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Changes in furosine and proteins of UHT-treated milks stored at high ambient temperatures

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Veränderungen im Furosingehalt und Proteinen in UHT-Milch bei Lagerung in hoher Umgebungstemperatur

Zusammenfassung. Uperisierte Milch wurde bei 90 Tage-Lagerung bei 20, 30, bzw. 40 °C auf Veränderungen im Furosingehalt, im Gehalt nicht denaturierter Molkenproteine und der Proteolyse untersucht. Mit höherer Temperatur und Lagerzeit hat das Furosin zugenommen. Die Peptidkonzentration hat während der Lagerung bei 30 °C stärker als bei 20 oder 40 °C zugenommen. Die Hochleistungsflüssigchromatographie-Analyse der nicht denaturierten Molkenproteine zeigte wesentliche Veränderungen der Peakformen während der Lagerung.

Abstract. Changes in furosine, undenatured whey protein content, and proteolysis during 90 days storage at 20, 30, and 40° C of UHT-processed milk were studied. Furosine increased as the temperature and storage time increased. The peptide concentration increased during the storage period, being faster at 30° C than at 20 or 40° C. High-performance liquid chromatographic analysis of undenatured whey proteins showed considerable changes in the shape of the peaks during storage.

Introduction

Proteolysis during storage of milk processed by ultra-high-temperature (UHT) treatment is one of the main factors limiting the shelf-life of the product. Although considerable work [1–3] has been carried out on protein breakdown caused by heat-stable proteinases of native or bacterial origin, the problem of controlling the gradual deterioration of UHT-treated milk during storage remains unsolved.

Changes in the protein fraction of UHT-treated milks during storage depend on several factors, such as the bacteriological quality of the raw milk, severity of the heating process and storage conditions. A satisfactory test for assessment of the quality of stored milk must deal with different changes in composition such as proteolysis, whey protein denaturation, and the Maillard reaction. These changes can be monitored by measuring different parameters, such as furosine [4], undenatured β-lactoglobulin [5], and free amino groups [6] which are indicators of the storage conditions of milk.

Analysis of peptides soluble in 4% trichloroacetic acid (TCA) is a method for following proteolysis during storage of milk; however, only data on storage at 20° C are available [7]. Because the rate of biochemical reactions depends on the storage temperature, which can be high in warm countries, the objective of this work was to study the changes in the protein fraction of UHT-treated milks during storage at high ambient temperatures.

Materials and methods

Milk samples. Samples UHT-treated milks that were heated indirectly (batches I and II; 141.5° C/22 s and 139° C/3 s respectively) and directly (batches III and IV; 144° C/6 s and 146° C/5 s, respectively), were supplied from commercial dairy plants. Containers were stored at 20, 30, and 40° C for 3 months. Samples were analysed for free amino groups and peptides at 0, 15, 45, 75, and 90 days of storage. Changes in furosine and undenatured whey proteins were evaluated at intervals of 0, 30, 60, and 90 days. The pH was measured with a Beckman pH meter.

Whey proteins. Whey proteins, separated from whole milk samples after the pH was adjusted to 4.6 with 2 **M** HCl, were processed by reversed-phase HPLC. A Beckman System Gold Chromatograph (Beckman Instruments, Fullerton, USA) composed of a programmable solvent module Model 126, variable-wavelength UV detector, was used with a System Gold Software data system (Beckman Instruments). Separations were performed on a PLRP 8 μm column (300 Å, 150 × 4.6 mm i. d.) (Polymer Laboratories, Church Stretton, UK) with a linear binary mobile phase gradient and wavelength set at 205 nm [8].

Calibrations were made using an external standard method. Standard curves of α-lactalbumin and β-lactoglobulin (Sigma, St. Louis, MO, USA) were linear at the same concentration range and chromatographic conditions at which milk samples were run.

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Furosine. The furosine standard was obtained by acid hydrolysis of ε-*N*-(1-deoxy-D-fructosyl)-L-lysine according to the procedure of Finot et al. [9]. Calibration was made using an external standard method.

