ELSEVIER

Journal of Chromatography A, 822 (1998) 225–232

JOURNAL OF CHROMATOGRAPHY A

Ultrarapid detection of bovine whey proteins in powdered soybean milk by perfusion reversed-phase high-performance liquid chromatography

M.C. García[a], M.L. Marina[a,b], M. Torre[a,*]

[a]*Departamento de Química Analítica, Facultad de Ciencias, Universidad de Alcalá, Ctra. Madrid–Barcelona Km. 33.600, Alcalá de Henares, 28871 Madrid, Spain*

[b]*Centro de Tecnología de los Alimentos y Servicios Biosanitarios, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, Alcalá de Henares, 28871 Madrid, Spain*

Received 18 March 1998; received in revised form 8 June 1998; accepted 27 July 1998

Abstract

A perfusion reversed-phase high-performance liquid chromatographic method has been developed to simultaneously separate soybean and bovine whey proteins (α-lactalbumin and β-lactoglobulins (A+B)) in a very short analysis time (~5 min). The method consisted of a linear binary gradient water–acetonitrile–0.10% trifluoroacetic acid at a flow-rate of 3 ml/min, with the column thermostated at 60°C, and ultraviolet detection at 254 nm. This method enables the rapid detection of adulterations of powdered soybean milks by addition of bovine whey proteins. When bovine whey proteins were too low to be detected by direct injection of the sample, a previous acidic precipitation step was required in order to concentrate these proteins. Quantitative analysis of bovine whey proteins was also successfully performed. In fact, it was possible to detect about 1% and 1.3% of α-lactalbumin and β-lactoglobulins, respectively, in a commercial powdered soybean milk in which these proteins were included in its formulation. Results were compared with those obtained by conventional reversed-phase high-performance liquid chromatography.

Keywords: Proteins; Whey proteins

1. Introduction

There are different factors that may cause an allergy against animal whey proteins (constituted mainly by α-lactalbumin and β-lactoglobulins (A+B)). Mostly (85%), a genetic factor is responsible for this disease, but there are others such as an excessive intake of milk or inadequate working conditions (e.g., milk factory workers) [1]. Allergy against animal whey proteins causes low intestinal absorption and produces vomiting, diarrhoea, and dehydration and, occasionally, respiratory, skin, and psychological diseases [1]. Soybean dairy-like products (soybean milk, soybean infant formulas, yogurt- and cheese-like products, etc.) are an alternative for people with this problem [2,3]. Nevertheless, in some cases, animal whey proteins are added to soybean dairy-like products to enrich them. Thus, it is obvious that there is a need to develop new analytical methods for the detection of these products to prevent the potential adulterations resulting from

*Corresponding author.

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00619-0

the addition of whey proteins to soybean dairy-like products and their adverse effects on allergic people.

Although soybean and animal whey proteins have been characterized and determined separately using different techniques, including capillary electrophoresis [4] and high-performance liquid chromatography (HPLC) [5–11], little has been achieved concerning the simultaneous separation of both soybean and animal whey proteins. Recently, a reversed-phase high-performance liquid chromatographic (RP-HPLC) method was designed to perform the simultaneous separation of soybean and bovine whey proteins in approximately 20 min [12]. This method was applied to quantitate and detect bovine whey proteins in dairy-like soybean products. However, a decrease in the analysis time would be desirable to achieve rapid quality control in routine analysis.

Perfusion chromatography is a promising technique in the separation of biopolymers due to the rapidity of its analysis [13–16]. This technique overcomes the mass transfer problems associated with conventional chromatography by using packing materials of cross-linked polystyrene-divinylbenzene matrix having a bidisperse porous structure constituted by a macroporous region with 6000–8000 Å transecting pores (throughpores) and a connected network of smaller size diffusive pores (800–1500 Å) that provide a large adsorption surface area [15,17,18]. In perfusion chromatography, solute is transported by convection to the interior of the particle by the throughpores and inside that, it diffuses through the diffusivepores. This combination of large and diffusive pores in the perfusion particles, maximizes intra-particle convection and pore diffusion of solutes thus enhancing and accelerating the mass transfer of large molecules such as proteins [15,17–19]. This technique has been recently applied to the quantitation of soybean proteins in commercial soybean products [20].

