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Furosine formation and proteolytic changes in carbohydrate reduced UHT milks

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The effect of carbohydrate reduction on furosine formation and proteolysis in lactose hydrolysed direct UHT-treated milks was studied during storage of 12 weeks. Carbohydrate reduction levels of 0, 23 and 96% were compared. Lactose unhydrolysed milk was used as a reference. Furosine formation and lysine blockage were best avoided in 96% carbohydrate reduced milk (carbohydrate free milk) and lactose unhydrolysed milk. Proteolysis was almost eliminated in carbohydrate free UHT-milk manufactured with chromatographic separation. Chromatographic separation of lactose from skim milk at 65 °C separated lactose from the proteins and probably caused thermal inactivation of the plasmin enzyme system. Release of γ-casein referring to action of plasmin during the storage was not significantly noticed in carbohydrate free milk like in lactose hydrolysed, carbohydrate reduced and hydrolysed or unhydrolysed milks. Colour formation at ambient or higher temperature was avoided in carbohydrate free milk.

Furosin-Bildung und proteolytische Veränderungen in Kohlenhydrat reduzierten UHT-Milchen

Im Verlauf einer zwölfwöchig Lagerung wurden die Auswirkungen einer Kohlenhydrat-Reduktion auf die Furosin-Bildung und Proteolyse in Laktose hydrolysierten, direkt UHT behandelten Milchen untersucht. Verglichen wurden Kohlenhydrat-Reduktionswerte von 0%, 23% und 96%. Als Referenz diente eine Milch ohne Laktose-Hydrolyse. Die Furosin-Bildung und die Blockierung von Lysin wurden am besten in einer Milch vermieden, in der die Kohlenhydrate um 96% reduziert waren („Kohlenhydrat freie Milch") und in Milch ohne Laktose-Hydrolyse. Eine Proteolyse wurde nahezu vollständig eliminiert in Kohlenhydrat freier UHT-Milch, hergestellt mit chromatographischer Abtrennung. Die chromatographische Abtrennung von Laktose aus Magermilch bei 65 °C führt zu einer Abtrennung der Laktose von den Proteinen und wahrscheinlich zu einer Inaktivierung des Plasmin-Enzymsystems. Die Freisetzung von γ-Casein in Folge der Plasminwirkung während der Lagerung wurde in Kohlenhydrat freier Milch nicht signifikant beobachtet im Vergleich zu Laktose hydrolysierter, Kohlenhydrat reduzierter sowie hydrolysierter oder nicht hydrolysierter Milch. Eine Farbbildung bei Umgebungstemperaturen oder höher wurde in Kohlenhydrat freier Milch vermieden.

34 UHT milk (carbohydrate reduction, furosine formation, proteolysis)

34 UHT-Milch (Kohlenhydrat-Reduktion, Furosinbildung, Proteolyse)

1. Introduction

The Maillard reaction in lactose hydrolysed and lactose free milks is a common problem (1, 2, 3). The reaction can cause deterioration of the nutritional value in several different ways (4, 5, 6, 7) as well as changes in colour (8, 9) and flavour (10) of the milk during heat treatment or storage of the product. In UHT-milks also proteolytic changes due to indigenous or contaminating microbial enzymes take place when stored at ambient temperature (11). In lactase treated UHT-milks proteolysis may be enhanced due to proteolytic side activities of the enzyme preparation (12). This may activate the Maillard reaction even further (13). The early phases of the Maillard reaction in milk are often studied by measuring furosine. This describes formation of lactulosyllysine or fructosyllysine which are intermediate products in the reaction (7). Furosine can be released from the protein by acid hydrolysis treatment and analysed with HPLC. Lysine is the amino acid in proteins which most readily reacts with reducing sugars.

Possibilities to reduce furosine formation and lysine blockage in lactose hydrolysed UHT-milk were investigated in this study by reducing the carbohydrate content in milk prior to enzymatic hydrolysis of the lactose. Changes in proteolysis and colour of the milks were followed throughout the storage period.