Furosine in samples was determined after hydrolysing 0.5 ml milk with 3 ml of 7 *M* HCl at 110° C for 24 h in evacuated and sealed tubes. The hydrolysate was evaporated to dryness in a Speed Vac concentrator A-160 (Savant) and was quantified by ion-pair reversed-phase HPLC according to the method of Delgado et al [10], using a Spherisorb ODS2 5-μm column (250 × 4.6 mm i.d.; Phenomenex, Torrance) operated at ambient temperature, a pump Model 510 (Waters Chromatography Division, Milford, Conn., USA), an injector (Model 7125, Rheodyne, Cotati) and a variable wavelength UV detector Model SM 4000 (LDC Analytical Ribiera Beach, FL, USA). The mobile phase system consisted of 5 m*M* sodium heptanesulphonate with 20% acetonitrile as organic modifier and 0.2% formic acid. The wavelength was set at 280 nm. The flow rate of the elution was 1.2 ml min^{-1}.

Peptides. Before HPLC analysis [7], samples were prepared according to the method of Olieman and van den Bedem [11], but the final TCA concentration was 4% (wt/vol). A 12% (wt/vol) TCA solution (2 ml) was slowly added to milk (4 ml) while it was stirred at 25° C. The mixture was held for 1 h at 25° C and then filtered through Whatman no. 1 filter paper (Whatman, Clitton, NJ, USA). The filtrate was used for HPLC analysis [25 μl; previously filtered through a Millipore 0.45-μm filter, (Millipore, Bedford, MA, USA)]. A Beckman System Gold chromatograph (Beckman Instruments) composed of two Model 115 pumps in combination with a Beckman System Organizer and a 168 Diode Array Detector Module, fitted to a Waters U6K injector, was used together with a System Gold Software data system. The wavelength was set at 210 nm. Separations were performed on a reversed-phase Beckman Ultrapore RPSC column (75 × 4.6 mm i. d.) at room temperature.

Native polyacrylamide gel electrophoresis (native PAGE) of whey proteins. Native-PAGE analysis of the fraction soluble at pH 4.6 followed the procedure of Andrews [12]. Bands were stained with Coomassie brilliant blue R-250 [13]. A Shimadzu (Kyoto, Japan) CS-930 densitometer was used for the densitometric readings.

Measurement of free amino groups. Free amino groups were determined with trinitrobenzenesulphonic acid (TNBS) (Sigma) by the method of McKellar [14]. To prepare samples, 24% TCA (2 ml) was slowly mixed with milk (2 ml) and, after 30 min at room temperature was filtered through Whatman no. 1 filter paper. The absorbance at 420 nm was measured using a Shimadzu spectrophotometer (Model UV-120-01; Kyoto, Japan). Absorbances were converted to micromoles of glycine per millilitre of milk using glycine standard solutions from 0.05 to 0.4 μ*M*.

Results and discussion

The pH of freshly processed UHT-treated milks were 6.50, 6.65, 6.75, and 6.64 for batches I, II, III, and IV, respectively. The lower pH for batch I may be attributable to the severity of the heat treatment undergone by this batch.

Levels of furosine and undenatured whey proteins in freshly processed UHT-treated batches are shown in Table 1. The furosine content in indirect UHT-treated batches was considerably higher than those of direct UHT-treated batches. Marked differences also existed between directly and indirectly heated batches in the remaining undenatured β-lactoglobulin and α-lactalbumin. These differences were mainly due to the comparatively longer equivalent holding periods during indirect heating [4, 5].

Table 1. Furosine and undenatured β-lactoglobulin and α-lactalbumin content in freshly prepared of batches UHT-treated milk (expressed in mg/l)

Batch	Furosine	β-Lactoglobulin	α-Lactalbumin
I	53.4	34.6	95.4
II	39.0	111.5	252.8
III	15.7	530.6	651.8
IV	17.8	348.0	534.7

The furosine and β-lactoglobulin contents of batch I were close to the levels corresponding to in-bottle sterilized milks. This points to the excessive heating applied during processing of this batch.