The aim of this work is to take advantage of perfusion chromatography to carry out the simultaneous separation of soybean and bovine whey proteins in shorter analysis times than by conventional reversed-phase high-performance liquid chromatography in order to apply this method to detect adulterations of bovine whey proteins in powdered soybean milks.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (ACN) (HPLC grade; Scharlau, Barcelona, Spain), trifluoroacetic acid (TFA) (HPLC grade; Pierce Europe, Oud Beijerland, Netherlands), and HPLC-grade water (Milli-Q system; Millipore, Bedford, MA, USA) were used in the preparation of mobile phases.

Soybean protein isolate, taken as a standard of soybean proteins, and β-lactoglobulins (β-LG) (A+B) from bovine milk were obtained from ICN (Aurora, OH, USA). α-Lactalbumin (α-LA) from bovine milk was obtained from Sigma (St. Louis, MO, USA). Two commercial powdered soybean milk products (industrially manufactured from soybean protein isolate) were purchased in local markets at Alcalá de Henares, Madrid, Spain. Before quantitative analysis by HPLC, dry matter content of the powdered milks was determined by drying at 102°C to a constant weight.

The protocol used for preparing all solutions of standards and samples was the following: the standard (soybean protein isolate) and the samples of powdered soybean milk were weighed and dissolved in distilled water, then the mixture was sonicated for 3 min and centrifuged (1450 *g*, 5 min, 3°C). The supernatants were removed and kept on ice until their injection on the chromatographic system (protocol A). When bovine whey proteins could not be directly detected when injecting the solution of powdered soybean milk prepared following protocol A into the chromatograph, a protocol to concentrate bovine whey proteins, if present, was used. Then, an acidic whey was obtained by precipitation at pH 4.6 (2 *M* HCl) of a freshly prepared solution corresponding to a glass of milk (~12 g of soybean milk in 250 ml of water) and subsequent centrifugation (2000 *g*, 20 min) to precipitate the major fraction of soybean proteins and, simultaneously, concentrate in the supernatant the bovine whey proteins (protocol B) [21]. Undiluted wheys were frozen at −70°C until use.

2.2. High-performance liquid chromatography

A Hewlett-Packard 1090 Series II liquid

chromatograph (Hewlett-Packard, Pittsburgh, PA, USA) equipped with a diode array detector and a HP 9153C data acquisition system was used. The volume injection was 20 μl. Proteins were detected by UV absorption at 254 nm, since at this wavelength sensitivity for soybean proteins is higher than that obtained at other wavelengths commonly used to detect proteins (215, 228, 240 and 280 nm). In addition, at 254 nm there was an acceptable baseline drift.

The separation was accomplished with a POROS R2/H (PerSeptive Biosystems; Framingham, MA, USA) perfusion column (50×4.6 mm I.D.) packed with crosslinked polystyrene–divinylbenzene beads (10 μm particle size). The columnś dead-time (0.234 min) and efficiency (1281 plates/m) were determined by using uracyl as the unretained solute.

Separation of soybean and animal whey proteins was performed using the following gradient: 5–25% B for 1.7 min, 25–34% B for 0.3 min, and 34–41% B for 3 min, followed by a linear reversed gradient from 41 to 5% B for 1 min and a 1 min step at 5% B to re-equilibrate the column to the initial conditions between runs. The flow-rate was 3 ml/min (linear flow velocity of 1058 cm/h) and the column was thermostated at 60°C. Mobile phases were: phase A, 0.10% TFA in water; phase B, 0.10% TFA in ACN. The mobile phases were filtered using 0.45 μm nylon filters and degassed with helium before use.

