Lait 82 (2002) 589–599

DOI: 10.1051/lait:2002035


**Original article**

# Effect of homogenisation on protein distribution and proteolysis during storage of indirectly heated UHT milk

Mónica R. García-Risco, Mar Villamiel, Rosina López-Fandiño*

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

(Received 20 July 2001; accepted 3 October 2001)

**Abstract** – This paper compares modifications in whey proteins and caseins and proteolysis during storage (120 days) at 20 ºC of unhomogenised and homogenised milks (2% fat) heated (137 ºC, 4 s) in a pilot-scale indirect UHT plant. Homogenisation resulted in the attachment of caseins and whey proteins to the milk fat globule membrane, changed the morphology of casein micelles and gave rise to small micellar particles, not easily sedimentable by centrifugation. It also promoted whey protein denaturation and slowed down enzymatic reactions that depend on residual proteinase, glycosidase and phosphatase activities. Homogenisation of milk before UHT treatment led to lower levels of the enzymes that contribute to deterioration during storage. This phenomenon, together with an increased complex formation between κ-casein and β-lactoglobulin, could be responsible for reduced proteolytic degradation.

**Homogenisation / UHT milk / protein distribution / proteolysis**

**Résumé** – **Effet de l'homogénéisation sur la distribution des protéines et la protéolyse pendant la conservation du lait UHT obtenu par chauffage indirect.** Dans ce travail on compare les modifications des protéines de lactosérum et des caséines et la protéolyse pendant la conservation (120 jours) à 20 ºC, dans du lait non-homogénéisé et homogénéisé (2 % matière grasse), chauffé (137 ºC, 4 s) dans une installation UHT indirect à l'échelle pilote. L'homogénéisation entraînait l'adsorption des caséines et des protéines de lactosérum à la membrane des globules gras, des changements morphologiques des micelles de caséine et donnait lieu à la formation de petites particules micellaires, difficilement sédimentables par centrifugation. L'homogénéisation occasionnait aussi la dénaturation des protéines de lactosérum et ralentissait les réactions enzymatiques dépendantes des activités protéinase, glycosidase et phosphatase résiduelles. L'homogénéisation du lait avant le traitement UHT a conduit à des teneurs plus faibles en enzymes qui contribuent à la détérioration pendant la conservation, ce qui, associé à un accroissement de la formation de complexes entre la caséine-κ et la β-lactoglobuline, peut être la cause de la moindre dégradation protéolytique observée.

**Homogénéisation / lait UHT / distribution des protéines / protéolyse**

* Correspondence and reprints
Tel.: 34 91 5622900; fax: 34 91 5644853; e-mail: rosina@ifi.csic.es

## 1. INTRODUCTION

Proteolysis in processed milk can be attributed to two main enzyme groups that exhibit considerable thermostability: (1) the native milk proteolytic system and (2) proteinases from psychrotrophic bacteria that grow during refrigerated storage of milk before processing. Even low residual or reactivated enzymatic activities can cause serious defects in UHT milk, which is approximately twice as sensitive as pasteurised milk to the action of proteolytic enzymes [15]. Although the relationship between residual proteolytic activity and gelation in UHT milk is controversial, milk with high proteinase levels is regarded as susceptible to destabilisation during storage [9]. In addition, the relationship between proteolysis and development of bitter off-flavours in UHT milk has been demonstrated [15, 16], and the appearance of astringent off-flavours is linked to the activity of plasmin and psychrotrophic proteinases towards β-casein and the production of γ-caseins [10].

In addition to proteases, other enzymes can be responsible for biochemical changes during storage of UHT milk. Recio et al. [22] and Belloque et al. [1] found, particularly in samples with high residual proteolytic activities, substantial increases in the contents of carbohydrates such as galactose, N-acetyl-galactosamine and N-acetyl-glucosamine, which were attributed to heat-stable phosphatases and/or glycosidases of microbial origin.

The extent of changes in UHT milk during storage not only depends on the enzymes present, but also on the milk composition and processing conditions [2], as well as temperature and length of storage [24]. It is known that the sequence of homogenisation and heating changes the structure of casein micelles by adsorption to the milk fat globule membrane (MFGM) and binding of whey proteins [3, 26]. Previous work revealed that homogenisation considerably alters the properties of proteins and affects their susceptibility to proteolysis, but the effects depend on whether it precedes or follows heating and vary with the fat content of the milk [7]. In fact, higher proteolytic degradation was found in skimmed milk homogenised after heating compared to whole milk, which could be, at least partially, attributed to an increased exposure of κ-casein to proteinase action. Conversely, homogenisation performed before heat treatment caused higher whey protein denaturation and lower proteolysis, particularly in whole milk samples, compared with homogenisation performed after heating [7].