2. Materials and methods

Test milks: (1) lactose unhydrolysed milk (UM) was normal skim milk received at the Valio UHT-plant, Turenki, Finland. (2) lactose hydrolysed milk (HM) was skim milk prehydrolysed in a tank. (3) carbohydrate reduced and hydrolysed milk (CRHM) was prepared using chromatographic separation to remove lactose from evaporated milk as described by HARJU (14). Separation was performed in a column at 65 °C lasting for 2.5-3.5 h using water as eluate. Skim milk was added to balance the composition so that milk had a normal protein content and 3.2% of lactose prior to hydrolysis. 4. carbohydrate free milk (CFM) was prepared by removing lactose almost totally with chromatographic separation. Composition of the milks is presented in Table 1. The same dosage of Godo YNL-2 lactase (Godo Shusei, Japan) per lactose quantity (1.9% of lactose) was used in all hydrolysed test milks. Hydrolysis time was 15 h at 5-10 °C. All milks were treated with direct-UHT process as described in an earlier article (12) and packed into 1 L TetraBrik cartons. The direct UHT treatment for all four test milks was identical.

The cartons were divided into four groups to be stored at temperatures of 5, 22, 30 and 45 °C, respectively. The cartons were stored for 12 weeks and samples were taken for analyses at least every 4 weeks. The samples were frozen at –70 °C and then transferred to –21 °C for storage.

Analyses: Samples were analysed every 4 weeks for furosine, tyrosine equivalent and α-amino-N. Samples for SDS-PAGE analyses were taken directly after packing of milk and after 4 and 12 weeks of storage. Furosine was analysed according to (15). Lysine blockage was estimated on basis of furosine according to EVAN-

GELISTI *et al*. (1). Colour of the milk samples was measured as described earlier (32). Lactose, glucose and galactose were analysed using high-performance anion-exchange chromatography with pulsed amperometric detection with a modified method of DE SLEGTE (16). Carbohydrate content was calculated as a difference between dry matter and sum of protein, fat and ash contents. Tyrosine equivalent content was analysed with Folin reagent according to MATSUBARA *et al*. (17) in four replicates. α-Amino-N was analysed according to LIESKE and KONRAD (18). Analysis was performed in four replicates. SDS-PAGE analyses were done according to (19) using 10 µg protein per sample well. Ready-made 18% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA, USA) were used. Protein bands were visualized by staining with Coomassie G-250 (GelCode® Blue Stain Reagent, Pierce, USA) and compared with molecular weight markers (Prestained SDS-PAGE standards, broad range, Bio-Rad, USA). Milk samples were analysed for fat (20), total nitrogen and protein (21), dry matter (22), ash (34), standard plate count (23), coliforms (24), *Bacillus cereus* (25), psychrotrophs (26) and thermophilic bacteria (27). Temperature was measured with a calibrated thermometer.

3. Results

Composition of the UHT-milks is shown in Table 1. The carbohydrate content of the milks varied from 0.2 to 4.5%. In lactose hydrolysed milks carbohydrate reduction levels were 23% (CRHM) and 96% (CFM) as compared to HM. In UM the carbohydrate was lactose and in other milks it was hydrolysed lactose i.e. mainly glucose, galactose and residual lactose.

Table 1: Composition of the test UHT milks

	Unhydro-lysed	Hydro-lysed	Carbohydr. red. and hydrolysed	Carbohydr. free
Dry matter (%)	8.7	8.2	7.6	4.4
Protein (%)	3.4	3.2	3.5	3.5
Fat (%)	0.08	0.2	0.2	0.05
Carbohydrate (%)	4.5	4.2	3.2	0.2
Lactose (%)	4.5	0.1	0.1	<0.01
Ash (%)	0.7	0.6	0.7	0.6

Table 2 shows the microbiological quality of the milks before the UHT-treatment. The microbial count varies due to different production methods. After the UHT-treatment the milks were sterile with no microbes found.

Table 2: Microbial counts (cfu/g) of the milks before the UHT-treatment.