2.3. Calibration

Calibration using the multipoint external standard method was individually performed for each bovine whey protein. For this purpose, standard solutions that contained known concentrations of α-LA (0.01–0.10 mg/ml) or β-LG (A+B) (0.17–0.89 mg/ml) were prepared by dilution of stock solutions of each bovine whey protein. A fixed volume (20 μl) of each standard solution was injected into the chromatographic system. The peak area corresponding to α-LA was integrated by setting the baseline from valley to valley. As β-LG (A+B) elutes in two unresolved peaks, the whole area, integrated as a cluster from the start point of the first peak to the end point of the second peak, was considered. The calibration curve was obtained by plotting peak area against protein concentration. Bovine whey proteins were quantitated in aqueous solutions and wheys of powdered soybean milk using the calibration curve. All solutions and samples were injected three times. During the calibration several blank gradients were run to check that there were no baseline problems.

2.4. Data treatment

Statistical analysis was performed by using the Statgraphics Plus program. The linearity in the relationship between peak area and the concentration of bovine whey proteins injected in the calibration was obtained by least-squares regression analysis carried out with a Univariate Linear Calibration program [22]. The linear model was validated by means of the analysis of the residual and the analysis of variance [23].

3. Results and discussion

3.1. Chromatographic separation

A solution prepared by mixing soybean protein isolate (prepared following protocol A), α-LA, and β-LG (A+B) was used to develop the method. Simultaneous separation of soybean and bovine whey proteins was first tried using a previously optimized gradient used to separate soybean proteins (5–25% B for 1.7 min and 25–43% B for 1.3 min) [20]. However, this gradient did not enable the separation of both kinds of proteins, since α-LA and β-LG (A+B) eluted at the same retention times found for the last peaks of the soybean protein isolate. In order to separate both kinds of proteins, the gradient range (%/min) was decreased in the last step of the gradient. Among the different gradients attempted, the best separation of soybean proteins from bovine whey proteins in a short analysis time was performed using a three-step linear binary gradient consisted of: 5–25% B for 1.7 min, 25–34% B for 0.3 min, 34–41% B for 3 min, followed by a linear reversed gradient from 41 to 5% B for 1 min and 1 min at 5% B to re-equilibrate the column to the initial conditions. In Fig. 1 the chromatogram obtained for the separation of soybean and bovine whey proteins from a simulated sample composed by soybean protein isolate (0.9456 mg/ml) (prepared

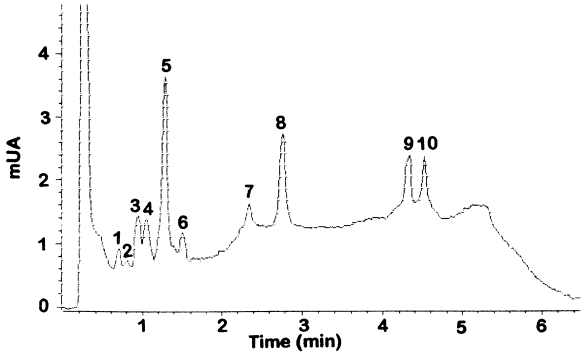

Fig. 1. Chromatogram corresponding to a simultaneous separation of soybean and bovine whey proteins. Conditions: 0.9456 mg/ml of soybean protein isolate, 0.0518 mg/ml of α-LA, and 0.4413 mg/ml of β-LG (A+B); temperature: 60°C; flow-rate: 3 ml/min; gradient: 5–25% B for 1.7 min, 25–34% B for 0.3 min, and 34–41% B for 3 min, followed by a linear reversed gradient 41–5% B for 1 min and 1 min at 5% B to re-equilibrate the column to the initial conditions; mobile phases: A, 0.10% TFA in water; B, 0.10% TFA in ACN; injection volume: 20 μl; detection: 254 nm. Peak identification: 1–7, soybean proteins; 8, α-LA; 9 and 10, β-LG (A+B).

following protocol A) to which the standards of α-LA (0.0518 mg/ml) and β-LG (A+B) (0.4413 mg/ml) were added, is shown. With this gradient soybean proteins (peaks 1–7), α-LA (peak 8) and β-LG (A+B) (peaks 9–10) were separated in less than 5 min. The assignment of these peaks was performed by comparing their retention times with those of the individual standards injected in the chromatographic system in the same conditions. The ratio (polar+ionic amino acids)/ nonpolar amino acids for soybean (~1.75) and bovine whey proteins (~1.2) could explain the order of elution of these proteins that in addition coincides with the elution order obtained with a reversed-phase conventional column [12].