In this work, modifications of whey proteins and caseins as a result of processing, as well as subsequent proteolysis during storage at room temperature, were studied in milk (2% fat) heated in a pilot-scale indirect UHT plant, following homogenisation at 20 MPa. As a control treatment, homogenisation preceding heat treatment was performed at 0 MPa. Special attention was paid to residual enzymes remaining in the milk after processing, such as proteinases, phosphatases and/or glycosidases, whose activities were indirectly measured, as well as to the proteolytic degradation of κ-casein. The main aim was to estimate the contribution of homogenisation to survival of proteinases and changes in susceptibility of proteins towards proteolysis.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of UHT milk

Two experimental UHT milk batches (H and U) were produced in a pilot plant using 320 L of partially skimmed milk (2.0 ± 0.2% w/v fat). With the aim of promoting the growth of psychrotrophs and thus the concentration of enzymes associated with such bacteria, raw milk was held at 4 $^{o}$C for 24 h before UHT processing.

Our intention was to ensure enough proteolytic activity so as to estimate the effect of homogenisation on proteolysis. The total mesophilic and psychrotrophic counts of the raw material, assessed by standard methods [14], were 7.04 and 4.86 log CFU·mL$^{-1}$, respectively.

Half of the milk was submitted to one-stage homogenisation at 20 MPa and 65 ºC and then heat treated at 137 ºC for 4 s in a pilot UHT plant using a tubular heat exchanger (Finamat 6500/010 Gea Finnah, Bochum, Germany) at a flow rate of 1 000 L·h$^{-1}$ ("Batch H"). The other half was subjected to the same process but without any homogenisation pressure (0 MPa) ("Batch U"). Both batches (H and U) were packed under aseptic conditions in 200 mL Tetra-Pack® sterile containers and stored at 20 ºC for up to 120 d. At different times of storage, two different milk containers, randomly chosen, were opened for duplicate analyses.

### 2.2. Separation of milk fractions

Milk samples were ultracentrifuged sequentially at 7 000 *g* for 30 min, 20 000 *g* for 30 min and 50 000 *g* for 120 min at 20 ºC [13] in a Beckman L-70 preparative ultracentrifuge, using a type 70 Ti rotor (Beckman Instruments Inc., San Ramon, CA, USA). A sample of the supernatant was collected for analysis after each centrifugation step and the final cream layers and casein pellets were carefully separated. The cream was washed in 5 mmol·L$^{-1}$ $CaCl_2$, 50 mmol·L$^{-1}$ NaCl and 20 mmol·L$^{-1}$ imidazole by stirring at 32 ºC for 30 min, centrifuged at 60 000 *g*, 20 min, 20 ºC, spread on a filter paper to remove the excess of aqueous phase, frozen and lyophilised. This treatment preserves the original coating of casein micelles and micellar fragments on the fat globules [26]. The casein precipitates were washed 3 times with buffer consisting of 1 mol·L$^{-1}$ sodium acetate and 100 mL·L$^{-1}$ acetic acid, pH 4.6, and twice with a 1:1 mixture of buffer:dichloromethane, centrifuging after each wash (4 500 *g*, 5 ºC, 5 min) [21], and finally, were lyophilised.

The pH 4.6 soluble fractions were prepared by precipitation of milk by adding 2 mol·L$^{-1}$ HCl to pH 4.6, followed by centrifugation at 4 500 *g* for 15 min. The supernatants were filtered through Whatman No 40 paper and kept frozen until analysed.

### 2.3. Measurement of denaturation of whey proteins

Native whey proteins were determined in the pH 4.6 soluble fractions by reversed phase HPLC on a PLRP 8-μm column (300 Å, 150 × 4.6 mm i.d.; Polymer Laboratories, Church Stretton, England) with a linear binary gradient [25]. Denaturation of α-lactalbumin and β-lactoglobulin was calculated relative to the initial levels in the raw milk.

### 2.4. Determination of lactulose

Lactulose was determined by gas-liquid chromatography (GLC) analysis of the trimethylsilyl derivatives of the free carbohydrate fraction using a 3 m × 1.0 mm i.d., stainless steel column (Chrompack, Middelburg, The Netherlands) packed with 2% OV-17 on nonsilanised 120/140 Volaspher A-2 (Merck, Damstadt, Germany), following the method described in [17]. Phenyl-β-D-glucoside (Sigma Chemical Co., St Louis, MO, USA) was used as an internal standard.

### 2.5. Analysis of monosaccharides

Free monosaccharides were analysed by GLC as their trimethylsilyl derivatives using a fused silica column (25 m × 0.2 mm) coated with methyl silicone, following [28]. Methyl-α-D-galactopyranoside (Sigma Chemicals Co.) was used as an internal standard.

### 2.6. Electron microscopy

Milk samples (5 mL) were warmed to 45 ºC and mixed with 5 mL of 3% w/v agar solution. The mixture was poured on a Petri dish, allowed to solidify and cut into strips of $1 \times 1 \times 10$ mm$^3$ approximately. The strips were fixed for 3–4 h in 2.5% v/v glutaraldehyde in phosphate buffer saline (PBS), pH 7.0, at 4 ºC, rinsed 3 times with PBS and kept overnight in PBS at 5 ºC. The strips were then postfixed in 1% w/v osmium tetroxide (Sigma Chemicals Co.) for 1 h, rinsed 3 times in bidistilled water and dehydrated using increasing concentrations of acetone (40, 60, 70, 90, 95, 100% v/v). Finally, they were infiltrated 4 times with a solution containing acetone and Spurr's epoxi resin (Sigma Chemical Co.) in proportions ranging from 3:1 acetone:resin to 100% resin, hardened for 72 h at 60–70 ºC and cut into thin sections, which were poststained with 2% uranyl acetate and Reynolds lead citrate and examined with a transmission electron microscope (Zeiss 902, Carl Zeiss, Oberkochen, Germany) operated at 80 kV.