	Standard-plate count	Psychro-trophic microbes	Coli-form	B. cer-eus	Thermo-philic bacteria
Unhydrolysed	23000	0	0	<10	3
Hydrolysed	35	0	0	<10	3
Carboh. red. and hydrolysed	63000	90000	1000	200	20
Carbohydr. free	3500	35	40	4	520

3.1 Furosine and available lysine

Figure 1 shows the effect of carbohydrate content on furosine formation in UHT-milks stored at 5, 22, 30 or 45°C for 12 weeks. In CRHM reduction of carbohydrates by 23% as compared to HM lowered the furosine content by 9-24% depending on the storage temperature. Furosine level in CRHM was 2-3.7 times higher than in UM. In CRHM the lower content of reducing sugars lowered the formation of furosine almost at the same ratio as reduction of carbohydrate content. Taking into account that CRHM had a slightly higher heat load due to the chromatographic separation than HM it seemed that the content of reducing sugars had a significant effect on the rate of the Maillard reaction. By removing lactose and other reducing sugars almost totally from milk with chromatography it was possible to avoid furosine formation almost completely in CFM in all storage temperatures tested.

The relative amount of blocked lysine was estimated on the basis of furosine. Results are presented in Table 3. Lysine blockage remained at the lowest level in CFM and here the storage temperature did not have any significant effect.

Table 3: Estimated blocked lysine (% of initial lysine) during the storage at different temperatures

	Time (wk)	Unhydro-lysed	Hydro-lysed	Carbohydr. red. & hydrolysed	Carbo-hydrate-free
5°C	0	0.7±0.0	2.4±0.1	2.4±0.1	1.2±0.1
	4	1.0±0.1	2.6±0.1	2.5±0.1	1.2±0.1
	8	1.3±0.1	2.8±0.1	2.6±0.1	1.3±0.1
	12	1.3±0.1	2.9±0.1	2.8±0.1	1.2±0.1
22°C	0	0.7±0.0	2.4±0.1	2.4±0.1	1.2±0.1
	4	1.3±0.1	4.3±0.2	4.1±0.2	1.3±0.1
	8	2.1±0.1	6.1±0.3	5.5±0.3	1.3±0.1
	12	2.6±0.1	7.5±0.4	6.7±0.3	1.2±0.1
30°C	0	0.7±0.0	2.4±0.1	2.4±0.1	1.2±0.1
	4	2.2±0.1	9.4±0.5	8.4±0.4	1.2±0.1
	8	4.3±0.2	14±1	13±1	1.2±0.1
	12	5.5±0.3	19±1	16±1	1.1±0.1
45°C	0	0.7±0.0	2.4±0.1	2.4±0.1	1.2±0.1
	4	6.6±0.3	26±1	22±1	0.6±0.1
	8	13±1	37±2	32±2	0.4±0.0
	12	14±1	35±2	36±2	0.4±0.0

3.2 Changes in colour during the storage

The correlation between colour index change and furosine change in UHT-milks during the storage was analysed. As found in the earlier study by TOSSAVAINEN and KALLIOINEN (32) the correlation was highest among the milks stored at 45°C, then at 30°C, then 22°C and lowest at 5°C, where the colour changes were almost nonexistent. Again a high correlation (r^2 = 0,909, y = 30.72x+10.98, n = 60) among all milk samples between the colour index change (x) and furosine (mg/100 g protein) change (y) was found in spite of the different carbohydrate contents and composition in milks.

3.3 Proteolysis during storage

Proteolysis was clearly lowest in CFM at 5, 22 and 30°C both after storage of 4 weeks and 12 weeks (Fig. 2). Only negligible amounts of γ-casein was released in CFM referring that milk plasmin enzyme (PLM) was not active to any significant extent. Tyrosine equivalent analysis support this SDS-PAGE analysis result (Table 4). However, on the contrary at 45°C significant proteolysis took place also in CFM already after 4 weeks of

storage referring that some thermophilic proteolytic enzymes of microbial origin had survived the heating process (Table 4). Tyrosine equivalent analysis was preferred over the α-amino-N analysis due to the better repeatability of the tyrosine equivalent method (data not shown). In CRHM proteolysis was significant probably due to the active PLM derived from skim milk in the recipe and psychrotrophic bacteria found in milk before the UHT-treatment (Table 2).