A simulated sample of soybean protein isolate and bovine whey proteins, was used to check the precision of the method. In Table 1 the retention time, area percentage, and repeatability (expressed as Standard Deviation, S.D.) are grouped for the determination of both parameters on the same day (ten consecutive injections a day) and between two consecutive days (mean of the two mean values obtained from ten consecutive injections performed every day). Results demonstrate good precision of the method for evaluating the retention time and an acceptable precision of the method for evaluating the area percentage.

3.2. Application to edible samples

In Fig. 2 the chromatogram obtained when injecting a solution of a commercially available powdered soybean milk (prepared following protocol A) spiked with α-LA and β-LG (A+B) standards is shown. This chromatogram shows the same peaks that appeared in the chromatogram of Fig. 1 corresponding to a synthetic sample of soybean protein isolate and bovine whey protein standards.

The optimized method has been used to detect bovine whey proteins in commercial powdered soybean milk. As representative samples, two different powdered soybean milk products were chosen: one containing whey proteins in its formulation and another whose label did not specify the addition of whey proteins. Both products were diluted in distilled water following protocol A (at a concentration of approximately 1.5 mg/ml) and the supernatants were directly injected in triplicate into the chromatographic system. The resulting chromatograms showed no peaks corresponding to bovine whey proteins in either solution of soybean milks (Fig. 3a and b). Although this suggested the absence of bovine whey proteins in these products, it was necessary to carry out an acidic precipitation following protocol B to confirm this. When injecting the wheys obtained (without dilution) into the chromatographic system (Fig. 3c and d), it was possible to detect bovine whey proteins in the milk in which the addition of these proteins was specified in the label. Early peaks (peaks 1–5) in Fig. 3c and d correspond to those soybean proteins that have not precipitated at the precipitation pH. Peak 8 and peaks 9 and 10 in Fig. 3d corresponds to α-LA and β-LG (A+B), respectively.

3.3. Quantitative analysis of bovine whey proteins

The perfusion RP-HPLC method was used to quantitate bovine whey proteins in the powdered

Table 1
Retention time (t_R, min), peak area percentage (A, %), and repeatability, expressed as standard deviation (S.D.), for a simultaneous separation of proteins in a simulated sample of soybean protein isolate and standards of bovine whey proteins using a perfusion RP-HPLC method[a,b]

Assay	Soybean proteins														α-LA		β-LG (A+B)			
	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5		Peak 6		Peak 7		Peak 8		Peak 9		Peak 10	
	t_R	S.D.	t_R	S.D.	t_R	S.D.	t_R	S.D.	t_R	S.D.	t_R	S.D.	t_R	S.D.	t_R	S.D.	t_R	S.D.	t_R	S.D.
Same day[c]	0.70	0.00	0.81	0.01	0.95	0.00	1.06	0.00	1.29	0.00	1.51	0.00	2.35	0.00	2.78	0.00	4.32	0.02	4.54	0.03
Between days[d]	0.70	0.00	0.81	0.00	0.95	0.00	1.06	0.00	1.29	0.00	1.51	0.00	2.35	0.00	2.77	0.00	4.32	0.00	4.53	0.01
						Total A (%)[e]		S.D.							A (%)	S.D.		Total A (%)[f]		S.D.
Same day[c]						62.59		6.93							23.05	5.27		14.41		2.44
Between days[d]						61.85		1.05							22.34	1.01		15.84		2.02

[a] Protein concentration (mg/ml) in the simulated sample injected: 0.9456 soybean protein isolate+0.0518 α-LA+0.4413 β-LG (A+B).
[b] Experimental conditions as in Fig. 1.
[c] Number of injections in the same day, $n=10$.
[d] Data obtained as the mean value of two mean values of ten injections each one obtained on two consecutive days.
[e] Area percentage of soybeam proteins calculated as the ratio (total peak area of soybean proteins): (total peak area of soybean proteins+area of α-LA+total peak area of β-LG (A+B)).
[f] Area percentage of β-LG (A+B) calculated as the ratio (total peak area of β-LG (A+B)): (total peak area of soybean proteins+area of α-LA+total peak area of β-LG (A+B)).

