Protein Changes in Stored Ultra-High-Temperature-Treated Milks Studied by Capillary Electrophoresis and High-Performance Liquid Chromatography†

Isidra Recio, Mercedes de Frutos,‡ Agustín Olano, and Mercedes Ramos*

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

The evolution of direct and indirect ultra-high-temperature-treated milk during storage is studied. Capillary electrophoresis and HPLC analysis of whey is performed. Both techniques allow the quantitation of α-lactalbumin and β-lactoglobulin, providing comparable results at the beginning of the storage and making it possible to estimate the thermal treatment to which milk has been subjected. Besides, the capillary zone electrophoresis method allows differentiation between the proteolysis products caused by plasmin and by some proteases from psychrotrophic bacteria. Isoelectric focusing of the casein fraction allows identification of the degradation products originating from both types of proteases.

Keywords: *UHT milk; storage; proteolysis; capillary electrophoresis*

INTRODUCTION

Changes on the protein fraction in ultra-high-temperature (UHT)-treated milk have important significance. Measurement of whey protein denaturation may be used in assessing the severity of the process (Resmini et al., 1989). If it is suspected that UHT milk has been produced from raw milk of unacceptable poor quality, an indication of this can be obtained from the detection of peptides present in freshly processed milk (Mottar, 1981). Moreover, formation of peptides during storage of UHT milk may be an indication of the presence of heat resistant enzymes (López-Fandiño et al., 1993a).

Among the methods currently employed to determine the extent of whey protein denaturation, reversed-phase HPLC (RP-HPLC) has proved to be a reliable method with high resolving power (Resmini et al., 1989). RP-HPLC has also been applied to the study of proteolysis in UHT milk (López-Fandiño et al., 1993a).

Capillary zone electrophoresis (CZE) is an alternative separation technique to HPLC. The potential of CZE for the analysis of proteins has been demonstrated. Components of the whey protein fraction of milk can be separated in a relatively short time (Chen et al., 1992; Cifuentes et al., 1993; de Jong et al., 1993; Otte et al., 1994; Recio et al., 1995).

The present paper compares the HPLC and CZE analyses of whey proteins in stored UHT milks. In addition, the origin of the degradation products observed by CZE is studied and the heat treatment to which milk has been subjected is also evaluated. The extent of proteolysis is followed in the casein fraction by isoelectric focusing (IEF).

MATERIALS AND METHODS

Milk Samples. Whole UHT-treated milks heated directly and indirectly were supplied by commercial dairy plants. The containers were stored at 22 °C for 4 months, and samples were analyzed at 0, 30, 60, 90, and 120 days of storage. Duplicate samples were taken from two containers freshly opened at each analysis time.

Model Systems. Isoelectric casein was dissolved in 0.1 M potassium phosphate buffer to a concentration of 2.5% (w/v). Proteolysis with plasmin (EC 3.4.21.7, from Sigma Chemical Co., St. Louis, MO) or with *Pseudomonas fluorescens* B52 protease (López-Fandiño et al., 1993a) was carried out. Plasmin was added at an enzyme concentration of 6.4×10^{-4} units/mg of casein, and an adequate ratio was chosen for the digests with *P. fluorescens* B52 protease. Model systems were incubated at pH 6.5 and 37 °C for different periods (between 0 and 3 h). Aliquots were withdrawn from the mixtures at intervals, and reactions were stopped by heating at 100 °C for 10 min. The sample was cooled in ice, and the fraction soluble at pH 4.6 was obtained as follows.

Isolation of the Nitrogen Fractions. Milk and casein digests were acidified with 2 N HCl to pH 4.6 to precipitate the casein fraction. In the case of milk samples, the remaining fat in the casein precipitate was removed by washing three times with dichloromethane/water, 1:1 (v/v), and centrifuging at 4500*g*, 15 min, 20 °C. The final casein precipitate was lyophilized.

