a HPLC method.

Acknowledgements

This study was financed by the CeRTA (Centre de Referència en Tecnologia dels Aliments, Generalitat de Catalunya). Meritxell Romeu Nadal acknowledges financial support from a grant from Generalitat de Catalunya. We also thank mothers of "Alba" for their human milk and Robin Rycroft for revising the English manuscript.

References

[1] American Academy of Pediatrics: Work Group on Breastfeeding, Pediatrics 100 (1997) 1035.
[2] The Committee on Nutrition of the American Academy of Pediatrics, Breast-feeding, Pediatrics 62 (1978) 591.
[3] R.J. Schanler, N.M. Hurst, C. Lau, Clin. Perinatol. 26 (1999) 379.
[4] G.D. Georgeson, B.J. Szony, K. Streitman, Eur. J. Obstet. Gynecol. Reprod. Biol. 103 (2002) 136.
[5] G. Baydas, F. Karatas, M.F. Gursu, Antioxidant vitamin levels in term and preterm infants and their relation to maternal vitamin status, Arch Med Res 33 (2002) 276–280.
[6] N. Hanna, K. Ahmed, M. Anwar, A. Petrova, M. Hiatt, T. Heigyi, Arch. Dis. Child. Fetal Neonatal. 89 (2004) F518.
[7] D.W. Thibeault, Am. J. Perinatol. 17 (2000) 167.
[8] J. Lykkesfeldt, S. Loft, H.E. Poulsen, Anal. Biochem. 229 (1995) 329.
[9] L. Ahmed, S.K. Nazrul Islam, M.N.I. Khan, S.N. Nahid, Mal. J. Nutr. 10 (1) (2004) 1.
[10] L. Ahmed, S.K. Nazrul Islam, M.N.I. Khan, S. Huque, M. Ahsan, J. Trop. Pediatr. 50 (6) (2004) 357.
[11] S.K. Lee, A.A. Kader, Postharvest Biol. Technol. 20 (2000) 207.
[12] J.A. Tudela, J.C. Espín, M.I. Gil, Postharvest Biol. Technol. 26 (2002) 75.
[13] W.D. Graham, D. Annette, J. Chromatogr. 594 (1992) 187.
[14] W.R. Thomas, P.Z. Holth, Clin. Exp. Immunol. 32 (1978) 370.
[15] B.V. Siegel, Nutrition and immunology, Plenum Press, New York, 1993, p. 167.
[16] M.R. Bank, A. Kirksey, K. West, G. Giacoia, Am. J. Clin. Nutr. 41 (1985) 235.
[17] I.H. Buss, F. McGill, B.A. Darlow, C.C. Winterbourn, Acta Paediatr. 90 (2001) 813.
[18] S.P. Arya, M. Mahajan, P. Jain, Rev. Anal. Chim. Acta 417 (2000) 1.
[19] P. Wimalasiri, R.B.H. Wills, J. Chromatogr. 256 (1983) 368.
[20] S.H. Ashoor, W.C. Monte, J. Welty, J. Assoc. Off. Anal. Chem. 67 (1984) 78.
[21] M.J. Esteve, R. Farré, A. Frígola, J.C. López, J.M. Romera, M. Ramírez, A. Gil, Food Chem. 52 (1995) 99.
[22] M.A. Kall, C. Andersen, J. Chromatogr. B 730 (1999) 101.
[23] N. Furusawa, Food Control 12 (2001) 27.
[24] A. Pérez-Vicente, A. Gil-Izquierdo, C. García-Viguera, J. Agric. Food Chem. 50 (2002) 2308.
[25] C.L. Steffensen, H.J. Andersen, J.H. Nielsen, J. Agric. Food Chem. 50 (2002) 7392.
[26] C. Sánchez-Moreno, L. Plaza, B. Ancos, M.P. Cano, J. Agric. Food Chem. 51 (2003) 647.
[27] K.R. Dhariwal, P.W. Washko, M. Levine, Anal. Biochem. 189 (1990) 18.
[28] P.W. Washko, W.O. Hartzell, M. Levine, Anal. Biochem. 181 (1989) 276.
[29] B. Ancos, E.V. González, M.P. Cano, J. Agric. Food Chem. 48 (2000) 4565.
[30] H. Ostdal, H.J. Andersen, J.H. Nielsen, J. Agric. Food Chem. 48 (2000) 5588.
[31] J.H. Nielsen, G. Hald, L. Kjeldsen, H.J. Andersen, H. Ostdal, J. Agric. Food Chem. 49 (2001) 2998.
[32] M.J. Esteve, R. Farré, A. Frigola, J.M. Garcia-Cantabella, J. Chromatogr. B 688 (1997) 345.

[33] T.A. Bensinger, T.F. Zuck, B. Tolbert, S. McLaughlin, C.C. Peck, M. Knight, Biochem. Med. 19 (1) (1978) 118.
[34] T.Z. Liu, N. Chim, M.D. Kiser, W.N. Bigler, Clin. Chem. 28 (1982) 2225.
[35] W. Lee, S.M. Roberts, R.F. Labre, Clin. Chem. 43 (1) (1997) 154.
[36] C.D. Badrakhan, F. Petrat, M. Holzhauser, A. Fuchs, E.E. Lomonosova, H. de Groot, M. Kirsch, J. Biochem. Biophys. Methods 58 (2004) 207.
[37] The United States Pharmacopeia (USP XXIII), Mack Printing, Easton, 1989, p. 1711.
[38] W. Horwitz, Anal. Chem. 54 (1982) 67A.