### 2.7. SDS-PAGE

Proteins present in the milk fat globule membrane (MFGM) were studied by SDS-PAGE using the PhastSystem Electrophoresis apparatus, precast PhastGels Homogeneous 20%, and PhastGel SDS buffer strips (Pharmacia, Uppsala, Sweden). Lyophilised portions of cream layers, separated and washed as described in 2.2, were analysed. Sample treatment, electrophoretic conditions and staining with PhastGel Blue R followed the procedures of the manufacturer.

### 2.8. Proteolytic degradation

Proteolysis during storage was assessed by determining the increase in nitrogen soluble at pH 4.6, using the Kjeldahl method. As a measure of proteolysis, main proteins of milk samples and peptides present in the pH 4.6 soluble fractions were also analysed by capillary electrophoresis after certain periods of storage, as explained below.

### 2.9. Capillary electrophoresis

Capillary electrophoresis (CE) analyses were performed with a Beckman P/ACE System MDQ (Beckman Instruments Inc., Fullerton, CA, USA) using a hydrophilic-coated fused-silica capillary (CElect P1; Supelco, Bellefonte, PA, USA). Separation of individual proteins in the milk samples, ultracentrifugal supernatants and casein pellets was performed at pH 3.0, using a capillary of 0.60 m × 50 mm i.d., with a slit opening of 100 × 800 mm, (0.50 m to detection point), at a temperature of 45 ºC using a linear voltage gradient of 0–25 kV in 3 min, followed by a constant voltage of 25 kV [19]. Identification of individual proteins and degradation products was carried out according to [21].

CE analysis of peptides was carried out at pH 3.0, using a capillary of 0.37 m × 50 mm i.d., with a slit opening of 100 × 800 mm, (0.30 m to detection point), at a temperature of 45 ºC, using a linear current gradient of 0–50 μA in 10 min, followed by a constant current of 50 μA, with a final voltage of around 25 kV, as described by Recio et al. [20]. Samples were prepared as in [29] and peptide identification was performed according to Recio et al. [23].

## 3. RESULTS

### 3.1. Changes in protein distribution after processing of milk

Table I shows the levels of denaturation of α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) and formation of lactulose as a result

**Table I.** Effect of homogenisation performed before indirect UHT treatment of milk on whey protein denaturation and lactulose formation. Results are means of two determinations in two different Tetra-Pack® containers ($n = 4$), followed by SD in brackets.

| Milk* | % Denaturation | | Lactulose ($mg \cdot L^{-1}$) |
|---|---|---|---|
| | α-La | β-Lg | |
| U | 31.0 (0.78) | 73.6 (0.23) | 130.7 (14.8) |
| H | 39.6 (1.42) | 76.3 (0.29) | 150.1 (6.0) |

* U: unhomogenised (0 MPa) and heated (137 ºC, 4 s) milk;
H: homogenised (20 MPa) and heated (137 ºC, 4 s) milk.

of the UHT processes. Homogenisation decreased the levels of soluble α-La and β-Lg relative to the unhomogenised heat-treated milk by approximately 8 and 3%, respectively. This observation supports previous findings regarding the denaturing effect of homogenisation [7]. Another estimation of the intensity of heat damage was obtained from the determination of lactulose after processing. The values of lactulose in both batches H and U were consistent with the relatively mild indirect UHT treatment applied. As shown in Table I, lactulose levels were slightly higher in the homogenised milk.

Figure 1 shows transmission electron micrographs of milks from both batches immediately after processing. As expected, the most obvious effect of homogenisation was the reduction of fat globule size and the coverage of newly formed fat particles with casein micelles. Micelles linked to fat globules in the homogenised milk (Fig. 1d) were less round in shape and presented more irregular surfaces than those of the unhomogenised milk (Fig. 1b). It has been reported that spreading of casein particles occurs on the surface of homogenised fat globules [11]. It was difficult to estimate differences in micellar size distribution between batches U and H as judged from the micrographs.

Casein components and β-Lg were the major proteins present in the fat fractions of the homogenised milk, as detected by SDS-PAGE (results not shown). This is in agreement with the observations of Sharma and Dalgleish [26], who reported that, when homogenised milk is heated, whey proteins interact with micelles covering the fat globules. However, unlike the results of these authors, in our experiments α-La bound less than β-Lg. No protein bands were found attached to the fat globules of unhomogenised milk by Coomassie staining.