The soluble fraction obtained after precipitation of casein was first filtered through Whatman No. 1 filter paper and then through a 0.2 μm filter (sterile Acrodisc with HT Tuffryn membrane; Gelman Sciences, Ann Arbor, MI) before use in capillary electrophoresis and HPLC analyses. After filtering, samples for CZE were diluted 1:1 with a 0.22 mg/mL aqueous solution of the peptide Lys-Trp-Lys, used as internal standard.

Analytical Methods. Fractions soluble at pH 4.6 were analyzed by capillary electrophoresis using a P/ACE 2000 HPCE instrument controlled by System Gold Software (Beckman, Fullerton, CA). Separations were performed using an uncoated capillary column of 37 cm total length, 30 cm effective length, 75 μm i.d. (Polymicro Technologies Inc., Phoenix, AZ), at 25 °C and 7 kV. The injection was carried out at the anode using N_2 (0.5 psi) for 2 s. On-line detection was performed at 214 nm. The separation buffer used was 100 mM borate, pH 8.2, with 30 mM sodium sulfate added (Recio et al., 1995).

These samples were also analyzed by reversed-phase HPLC using a PLRP-S 8 μm column (300 Å, 150 × 4.6 mm) (Polymer Laboratories Ltd., Church Stretton, U.K.), with a linear binary mobile phase gradient (Resmini et al., 1989), on a Beckman apparatus with two pumps (Model 126), a variable wavelength UV detector (Model 166) operating at 205 nm, and an autoinjector (Model 502). System Gold Software data system (Beckman Instruments) was used to integrate the peaks.

† Part of this work has been presented at the Symposium on Heat Treatments and Alternative Methods, Vienna, Austria, Sept 6−8, 1995.

* Author to whom correspondence should be addressed (telephone, 34 1 5622900; fax, 34 1 5644853; e-mail, ifirg37@fresno.csic.es).

‡ Present address: Instituto de Química Orgánica (CSIC).

S0021-8561(96)00080-5 CCC: $12.00

Table 1. Content of the Major Whey Proteins Measured by Capillary Zone Electrophoresis (CZE) and Reversed-Phase HPLC (RP-HPLC)

	direct UHT				indirect UHT			
storage time (days)	α-La (mg/100 mL)	RSD	β-Lg (mg/100 mL)	RSD	α-La (mg/100 mL)	RSD	β-Lg (mg/100 mL)	RSD
				CZE				
0	64.96	0.005	82.68	0.004	25.38	1.58	21.34	5.10
30	73.37	2.01	93.14	1.97	25.21	0.97	21.93	3.28
60	66.60	4.03	84.73	3.94	25.06	4.94	21.12	0.19
90	78.61	2.09	99.64	2.05	24.71	3.19	21.50	4.52
120	74.30	3.34	94.30	3.28	24.93	3.59	21.06	4.56
				RP-HPLC[a]				
0	74.32	0.70	79.18	2.03	30.52	1.73	15.36	0.92
30	80.49	0.34	96.87	0.03	31.17	5.18	15.69	8.80
60	77.74	1.99	98.76	0.36	30.68	3.47	17.37	4.03
90	84.49	0.80	103.80	0.87	29.02	1.01	18.13	4.05
120	90.08	0.17	106.65	0.97	33.61	1.63	18.52	1.10
				RP-HPLC[b]				
0	61.50	0.26	73.92	2.08	25.46	1.24	14.20	4.39
30	57.66	0.67	79.89	0.07	24.70	5.16	12.89	8.24
60	57.97	1.57	73.58	0.39	19.01	1.99	12.07	4.77
90	57.49	1.02	71.55	0.53	17.83	2.52	11.20	4.23
120	58.59	1.03	65.70	0.72	18.33	2.14	9.34	0.89

[a] HPLC values including the shoulders of the α-lactalbumin and β-lactoglobulin peaks. [b] HPLC values excluding the shoulders of the α-lactalbumin and β-lactoglobulin peaks. RSD, relative standard deviation; $n = 4$.