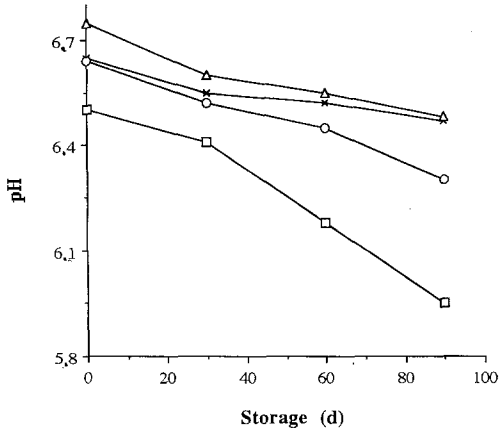

Fig. 1. Changes in pH value during storage at 40° C of samples UHT-treated milk: □, batch I; ×, batch II; △, batch III, ○, batch IV; d, days

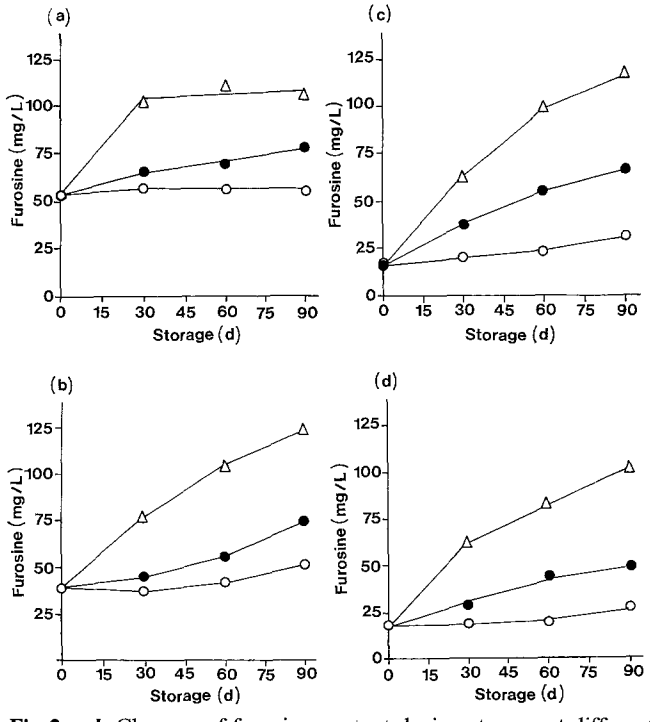

Fig. 2 a–d. Changes of furosine content during storage at different temperatures of UHT-treated milk samples: **a** Batch I. **b** Batch II. **c** Batch III. **d** Batch IV ○, 20° C; ●, 30° C; △, 40° C

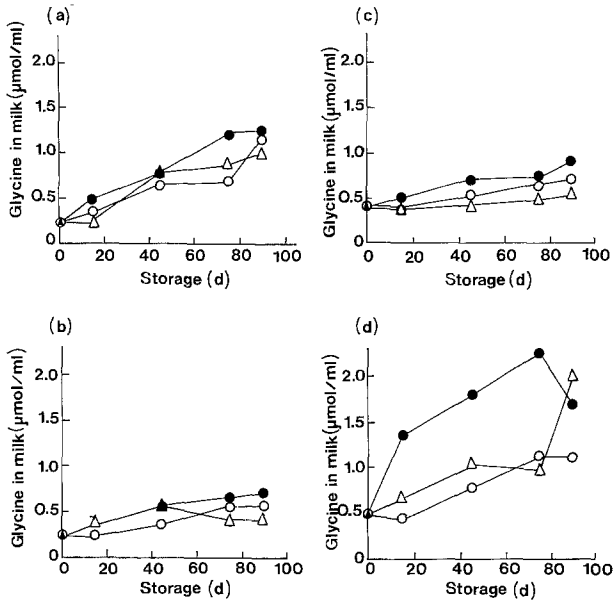

Fig. 3 a–d. Proteolysis [determined on the fraction soluble in 12% (wt/vol) of TCA by the TNBS method] during storage af different temperatures of UHT milk samples: batches and symbols as for Fig. 2

During storage at 20° C and 30° C no appreciable decrease in pH was observed in all batches. However, the pH of samples stored at 40° C dropped considerably. This effect was more pronounced in batch I than in the other batches (Fig. 1).