The main proteins present in the ultracentrifugation supernatants and pellets of milk samples centrifuged at different speeds were quantified by CE. In addition to whey proteins, non-sedimentable caseins were also found in the ultracentrifugation supernatants. As shown in Figure 2, maximum soluble casein levels were observed in the supernatants obtained at the lowest speed. Centrifugation at 7 000 *g* causes only the larger micelles to sediment, 20 000 *g* also causes sedimentation of medium-size micelles and, at 50 000 *g*, most micelles are in the pellet [13]. Comparing both batches U and H, the concentration of soluble casein was the highest in the homogenised milk (H) centrifuged at 7 000 and 20 000 *g* (Fig. 2), which might indicate the formation, on homogenisation, of smaller micellar particles that did not sediment easily on centrifugation, even if this was not clearly visible in the electron microscopy studies.

The individual protein contents of the different ultracentrifugation supernatants

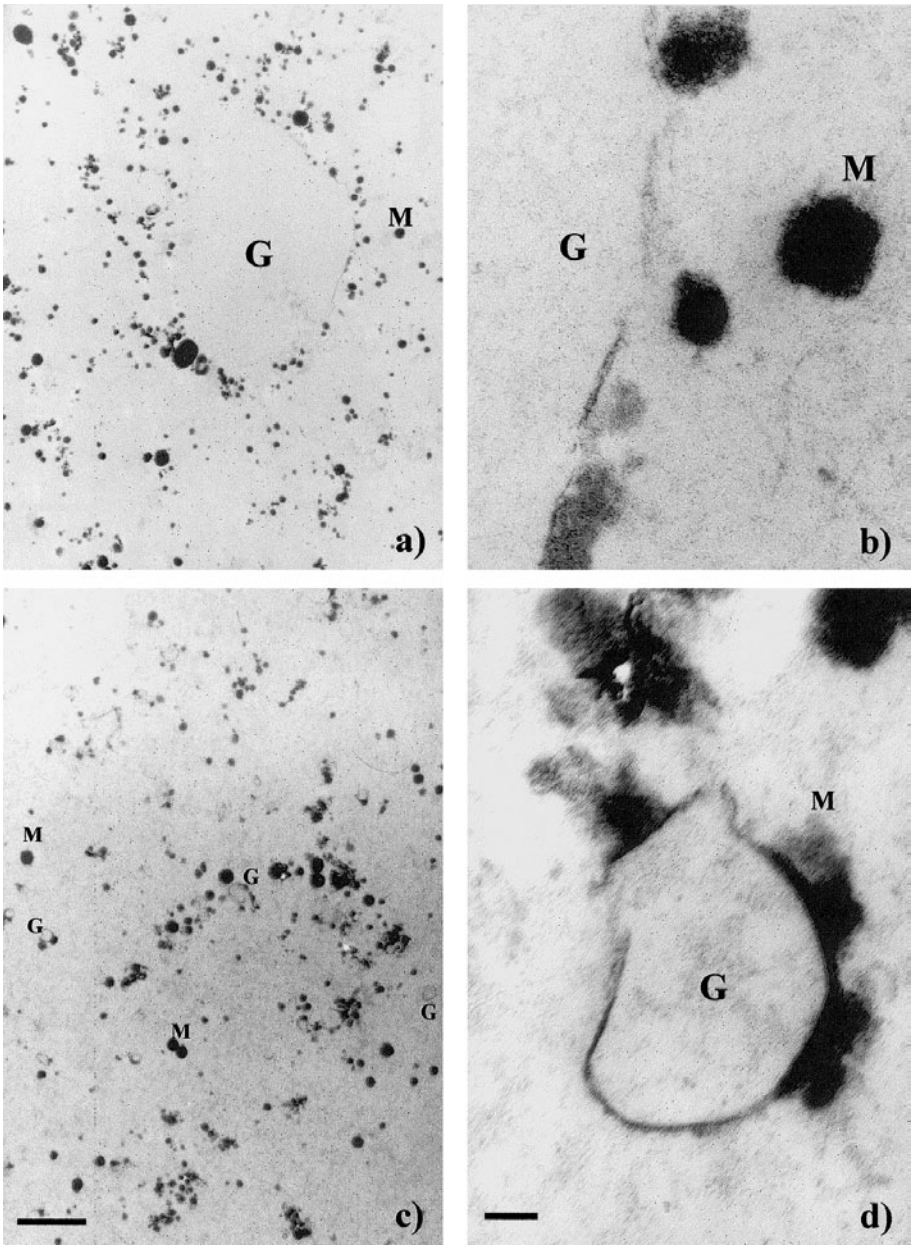


**Figure 1**. Transmission electron micrographs of unhomogenised (0 MPa) (**a** and **b**) and homogenised (20 MPa) (**c** and **d**) milk samples, both subsequently heated (137 ºC, 4 s). The bar corresponds either to 1 μm (**a** and **c**) or 50 nm (**b** and **d**). G: fat globule; M: casein micelle.