Caseins were analyzed by IEF following the EEC regulation (1992), using ultrathin (0.2 mm) layer plates, 3.5−9.5 pH gradient. Isoelectric point markers (Pharmacia LKB, Uppsala, Sweden) were amyloglucosidase (p*I* 3.5), soybean trypsin inhibitor (p*I* 4.55), β-lactoglobulin A (β-Lg A) (p*I* 5.20), bovine carbonic anhydrase B (p*I* 5.85), human carbonic anhydrase (p*I* 6.55), horse myoglobin-acidic band (p*I* 6.85), horse myoglobin-basic band (p*I* 7.35), lentil lectin-acidic band (p*I* 8.15), lentil lectin-middle band (p*I* 8.45), lentil lectin-basic band (p*I* 8.65), and trypsinogen (p*I* 9.30). Extent of proteolysis was also determined by measuring the content of nitrogen in the fraction soluble at pH 4.6 by the Kjeldahl method.

Lactulose determination was done by gas chromatographic analysis of the trimethylsilyl derivatives of the free carbohydrate fraction using a 3 m × 1.0 mm stainless steel column (Chrompack, Middelburg, The Netherlands), packed with 2% OV-17 on nonsilanized 120/140 Volaspher A-2 (Merck, Darmstadt, Germany) following the method described by Olano et al. (1986).

RESULTS AND DISCUSSION

Quantitative Analysis of Whey Proteins by CZE and RP-HPLC. The CZE analysis of the freshly prepared UHT milk samples permitted the quantitative determination of the major whey proteins, which can serve as an indicator of the heat treatment conditions to which the milks have been subjected. The use of a separation buffer of high ionic strength and pH higher than the isoelectric point of the major whey proteins diminishes the protein adsorption to the capillary wall. In addition, dilution of the sample with an aqueous solution of the internal standard caused lower conductivity of the sample than that of the separation buffer (stacking effect), providing an enrichment of the sample and enhanced sensitivity and resolution. β-Lg was almost completely denatured by the indirect UHT treatment (Table 1), being higher than the denaturation observed in the direct UHT samples. These results are consistent with a higher lactulose content found in indirect UHT milk (43.82 mg/100 mL) than in direct UHT milk (17.73 mg/100 mL) and can be explained by the more severe heat treatment undergone during the indirect UHT process.

CZE was used to evaluate changes in α-La (α-lactalbumin) and β-Lg during storage (Table 1). During storage, changes were negligible in the α-La and β-Lg areas in the indirect UHT milk. Nevertheless, slight variations were found in the content of α-La and β-Lg in the direct UHT milk. These variations could be due to proteolysis products which migrate close to the protein peaks, making the accurate quantification of the whey proteins difficult. This different behaviour observed in the direct and indirect UHT milks could be due to the lower proteolysis found in indirect UHT treated than in direct UHT treated milks.

RP-HPLC analysis of α-La and β-Lg revealed changes in the shape of the peaks during storage (Figure 1). These changes have been previously related to the progress of the Maillard reaction and to interactions among proteins (Corzo et al., 1994), giving rise to an increase of the corresponding peak areas during the storage of direct UHT samples. In these samples, in which distortion was seen even at the first month of storage, the apparent increases of α-La and β-Lg contents were significant ($p < 0.01$). No significant changes were found for indirect UHT samples. As mentioned above, the difference found between both heat treatments could be attributed to enzymatic reactions which take place mainly in the direct UHT milks. The overestimation of β-Lg in long-term-stored milks could be a source of error as β-Lg concentration is one of the parameters used for distinguishing between heat treatments given to milks. The shape of the peaks reveals the appearance of new shoulders and the increase of the existing ones during storage. If the contribution of these shoulders to the peak area is not considered and only the main peaks of each protein are taken into account, the apparent increase of concentration is not observed (Table 1). As shown in Figure 1, precise assignment of the zones of the bands corresponding to formed products and original proteins, avoiding the use of deconvolution peak programs, is difficult. For this reason, every modified shoulder, even if originally existing, was subtracted as indicated by the shaded zones in Figure 1. In this way, a decrease of the peak areas was observed with increasing storage time, indicating that distortion of the peaks can be partially due to the formation of complexes of α-La or β-Lg with other compounds.