Figure 2 shows the effect of storage temperature and time on the formation of furosine. As previously observed [4] furosine increased with time and temperature. Storage for 90 days at 20° C caused an increase in furosine in the range 2.7–15.8 mg/l in the four batches studied. At 30 and 40° C, the increase ranged from 25.2 to 51.1 mg/l and 54.1 to 101.4 mg/l, respectively. A similar increase in furosine was observed for batches II, III and IV for all temperatures whereas for batch I only a slight increase at 20° C and 30° C took place during storage. At 40° C an increase in furosine was only observable after 30 days storage. After this storage time no further increase was measured for batch I.

Taking into account that a small decrease in pH gives raise to a considerable decrease in Maillard reaction rate [15] the low formation of furosine during storage of batch I could be related to the lower pH observed in this batch. The formation of 12% TCA-soluble free amino groups increased gradually with storage time in all batches; the rate of increase was faster at 30° C than at 20 or 40° C (Fig. 3). Batches I and IV underwent greater proteolysis than batches II and III, which indicates differences between batches in the thermostable proteinase content.

The heating process for batch I was the more severe of the indirect UHT treatments, and batch IV was the more severely heat-treated of the direct UHT batches. The greater amount of free amino groups released during storage in the more severely heated batches (I and IV) suggests that the bacterial contents of the raw milks used for these batches were higher than those used for batches II and III or that the microbial populations in the raw milks may have contained proteases more thermoresistant than those of batches II and III.

Figure 4 shows the pattern of peptides of a reversed-phase HPLC chromatogram of the soluble fraction in 4% TCA of a indirect UHT-treated milk (batch I) stored at 30° C for 45 and 90 days. The major peptide components, corresponding to peaks 1–2 derived from the action of a *Pseudomonas* proteinase as was shown recently [7], increased progressively during storage; the increase in peak 1 (retention time 17.6 min) was the most pronounced. During the first 15 days of storage, no increase occurred in samples at 20° C, but at 30 and 40° C, an increase up to 27% was detected (Table 2). These results are in agreement with a previous study on storage at 20° C [7]. At 40° C, the maximum increase occurred after 45–75 days of storage and decreased at the end of the period studied. The rate of increase was faster at 30° C than at 20 or 40° C. Formation of peak 1 was greater in samples I and IV than in samples II and III, which agrees with the data on proteolysis.

HPLC analysis of β-lactoglobulin and α-lactalbumin revealed changes on the shape of the peaks during storage (Fig. 5). These changes were negligible during the first 30 days of storage at 20° C but noticeable after 60 and 90 days, increasing considerably with the storage tempera-

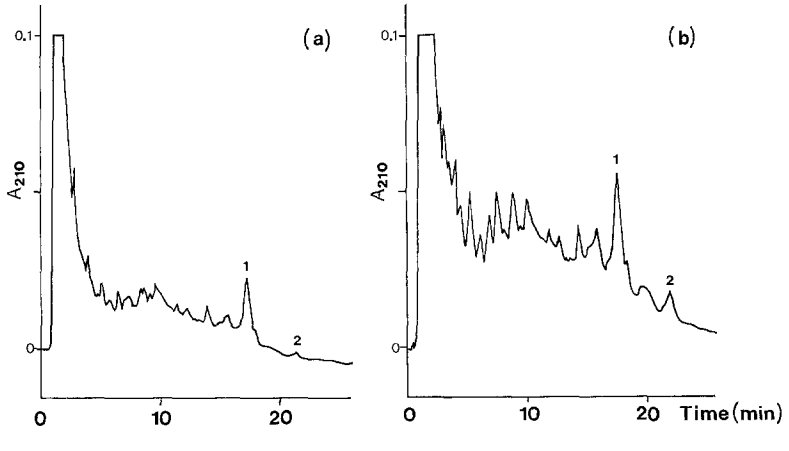

Fig. 4a, b. Reversed-phase HPLC chromatograms of the fraction soluble in 4% TCA from an indirectly heated UHT-treated milk (batch I) stored at 30° C for **a** 45 days or **b** 90 days. A_{210} absorbance at 210 nm wavelength; retention time, 17.6 min for peak 1