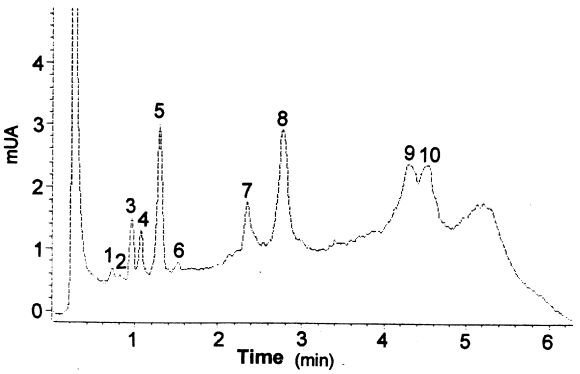

Fig. 2. Chromatogram corresponding to the direct injection of a solution of powdered soybean milk (1.9872 mg/ml) spiked with α-LA (0.0605 mg/ml), and β-LG (A+B) (0.5154 mg/ml). Experimental conditions and peak identification as in Fig. 1.

soybean milk in which bovine whey proteins were added to its formulation. Calibration of α-LA and β-LG (A+B) proteins by the external standard method was performed using standards prepared as suggested in the Experimental section (protocol A).

In Table 2 the values of the linear concentration range, slope, intercept, standard error, squared correlation coefficient, and detection limit for every calibration are shown. Good linear correlation ($r^2 \geq 0.999$) was found in both cases between the peak area of α-LA and the total peak area of β-LG (A+B) and the concentration of the respective standard injected in the chromatographic system. It is also important to note the possibility of detecting up to 47.1 and 4.3 μg/ml of β-LG (A+B) and α-LA, respectively, concentrations which are much lower