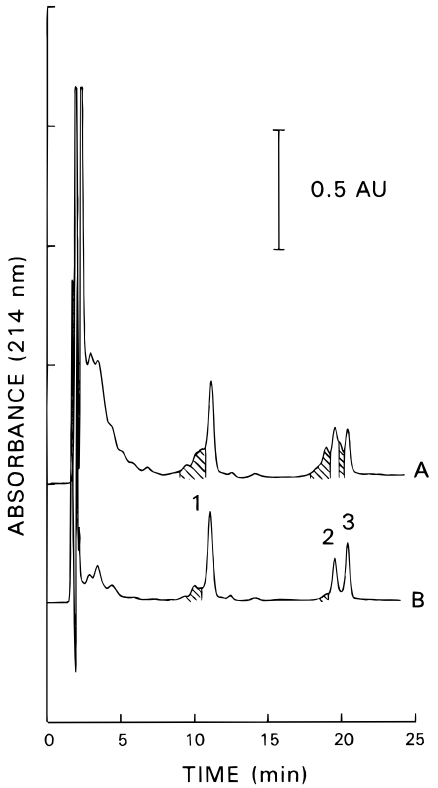

Figure 1. Chromatograms of direct UHT milk samples: (A) at 0 days and (B) stored for 120 days. Shaded zones correspond to the subtracted shoulders from α-lactalbumin and β-lactoglobulin peaks. Peak identities: 1, α-lactalbumin; 2, β-lactoglobulin variant B; and 3, β-lactoglobulin variant A (PLRP-S 8 μm column, 300 Å, 150 × 4.6 mm).

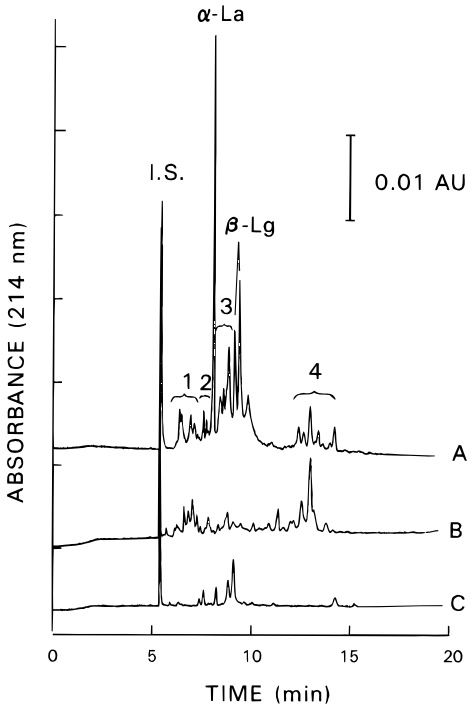

Figure 2. Electropherograms of (A) a direct UHT milk sample stored for 30 days at 22 °C, (B) caseins incubated with B52 proteinase from *P. fluorescens* for 1 h at 37 °C; and (C) caseins incubated with plasmin for 1 h at 37 °C. Peak identities: I.S., internal standard; α-La, α-lactalbumin; β-Lg, β-lactoglobulin; 1 and 4, peaks from the action of *P. fluorescens* B52 protease on caseins; and 2 and 3, peaks from the action of plasmin on caseins (uncoated capillary column, 37 cm total length, 30 cm effective length, 100 μm i.d.; 7 kV, 25 °C).