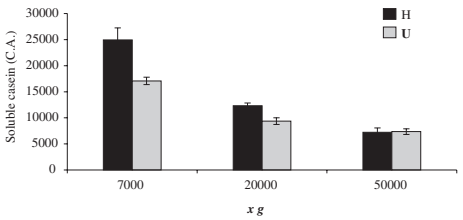


**Figure 2.** Corrected area (C.A.) of the main casein peaks (sum of κ-casein, $\alpha_{S1}$-casein and β-casein), present in the electrophoregrams (CE) of the supernatants obtained by ultracentrifugation at 7 000, 20 000 and 50 000 *g* of the milk samples. U: unhomogenised (0 MPa) and heated (137 ºC, 4 s) milk; H: homogenised (20 MPa) and heated (137 ºC, 4 s) milk. Results are means of two determinations in two different Tetra-Pack® containers ($n = 4$) ± SD.

and pellets, expressed as percentages of the total proteins in each fraction (which also included $\alpha_{S2}$-casein, $\alpha_{S0}$-casein, γ-caseins and other minor degradation products) are shown in Table II. A lower proportion of β-Lg was found in the supernatants of the homogenised milk at the three ultracentrifugation speeds, compared to the unhomogenised milk. This probably reflected the increased levels of soluble casein in the former, although it could also be partially attributed to the slightly higher level of whey protein denaturation in batch H (Tab. I). Indeed, an increased proportion of β-Lg was found in the micellar pellet of the homogenised milk and in the MFGM.

Non-sedimentable caseins of milks from both batches were particularly rich in β-casein. However, taking into account the initial contents of each individual protein in milk, the amount of κ-casein in the serum fraction was comparatively the highest. This agreed with previous studies on the composition of the ultracentrifugation supernatants of freshly processed UHT milk [6, 12]. It has been suggested that about 10–15% of the total whey protein-κ-casein complexes remain in the supernatant during heating of milk [27]. Furthermore, as shown in Table II, the levels of soluble β- and $\alpha_{S1}$-casein decreased markedly on increasing the centrifugation speed but, proportionately, higher levels of non-sedimentable κ-casein remained soluble. This could be indicative of the sedimentation

**Table II.** Contents of α-lactalbumin (α-La), β-lactoglobulin (β-Lg), $\alpha_{S1}$-casein ($\alpha_{S1}$-CN), κ-casein (κ-CN) and β-casein (β-CN), expressed as percentages of the total proteins in each fraction, present in the supernatants obtained by ultracentrifugation at 7 000, 20 000 and 50 000 *g*, and final pellets, from unhomogenised (U) (0 MPa) and homogenised (H) (20 MPa) milk samples, both subsequently heated (137 ºC, 4 s). Results are means of two determinations in two different Tetra-Pack® containers ($n = 4$), followed by SD in brackets.

| | U | | | | | H | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | α-La | β-Lg | $\alpha_{S1}$-CN | κ-CN | β-CN | α-La | β-Lg | $\alpha_{S1}$-CN | κ-CN | β-CN |
| 7 000 | 8.88 (0.64) | 23.36 (1.08) | 12.49 (0.80) | 5.50 (0.15) | 29.58 (0.94) | 6.92 (0.69) | 21.20 (1.24) | 13.98 (0.40) | 5.07 (0.13) | 32.32 (0.98) |
| 20 000 | 11.81 (0.47) | 36.58 (1.43) | 5.98 (1.40) | 6.25 (0.78) | 23.87 (2.61) | 10.54 (0.37) | 29.23 (1.15) | 8.99 (0.34) | 6.07 (0.28) | 28.49 (1.55) |
| 50 000 | 14.53 (1.80) | 40.10 (2.79) | 1.99 (0.43) | 7.26 (1.25) | 18.67 (2.10) | 13.60 (0.77) | 38.66 (0.87) | 4.67 (0.33) | 6.77 (0.33) | 21.28 (2.27) |
| Pellet | n.d.* | 2.16 (0.14) | 18.45 (0.46) | 1.48 (0.19) | 48.30 (1.04) | n.d.* | 3.07 (0.08) | 18.56 (0.39) | 1.64 (0.03) | 48.07 (0.90) |

n.d.*: not determined.

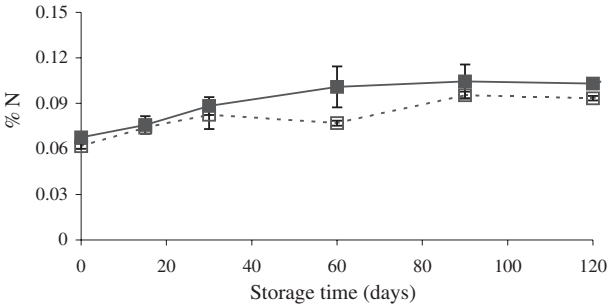


**Figure 3.** Changes in the percentage of nitrogen soluble at pH 4.6 during storage of unhomogenised (0 MPa) (—■—) and homogenised (20 MPa) (---□---) milk samples, both subsequently heated (137 ºC, 4 s). Results are means of two determinations in two different Tetra-Pack® containers ($n$ = 4) ± SD.

of large micelles that contained lower amounts of κ-casein [13]. Comparatively lower relative amounts of soluble κ-casein were found in the serum fractions of the homogenised milk, probably because the higher β-Lg denaturation kept a bigger fraction of κ-casein attached to the micelle surface or the MFGM, through the formation of β-Lg-κ-casein complexes (Tab. II).