Table 4: Tyrosine equivalent content (mg/100 g protein) in UHT-milks during the storage

	Storage time (weeks)			
	0	4	8	12
Unhydrolysed				
5℃	287±3	275±1	309±1	286±1
22℃	287±3	330±3	416±3	462±1
30℃	287±3	403±12	601±3	827±9
45℃	287±3	421±3	546±42	628±3
Prehydrolysed				
5℃	297±3	372±1	418±3	443±3
22℃	297±3	553±6	838±3	1155±6
30℃	297±3	933±12	1423±24	1909±12
45℃	297±3	1132±3	1325±12	1537±15
Carbohydrate reduced and hydrolysed				
5℃	346±3	353±1	364±3	435±9
22℃	346±3	589±6	879±3	1269±1
30℃	346±3	1003±6	1679±9	2173±9
45℃	346±3	1138±3	1323±12	1409±12
Carbohydrate free				
5℃	147±1	176±3	189±1	187±3
22℃	147±1	203±3	250±9	266±3
30℃	147±1	239±1	330±6	477±1
45℃	147±1	808±6	1112±3	1667±30

4. Discussion

In a previous article TOSSAVAINEN and KALLIOINEN (32) concluded that the storage temperature is a more significant factor than the order of the hydrolysis and UHT treatment steps for the formation of furosine. In this study the quantity of the reducing sugars has a significant effect on furosine formation. By reducing the amount of glucose and galactose close to zero it was possible to avoid the Maillard reaction almost completely.

As we noticed in our earlier work (12) also in this study proteolysis was more enhanced in HM than in UM (Table 4). However, in CFM produced with chromatographic separation technique both the Maillard reaction and proteolysis were efficiently prevented during storage at temperatures up to 30℃.

Chromatographic separation of lactose from milk seems to be a suitable process for producing protein+minerals fraction, where the proteolytic activity is very low. Furthermore this fractionation makes it possible to produce UHT-milk which has marginal proteolytic changes during the entire storage period of 12 weeks. This is probably due to the long heat treatment (2.5-3.5 h) at 65℃ during chromatographic separation which destroyed most of both the indigenous and microbial proteolytic activities in milk. In other lactose hydrolysed milks normal skim milk was part of the recipe and therefore PLM and added lactase enzyme with its side activities remained at least partly active in them.

The activity of PLM in milk is controlled by a system of enzyme activators (PAs) and inhibitors (PIs). PLM has an inactive precursor plasminogen (PLG) in milk, which is activated by PAs. Heat treatment of milk alters the natural balance between PAs and PIs in favour of PAs, which can lead to enhanced proteolysis in UHT milk (11). Therefore, if proteolysis elimination is the target, denaturation of whole PLM+PLG system should be the goal.