Comparison of the data obtained by HPLC and CZE at the beginning of the storage shows that both techniques provide quite similar values. Comparable values of α-La and β-Lg were obtained at zero days by both techniques, although the β-Lg content determined by CZE was sligthly higher than that determined by HPLC. The CZE α-La content was lower than the value determined by HPLC when the shoulder was included, but it was similar than the value obtained when the shoulder was subtracted. In general, as known for other proteins, HPLC showed better reproducibility than CZE. The screening performed on 28 different UHT milks has shown that high levels of proteolysis products migrating close to the β-Lg peaks make it difficult to quantitate, or even preclude the CZE quantitation of, the low level of β-Lg present in UHT milk (data not shown). The aqueous dilution of the sample included in the method enhances the resolution, making, in some cases, the analysis of controversial samples easier. Even in those unfavored cases, α-La determination by CZE agrees with the data obtained by the RP-HPLC method.

Proteolysis Assessment. As seen in the CZE analysis, simultaneously to the major whey proteins peaks, other peaks presumably corresponding to degradation products could be detected. In order to know whether these peaks arose from the action of native or exogenous proteases on caseins, model systems of caseins digested with enzymes were studied. Figure 2 shows that the degradation products soluble at pH 4.6 resulting from the action of plasmin on whole casein consisted mainly of peptides migrating in front of and after the α-La peak (group of peaks numbered as 2 and 3). The fraction soluble at pH 4.6 of the psychrotrophic enzyme digest of casein gave a very different elution pattern with many peaks. The major peaks due to the action of psychrotrophic proteinase migrated between the internal standard and α-La peaks (group of peaks numbered as 1) or were well separated after the peaks corresponding to β-Lg (group of peaks numbered as 4). Assignment of the peaks was done by spiking.

The capability of this CZE method to obtain electrophoretic profiles that include peaks corresponding to proteolysis products and major whey proteins has advantages and drawbacks. This procedure is able to distinguish between those peptides produced from the action of plasmin and from proteolytic enzymes of psychrotrophic bacteria and at the same time, in a single run, to evaluate the heat treatment intensity. However, high levels of proteolysis products make the quantitation of low amounts of β-Lg difficult.

The progress of proteolysis caused either by the action of psychrotrophic bacteria enzymes or by plasmin could be measured by quantifying those peaks. The increase of the area of those peaks and the increase in the content of nitrogen in the fraction soluble at pH 4.6 showed the same trend during the storage (Figure 3). Lower proteolysis was found in indirectly UHT- than in directly UHT-treated milks, probably due to the more severe heat treatment undergone by the indirect UHT milks which could have caused a higher level of enzyme inactivation (López-Fandiño et al., 1993b).

The extent of casein degradation upon storage of the UHT milk samples was also studied by IEF of the casein fraction (Figure 4). γ_2- and γ_3-caseins were identified as degradation products from the action of the plasmin by using a model system of casein digested with this enzyme. In addition, other minor bands arising from the action of plasmin on caseins could be detected. A

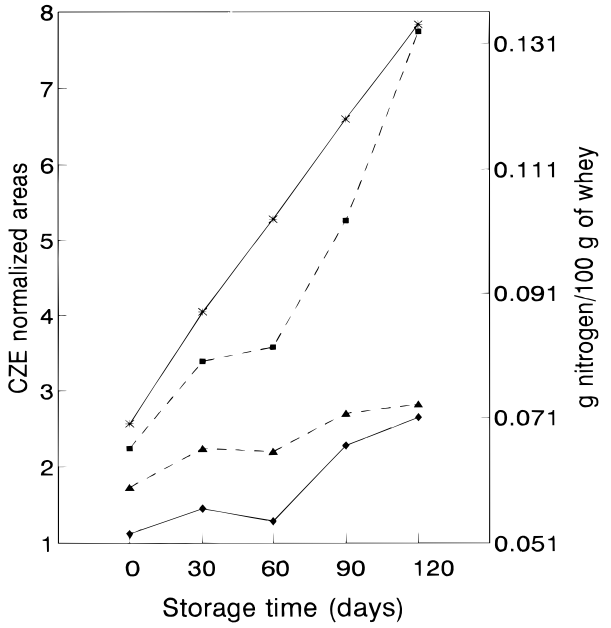

Figure 3. Capillary electrophoresis areas of peptides arising from proteolysis of casein and nitrogen content in the soluble fraction at pH 4.6 versus storage time: (■) CZE areas, direct UHT, (▲) CZE areas, indirect UHT, (*) percent nitrogen, direct UHT, and (♦) percent nitrogen, indirect UHT.