### 3.2. Changes during storage

Proteolysis, assessed by changes in the nitrogen soluble at pH 4.6, as shown in Figure 3, increased during storage. Homogenised milk exhibited the least extensive proteolytic degradation. The CE patterns of stored milk samples (results not shown) indicated a decrease in the concentrations of κ- and β-casein, which were more thoroughly

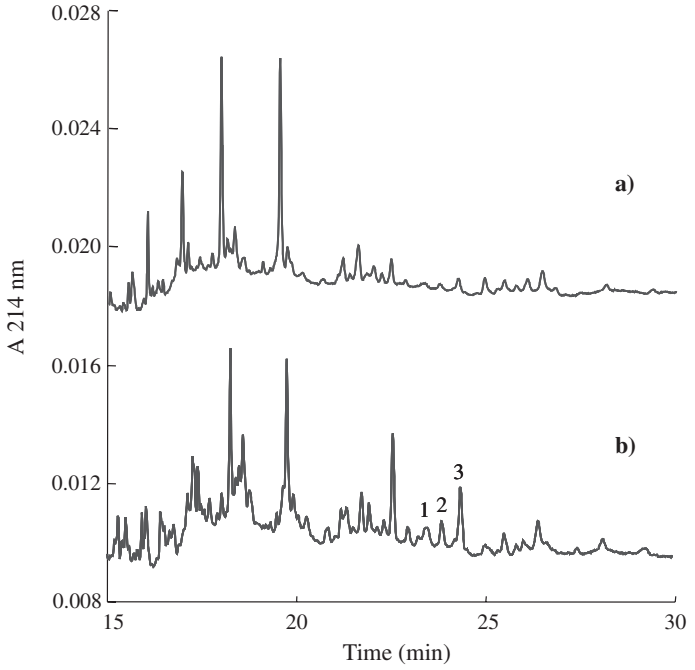


**Figure 4.** CE electrophoregrams of deproteinised wheys obtained from homogenised (20 MPa) and heated (137 ºC, 4 s) milk immediately after processing (**a**) and after 30 d of storage at 20 ºC (**b**). 1: CMP1, 2: CMP2, 3: CMP3 (for peak identification, see text).

**Table III.** Corrected areas of CE peaks CMP1, CMP2 and CMP3 during storage at 20 ºC of unhomogenised (U) (0 MPa) and homogenised (H) (20 MPa) milk samples, both subsequently heated (137 ºC, 4 s). Results are means of two determinations in two different Tetra-Pack® containers ($n$ = 4), followed by SD in brackets.

| Storage time (days) | U | | | H | | |
|---|---|---|---|---|---|---|
| | CMP1 | CMP2 | CMP3 | CMP1 | CMP2 | CMP3 |
| 0 | 0.0562 (0.003) | 0.0578 (0.004) | 0.1169 (0.003) | 0.0305 (0.004) | 0.0294 (0.004) | 0.0852 (0.009) |
| 15 | 0.1158 (0.011) | 0.0806 (0.014) | 0.2155 (0.010) | 0.1235 (0.024) | 0.0804 (0.001) | 0.2076 (0.027) |
| 30 | 0.1659 (0.003) | 0.111 (0.002) | 0.2777 (0.005) | 0.1621 (0.006) | 0.1011 (0.003) | 0.2632 (0.008) |
| 60 | 0.1754 (0.004) | 0.1485 (0.002) | 0.3369 (0.005) | 0.2593 (0.025) | 0.1296 (0.008) | 0.3419 (0.043) |
| 90 | 0.228 (0.035) | 0.1392 (0.008) | 0.3236 (0.001) | 0.3153 (0.006) | 0.1238 (0.001) | 0.3171 (0.010) |
| 120 | 0.4493 (0.012) | 0.1865 (0.005) | 0.3420 (0.012) | 0.3521 (0.007) | 0.1242 (0.004) | 0.3091 (0.009) |

degraded in batch U (58 and 26%, respectively) than in batch H (53 and 17%, respectively) after 120 d of storage.

Since κ-casein was the main protein affected, specific κ-casein breakdown products were studied by CE. Previous investigations pointed out that a characteristic three CE peak pattern could appear during storage of UHT milk samples due to the action of *Pseudomonas fluorescens* proteinases on κ-casein [20]. These peaks were further identified as κ-casein$_{A, B}$ f(106-169) 1P (CMP2), a mixture of κ-casein$_{A, B}$ f(107-169) 1P and κ-casein$_{A, B}$ f(108-169) 1P (CMP1), and κ-casein$_{A, B}$ f(105-169) 1P (CMP3) [23]. The raw milk used in this study was deliberately stored at 4 ºC for 24 h to promote high initial psychrotrophic counts, so that high enzymatic activities of bacterial origin could be expected. In fact, when peptides of milks from batches H and U were analysed by CE during storage, the three peak profile was found (Fig. 4). These κ-casein degradation products increased continuously during storage of batches U and H at 20 ºC, as shown in Table III. In agreement with the other proteolysis indices, the degradation of κ-casein was more extensive in batch U than in batch H at the end of the storage period studied.