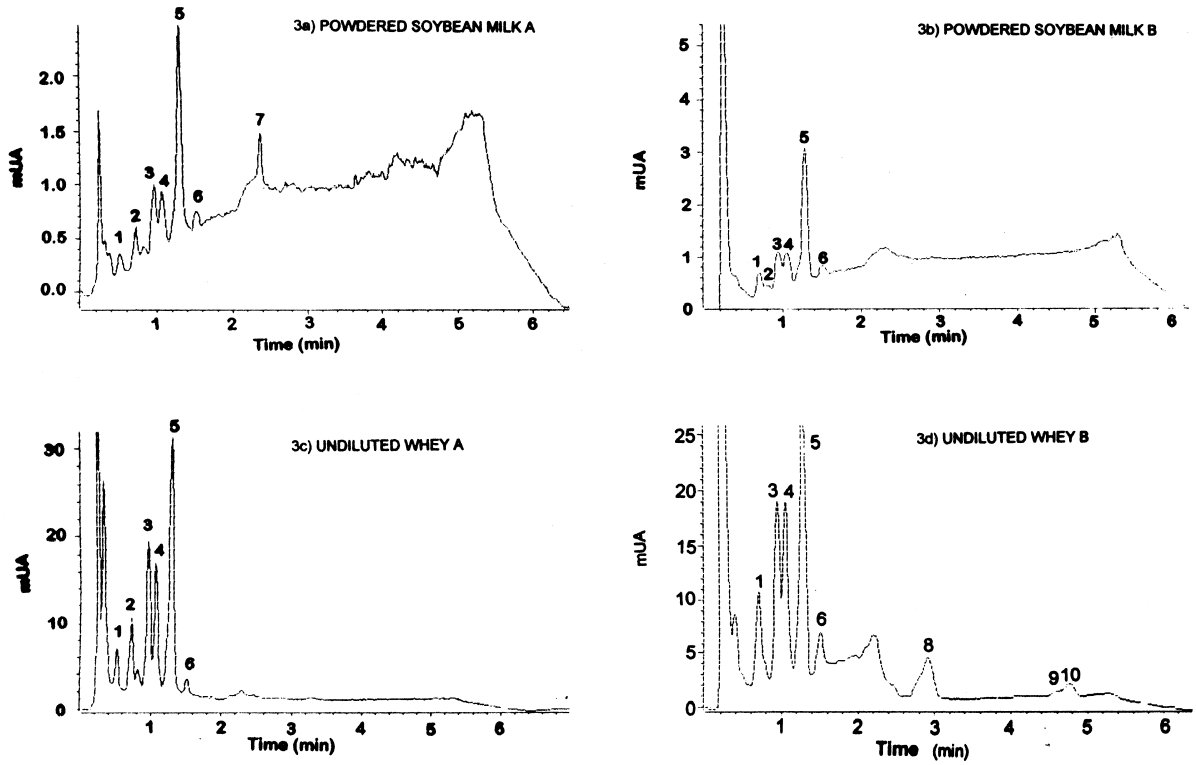

Fig. 3. Perfusion RP-HPLC of aqueous solutions of powdered soybean milks (3a: 1.6347 mg/ml; 3b: 1.5676 mg/ml) and the corresponding wheys obtained by acidic precipitation from a solution freshly prepared (corresponding to a glass of milk, approximately 50 mg/ml) of each powdered soybean milk. Experimental conditions and peak identification as in Fig. 1.

Table 2
Calibration by the multipoint external standard method of bovine whey proteins (α-LA and β-LG (A+B)) by perfusion RP-HPLC[a]

Protein	Linear conc. range (mg/ml)	n[b]	Slope[c]	Intercept[c]	Standard error	r^2[d]	Detection limit (mg/mml)[e]
β-LG (A+B)	0.1738–0.8347	4	39.09 (4.85)	−3.78 (2.59)	0.55	0.999	0.0471
α-LA	0.0173–0.1020	5	224.5 (14.73)	−1.95 (1.01)	0.32	0.999	0.0043

[a] Experimental conditions as in Fig. 1.
[b] Number of points considered for the regression. Each point represents the average of three consecutive injections of each standard solution.
[c] Errors in the slope and intercept of the regression line are given in parentheses.
[d] Squared correlation coefficient.
[e] The definition of the detection limit corresponds to the analyte concentration giving a signal equal to the intercept of the regression line, plus three times the standard error of the calibration plot.

than those detected by conventional reversed-phase high-performance liquid chromatography (86 μg/ml for β-LG (A+B) and 17 μg/ml for α-LA) [12].

These calibrations were used to determine the concentration of α-LA and β-LG (A+B) in the commercial powdered soybean milk where those proteins had been added to the formulation. The undiluted acidic whey prepared from this sample (Protocol B) was injected for the determination of β-LG (A+B) and the whey diluted (1:1) in water was analyzed for the quantitation of α-LA. The concentration of α-LA in the powdered milk was 0.355±0.015 mg in 100 mg of the powdered milk and the concentration of β-LG (A+B) was 0.453±0.114 mg in 100 mg of the powdered milk (on a dry-weight basis), which constitutes approximately 1% and 1.3% respectively of the total protein content (soybean protein+whey proteins) given by the manufacturer (35%). The high error obtained in the determination of the concentration of β-LG (A+B) could be due to the shape of these peaks and resolution between them in the whey chromatogram. When comparing these results with those obtained by conventional reversed-phase high-performance liquid chromatography for the same milk [12], no statistical difference was found either between the content yielded for both proteins by both techniques (*t*-tests, $P<0.05$) [22,23] or in the precision obtained for these analyses by both techniques (F-tests to compare variances, $P<0.05$) [22,23].