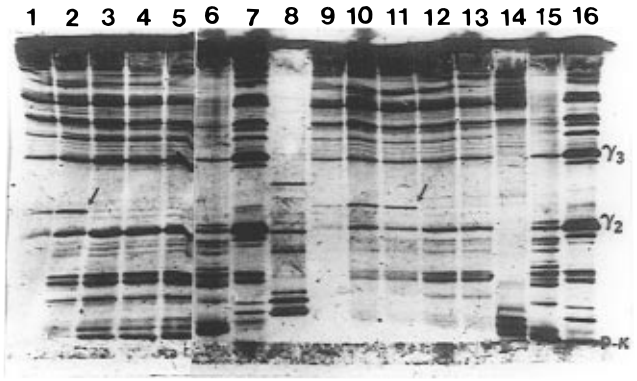

Figure 4. Isoelectric focusing of the casein fraction from UHT milk samples in ultrathin layer gel (pH 3.5−9.5); sample application at the cathodic end (top of the figure). Lanes 1−5 correspond to the casein fraction from direct UHT milk samples stored for 0, 30, 60, 90, and 120 days, respectively. Lanes 9−13 correspond to the casein fraction from indirect UHT milk samples stored for 0, 30, 60, 90, and 120 days, respectively. Lanes 6 and 15 correspond to the model system of casein incubated with the protease B52 from *P. fluorescens*. Lanes 7 and 16 correspond to the model system of casein incubated with plasmin. Lanes 8 and 14 correspond to the isoelectric point markers. γ_2 and γ_3 indicate respectively γ_2-casein and γ_3-casein. *p*-κ indicates *p*-κ-casein. The band pointed with an arrow corresponds to a proteolysis product with an approximate p*I* of 6.4.

band, migrating between γ_2- and γ_3-caseins, with approximate p*I* 6.4 and tentatively assigned to a degradation product originated by plasmin, was noticeable. This band was present in the direct UHT samples at 0 and 30 days, disappearing after 60 days. In the indirect UHT samples its intensity was low at 0 days, but it was clearly observed at 30 and 60 days. This degradation product is probably further proteolyzed by plasmin or exogenous enzymes during storage. This band could be another useful indicator of the proteolytic state of a UHT milk. The indirect UHT milk showed a lower level of plasmin activity due to the higher heat inactivation and the higher content of denatured β-Lg which inhibits plasmin via thiol−disulfide interchange (Grufferty and Fox, 1986).

Caseins incubated with B52 protease from *P. fluorescens* were used to identify those products arising from the action of bacterial proteases. Most of the products showed higher p*I* values than the γ_3-casein, although bands with the same pI value as the γ_2- and γ_3-caseins were observed (Figure 4). As known, bacterial proteases are also able to proteolyze β-casein, although in a minor grade than plasmin does (Grieve and Kitchen, 1985). One of the products due to the action of the bacterial proteases on κ-casein is the *p*-κ-casein which migrates close to the negative electrode due to its high p*I*. κ-Casein is known to be the most sensitive casein component to bacterial proteases (Miranda and Gripon, 1986).

CONCLUSIONS

The present CZE method allows the separation and quantitation of whey proteins and other peptides which arise from the action of thermostable proteases. This method allows differentiation between peptides produced by the action of plasmin and those arising from proteolytic enzymes of psycrotrophic bacteria. Therefore, the method provides information about the quality of the raw milk or the unknown technological history. Simultaneously, the heat treatment to which milk has been subjected can be evaluated.

Due to its high resolution, easy performance, and short analysis time, this method could find application both in research work and in routine quality control. Moreover, the CZE method provides increased resolution, compared with the HPLC method, allowing the quantitative analysis of the whey proteins even at the end of the shelf life. Nevertheless, the simultaneous use of both techniques can provide additional information about the changes that take place during storage of UHT milks, both techniques being complementary tools for the analysis of milk proteins.