The concentrations of monosaccharides found in both batches immediately after processing (Fig. 5) were close to those reported in the literature for commercial UHT milks [1, 22]. Galactose, N-acetyl-galactosamine and N-acetyl-glucosamine increased faster in batch U than in batch H during storage. Previous work showed substantial increases in these monosaccharides during storage of commercial UHT milk [22], which were attributed to the presence of residual glycosidase activities from psychrotrophs, although other processes such as the dephosphorylation of phosphorylated sugars could also contribute [1]. The observation that homogenised milk exhibited a less extensive proteolytic and glycolytic degradation suggested that homogenisation before heating led to an enhanced inactivation of enzymes of either bacterial or native origin.

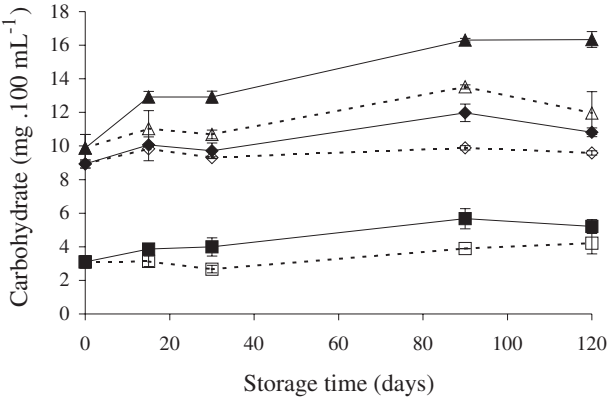


**Figure 5.** Changes in the concentration (mg·100 $mL^{-1}$) of N-acetyl-glucosamine (▲,△), galactose (◆,◇) and N-acetyl-galactosamine (■,□) during storage of unhomogenised (0 MPa) (—, closed symbols) and homogenised (20 MPa) (---, open symbols) milk samples, both subsequently heated (137 ºC, 4 s). Results are means of two determinations in two different Tetra-Pack® containers ($n = 4$) ± SD.

## 4. DISCUSSION

Our results showed that homogenisation of milk (2% fat), preceding an indirect heat treatment on a pilot scale, led to a product less prone to proteolysis than its unhomogenised counterpart, thus confirming previous experiments performed on a laboratory scale [7]. On the one hand, the promoting effect of homogenisation on whey protein denaturation and the observed reduction in enzymatic reactions that depend on residual proteinase, glycosidase and phosphatase activities, suggested that homogenisation of milk before UHT treatment can lead to lower levels of the enzymes that contribute to deterioration during storage.

On the other hand, homogenisation induced modifications in proteins that might have played a role in their susceptibility to proteolytic attack: it brought about the attachment of caseins and whey proteins to the MFGM, produced changes in the morphology of the casein micelles and gave rise to smaller micellar particles not easily sedimentable by centrifugation. Although the overall contribution of these phenomena to proteolysis is difficult to estimate, it should be noted that homogenisation preceding heat treatment increased the concentration of micellar β-Lg and did not have an obvious effect on the release of κ-casein to the serum phase. In fact, a lower proteolysis of κ-casein was found during storage of the homogenised milk, when its specific degradation products were investigated by CE. This could benefit product stability, since it is believed that high proportions of β-Lg-κ-casein complex present at the surface of casein micelles (favoured by high concentrations of denatured β-Lg, and by indirect over direct heating systems [18]), make the access of proteinases to caseins more difficult and so reduce the degree of proteolysis [4, 5] and retard gelation [6, 8].

## ACKNOWLEDGEMENTS

The authors wish to acknowledge financial support from the project AGL2000/1480 (CICYT). We also thank C. Talavera for technical assistance.

## REFERENCES

[1] Belloque J., Villamiel M., López-Fandiño R., Olano A., Release of galactose and N-acetyl-glucosamine during the storage of UHT milk, Food Chem. 72 (2000) 407–412.

[2] Burton H., Reviews of the progress of dairy science: the bacteriological, chemical, biochemical and physical changes that occur in milk at temperatures of 100–150 ºC, J. Dairy Res. 51 (1984) 341–363.

[3] Cano-Ruiz M.E., Richter R.L., Effect of homogenisation pressure on the milk fat globule

membrane proteins, J. Dairy Sci. 80 (1997) 2732–2739.

[4] Celestino E.L., Iyer M., Roginski H., Reconstituted UHT-treated milk: effects of raw milk, powder quality and storage conditions of UHT milk on its physico-chemical attributes and flavour, Int. Dairy J. 7 (1997) 129–140.

[5] Enright E., Kelly A.L., The influence of heat treatment of milk on susceptibility of casein to proteolytic attack by plasmin, Milchwissenschaft 54 (1999) 491–493.