Table 2. Effect of time and temperature of storage on formation of peak 1[a] in UHT-treated milk

Sample	Storage (days)	Peak 1_t/Peak 1_{t_0}		
		20° C	30° C	40° C
I	15	1.00	1.10	1.10
	45	2.25	3.25	1.50
	75	2.37	5.87	0.87
	90	4.37	6.25	1.12
II	15	0.80	1.00	1.20
	45	1.40	1.40	2.60
	75	2.40	2.40	1.00
	90	2.60	2.20	1.60
III	15	0.75	1.25	1.25
	45	1.00	1.75	3.25
	75	1.75	3.75	1.25
	90	2.25	4.00	1.50
IV	15	1.00	1.00	1.27
	45	1.80	8.90	1.90
	75	3.45	10.80	2.45
	90	4.45	10.40	1.90

[a] Peak height at 17.6 min in the HPLC chromatogram of proteins soluble in 4% (wt/vol) of trichloroacetic acid; t, time of storage; t_0, initial time

ture. The modification of the shapes of the peaks could be in part attributable to the progress of the Maillard reaction and also to interactions among proteins. Melo and Hansen [16] found a new component with mobility intermediate between α-lactalbumin and β-lactoglobulin after UHT processing of serum proteins in model systems. Analysis indicated that it was a complex of α-lactalbumin and β-lactoglobulin, probably linked by sulphhydryl groups. Native-PAGE analysis of samples stored

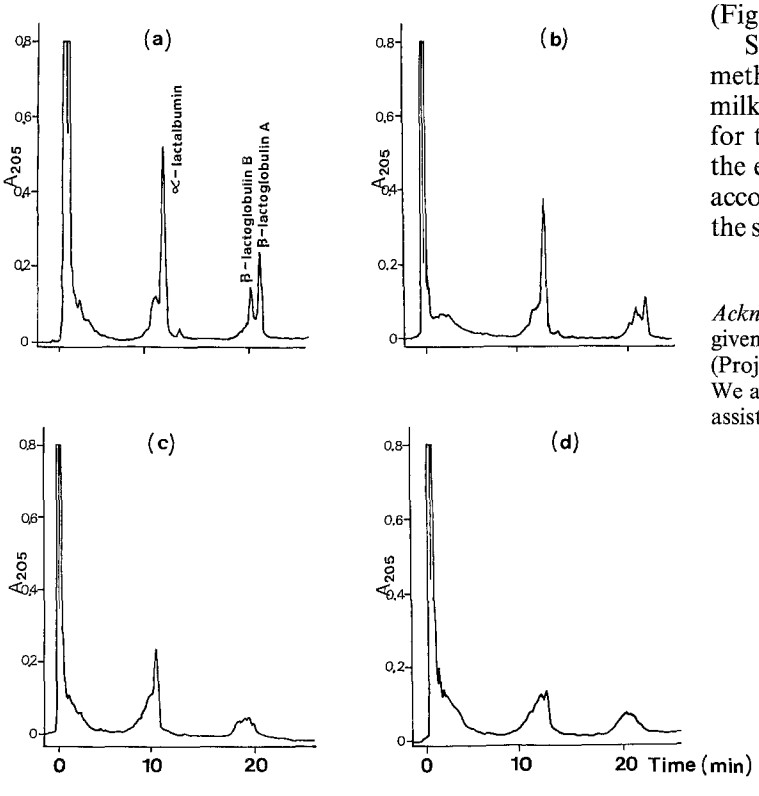

Fig. 5 a–d. Reversed-phase HPLC chromatograms of the fraction soluble in pH 4.6 from a directly heated UHT-treated milk (batch III): A_{205} = absorbance at 205 nm. **a** Freshly processed. **b** After 90 days storage at 20° C. **c** After 90 days storage at 30° C. **d** After 90 days storage at 40° C