ACKNOWLEDGMENT

We thank Dr. C. San José for kindly providing the *P. fluorescens* B52 protease. Ms. C. Talavera is thanked for excellent technical assistance.

LITERATURE CITED

Chen, F. A.; Kelly, L.; Palmieri, R.; Biehler, R.; Schwartz, H. Use of high ionic strength buffers for the separation of proteins and peptides with capillary electrophoresis. *J. Liq. Chromatogr.* **1992**, *15*, 1143−1161.

Cifuentes, A.; de Frutos, M.; Díez-Masa, J. C. Analysis of whey proteins by capillary electrophoresis using buffer-containing polymeric additives. *J. Dairy Sci.* **1993**, *76*, 1870−1875.

Corzo, N.; López-Fandiño, R.; Delgado, T.; Ramos, M.; Olano, A. Changes in furosine and proteins of UHT-treated milks stored at high ambient temperatures. *Z. Lebensm.-Unters. Forsch.* **1994**, *198*, 302−306.

EEC Regulations of the Community. A reference method for the detection of cow's milk casein in cheeses made from ewe's milk. *Off. J. Eur. Comm.* **1992**, *690/92*, 23−32.

Grieve, P. A.; Kitchen, B. J. Proteolysis in milk: the significance of proteinases originating from milk leucocytes and a comparison of the action of leucocyte, bacterial and natural milk proteinases on casein. *J. Dairy Res.* **1985**, *52*, 101−112.

Grufferty, M. B.; Fox, P. Milk alkaline proteinase. *J. Dairy Res.* **1986**, *55*, 609−630.

de Jong, N.; Visser, S.; Olieman, C. Determination of milk proteins by capillary electrophoresis. *J. Chromatogr.* **1993**, *652*, 207−213.

López-Fandiño, R.; Olano, A.; San José, C.; Ramos, M. Application of reversed-phase HPLC to the study of proteolysis in UHT milk. *J. Dairy Res.* **1993a**, *60*, 111−116.

López-Fandiño, R.; Olano, A.; Corzo, N.; Ramos, M. Proteolysis during storage of UHT milk differences between whole and skim milk. *J. Dairy Res.* **1993b**, *60*, 339−347.

Miranda, G.; Gripon, J. C. Origine, nature et incidences technologiques de la protéolyse dans le lait (Origin, nature and technological significance of proteolysis in milk). *Lait* **1986**, *66*, 1−18.

Mottar, J. Heat resistant enzymes in UHT milk and their influence on sensoric changes during uncooled storage. *Milchwissenchaft* **1981**, *36*, 87−91.

Olano, A.; Calvo, M. M.; Reglero, G. Analysis of free carbohydrates in milk using micropacked columns. *Chromatographia* **1986**, *21*, 538−540.

Otte, J. A. H. J.; Kristiansen, K. R.; Zakora, M.; Quist, K. Separation of individual whey proteins and measurement of α-lactalbumin and β-lactoglobulin by capillary zone electrophoresis. *Neth. Milk Dairy J.* **1994**, *48*, 81−97.

Recio, I.; Molina, E.; Ramos, M.; de Frutos, M. Quantitative analysis of major whey proteins by capillary electrophoresis using uncoated capillaries. *Electrophoresis* **1995**, *16*, 654−658.

Resmini, P.; Pellegrino, L.; Andreini, R.; Prati, F. Determinazione delle siero proteine solubili del latte per HPLC in fase inversa (Evaluation of milk whey proteins by reversed-phase HPLC). *Sci. Tec. Latt.-Casearia* **1989**, *40*, 7−23.

Received for review February 6, 1996. Revised manuscript received August 22, 1996. Accepted August 28, 1996.[⊗] We acknowledge financial support from Project ALI-95-0046-CO2-02 and CEE Contract 1116/92 ESP4-II. I.R. acknowledges the Spanish MEC for a scholarship.

JF960080J

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.