4. Conclusions

The new perfusion reversed-phase high-performance liquid chromatographic method developed in this work enables a very rapid and simultaneous separation of soybean and bovine whey proteins (~5 min) and represents an improvement in terms of routine analysis (205 samples in 24 h) of soybean products, especially in the detection of bovine whey proteins in soybean dairy-like products. The possibility that the sample could be directly injected by diluting it in distilled water or after acidic precipitation with hydrochloric acid also contributes to reducing the cost and time necessary to carry out every analysis. Perfusion chromatography enables the detection of smaller concentrations of bovine whey proteins than methods available up to now and allows the discrimination between soybean and bovine whey proteins and the determination of the content in every group of proteins.

Acknowledgements

The authors thank the Comunidad Autónoma de Madrid (Spain) for Projects COR 0035/94 and 06G/047/96 and C. Marina for linguistic assistance.

References

[1] J.C. Cheftel, in J.C. Cheftel, J.L. Cuq, D. Lorient (Editors), Proteínas Alimentarias. Bioquímica. Propiedades Funcionales. Valor Nutritivo. Modificaciones Químicas, Acribia, Zaragoza, 1989, p. 125.
[2] Soy Protein Products. Characteristics, Nutritional Aspects and Utilization, Soy Protein Council, Washington, 1987.
[3] M.C. García, M. Torre, M.L. Marina, F. Laborda, CRC Crit. Rev. Food Sci. Nutr. 37 (1997) 361.

[4] M. Kanning, M. Casella, C. Olieman, LC–GC Int. 6 (1993) 701.
[5] M.C. García, M. Torre, F. Laborda, M.L. Marina, J. Chromatogr. A 758 (1997) 75.
[6] R.E. Buehler, T.T. Van Toai, M.B. McDonald Jr., Seed Sci. Technol. 17 (1989) 193.
[7] R.E. Peterson, W.J. Wolf, Cereal Chem. 69 (1992) 101.
[8] R.J. Pearce, Aust. J. Dairy Technol. 38 (1983) 114.
[9] P. Resmini, L. Pellegrino, R. Andreini, F. Prati, Sci. Tec. Latt.-Cas. 40 (1989) 7.
[10] M. de Frutos, A. Cifuentes, L. Amigo, M. Ramos, J.C. Diez-Masa, Z. Lebensm. Unters. Forsch. 326 (1992) 331.
[11] M. Torre, M.E. Cohen, N. Corzo, M.A. Rodríguez, J.C. Diez-Masa, J. Chromatogr. A 729 (1996) 99.
[12] M.C. García, M.L. Marina, M. Torre, Anal. Chem. 69 (1997) 2217.
[13] N.B. Afeyan, S.P. Fulton, N.F. Gordon, I. Mazsaroff, L. Varady, F.E. Regnier, Biotechnology 8 (1990) 203.
[14] N.B. Afeyan, S.P. Fulton, F.E. Regnier, J. Chromatogr. 544 (1991) 267.
[15] N.B. Afeyan, N.F. Gordon, I. Mazsaroff, L. Varady, S.P. Fulton, Y. Yang, F.E. Regnier, J. Chromatogr. 519 (1990) 1.
[16] S.P. Fulton, N.B. Afeyan, N.F. Gordon, F.E. Regnier, J. Chromatogr. 547 (1991) 452.
[17] A.I. Liapis, M.A. McCoy, J. Chromatogr. 599 (1992) 87.
[18] M.A. McCoy, K. Kalghatgi, F.E. Regnier, N.B. Afeyan, J. Chromatogr. A 743 (1996) 221.
[19] Y. Xu, A.I. Liapis, J. Chromatogr. A 724 (1996) 13.
[20] M.C. García, M. Torre, M.L. Marina, J. Chromatogr. Sci. in press.
[21] International Dairy Federation, Questionnaire 3293/E, October 1993.
[22] M. Blanco, R. Boqué, R. Cela, J. Coello, S. Maspoch, M.C. Ortíz, J. Riba, F.X. Rius, A. Ruíz, L.A. Sarabia, X. Tomás, Avances en Quimiometría Práctica; Servicio de Publicación e Intercambio Científico, Santiago de Compostela, 1994.
[23] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, in B.G.M. Vandeginste, Y. Michotte (Editors), Chemometrics: A Textbook, Data Handling in Science and Technology 2, Elsevier, Amsterdam, 1988.