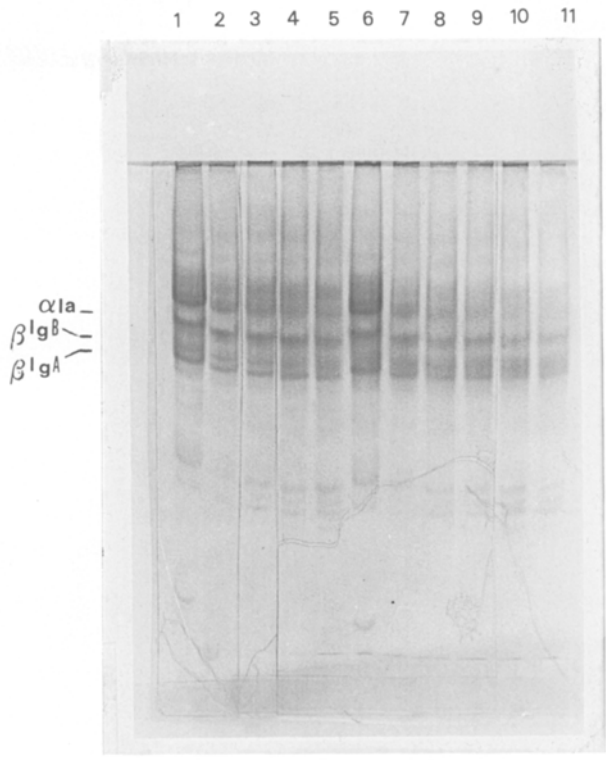

Fig. 6. Native PAGE analysis of whey proteins of milk sample I freshly prepared (*lanes 1* and *6*) and stored at 20° C for 15 (*lane 2*), 45 (*lane 3*), 75 (*lane 4*) and 90 (*lane 5*) days; and at 30° C for 15 (*lane 7*), 30 (*lane 8*), 45 (*9 lane*), 75 (*lane 10*) and 90 (*lane 11*) days. α-La, α-Lactalbumin; β-Lg, β-Lactoglobulin A resp. B

at 30° C showed that bands corresponding to β-lactoglobulins A and B lost sharpness and three or four less-defined bands appeared in the electrophoretogram (Fig. 6).

Since β-lactoglobulin determination is used as a method for distinguishing between heat treatments of milks, variations during storage could have implications for the interpretation of the results. On the other hand, the elution profile of whey proteins has to be taken into account since it can provide additional information about the storage conditions of the milk samples.

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References

1. Cousin MA (1982) J Food Prot 45:172–207
2. Law BA (1979) J Dairy Res 46:573–588
3. Miranda G, Gripon JC (1986) Lait 66:1–18
4. Nangpal A, Reuter H (1990) Kiel Milchwirtsch Forschungsber 42:77–86
5. Andreini R, Chiodi J, De Noni I, Resmini P, Batelli G, Cecchi L, Todesco R, Cattaneo TMP, Rampilli M, Foschino R (1990) Sci Tecn Latt Cas 41:472–492
6. Manji B, Kakuda Y, Arnott DR (1986) J Dairy Sci 69:2994–3001
7. Lopez-Fandiño R, Olano A, San José C, Ramos M (1993) J Dairy Res 60:111–116
8. Resmini P, Pellegrino L, Andreini R, Prati F (1989) Sci Tecn Latt Cas 40:7–23
9. Finot PA, Bricout J, Viani R, Mauron J (1968) Experientia 24:1097–1099
10. Delgado T, Corzo N, Santa-María G, Jimeno ML, Olano A (1992) Chromatographia 33:374–376
11. Olieman C, van den Bedem JW (1983) Neth Milk Dairy J 37:27–36
12. Andrews AT (1983) J Dairy Res 50:45–55
13. Winter A, EK K, Andersson V (1977) Bremma Sweden: LKB-Produkter (LKB Application Note 250)
14. McKellar RC (1981) J Dairy Sci 64:2138–2145
15. Ashoor SH, Zent JB (1984) J Food Sci 49:1206–1207
16. Melo TS, Hansen AP (1978) J Dairy Sci 61:710–713