[6] García-Risco M.R., Ramos M., López-Fandiño R., Proteolysis, protein distribution and stability of UHT milk during storage at room temperature, J. Sci. Food Agric. 79 (1999) 1171–1178.

[7] García-Risco M.R., Ramos M., López-Fandiño R., Modifications in milk proteins induced by heat treatment and homogenisation and their influence on susceptibility to proteolysis, Int. Dairy J. 12 (2002) 679–688.

[8] Hardham J.F., Effect of increased β-lactoglobulin and whey protein concentration on the storage stability of UHT processed milk, Aust. J. Dairy Technol. 54 (1999) 77–83.

[9] Harwalkar V.R., Age gelation of sterilized milks, in: Fox P.F. (Ed.), Advanced dairy Chemistry-1: proteins, London and New York, Elsevier Applied Science, 1992, pp. 691–734.

[10] Harwalkar V.R., Cholette H., McKellar R.C., Emmons D.B., Relation between proteolysis and astringent off-flavor in milk, J. Dairy Sci. 76 (1993) 2521–2527.

[11] Hillbrick G.C., McMahon D.J., McManus W.R., Microstructure of indirectly and directly heated ultra-high temperature (UHT) processed milk examined using transmission electron microscopy and immunogold labelling, Lebensm. Wiss. Technol. 32 (1999) 486–494.

[12] Law A.J.R., Effects of heat treatment and acidification on the dissociation of bovine casein micelles, J. Dairy Res. 63 (1996) 35–48.

[13] Law A.J.R., Leaver J., Felipe X., Ferragut V., Pla R., Guamis B., Comparison of the effects of high pressure and thermal treatments on the casein micelles in goat's milk, J. Agric. Food Chem. 46 (1998) 2523–2350.

[14] Marshall R.T., Standard methods for the examination of dairy products, Washington DC: American Public Health Association, 1993.

[15] McKellar R.C., Development of off-flavors in ultra-high temperature and pasteurized milk as a function of proteolysis, J. Dairy Sci. 64 (1981) 2138–2145.

[16] McKellar R.C., Froehlich D.A., Butler G., Cholette H., Campbell C., The effect of uncooled storage on proteolysis, bitterness and apparent viscosity in ultra-high-temperature milk, Can. Inst. Food Sci. Technol. 17 (1984) 14–17.

[17] Olano A., Calvo M.M., Reglero G., Analysis of free carbohydrates in milk using micropacked columns, Chromatographia 21 (1986) 538–540.

[18] Oldfield D.J., Singh H., Taylor M.W., Association of β-lactoglobulin and α-lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant, Int. Dairy J. 8 (1998) 765–770.

[19] Recio I., Olieman, C., Determination of denatured serum proteins in the casein fraction of heat-treated milk by capillary zone electrophoresis, Electrophoresis 17 (1996) 1228–1233.

[20] Recio I., López-Fandiño R., Olano A., Olieman C., Ramos M., Study of the formation of caseinomacropeptides in stored ultra-high-temperature-treated milk by capillary electrophoresis, J. Agric. Food Chem. 44 (1996) 3845–3848.

[21] Recio I., Amigo L. Ramos M., López-Fandiño R., Application of capillary electrophoresis to the study of proteolysis of caseins, J. Dairy Res. 64 (1997) 221–230.

[22] Recio I., Villamiel M., Martínez-Castro I., Olano A., Changes in free monosaccharides during storage of some UHT milks: a preliminary study, Z. Lebensmittel Unters. Forsch. 207 (1998) 180–181.

[23] Recio I., García-Risco M.R., Ramos M., López-Fandiño R., Characterization of peptides produced by the action of psychrotrophic proteinases on κ-casein, J. Dairy Res. 67 (2001) 625–630.

[24] Renner E., Storage stability and some nutritional aspects of milk powders and ultra high temperature products at high ambient temperatures, J. Dairy Res. 55 (1988) 125–142.

[25] Resmini P., Pellegrino L., Andreini R., Prati F., Evaluation of milk whey proteins by reversed-phase HPLC, Sci. Tecn. Latt. Casearia 40 (1989) 7–23.

[26] Sharma S.K., Dalgleish D.G., Interactions between milk serum proteins and synthetic fat globule membrane during heating of homogenised whole milk, J. Agric. Food Chem. 41 (1993) 1407–1412.

[27] Singh H., Latham J.M., Heat stability of milk: aggregation and dissociation of protein at ultra-high temperatures, Int. Dairy J. 3 (1993) 225–237.

[28] Troyano E., Olano A., Fernández-Díaz M., Sanz J., Martínez-Castro I., Gas chromatographic analysis of free monosaccharides in milk, Chromatographia 32 (1991) 379–382.

[29] van Riel J.A.M., Olieman C., Determination of caseinomacropeptide with capillary zone electrophoresis and its application to the detection and estimation of rennet whey solids in milk and buttermilk powder, Electrophoresis 16 (1995) 529–533.