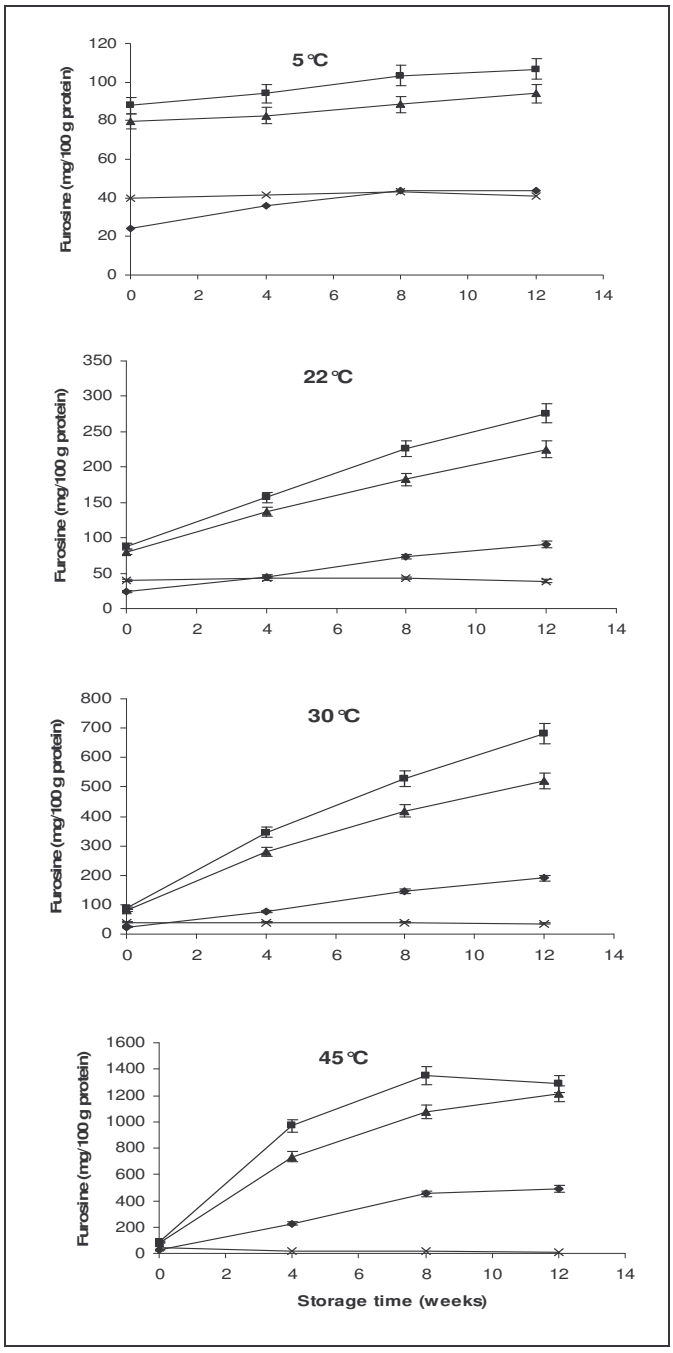

Fig. 1: Furosine formation in different lactose hydrolysed UHT-milks during storage. Symbols: (◆) unhydrolysed, (■) hydrolysed, (▲) carbohydrate reduced and hydrolysed milk, (X) carbohydrate free skim milk.

The heat treatment needed to inactivate PLM system depends on the availability of free SH-groups (denatured β-lactoglobulin) (28). In milk for PLM and PLG decimal reduction times (time needed for 90% loss of activity) of 195 min and 134 min respectively at 70℃ have been reported (28). Driessen (29) reported decimal reduction time of 55.6 min for PLM at 67.5℃.

These results support that the PLM system was significantly inactivated during the chromatographic separation.

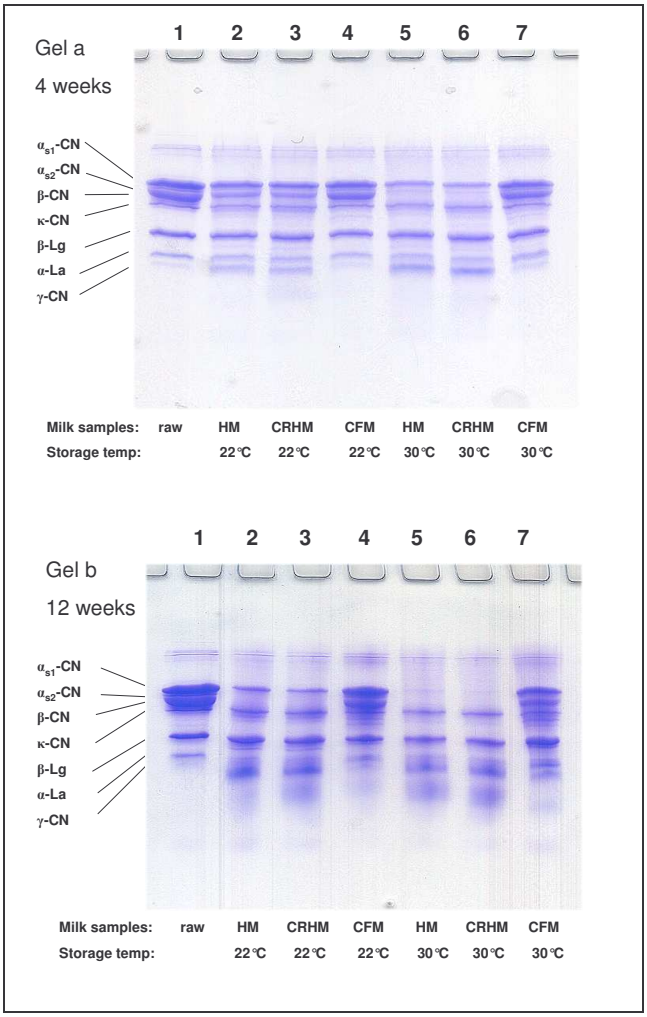

Fig. 2: SDS-PAGE analyses of the UHT-milks at different storage temperatures and times. Symbols: raw = separated raw milk, HM = hydrolysed milk, CRHM = carbohydrate reduced and hydrolysed milk, CFM = carbohydrate free milk.

On the other hand extracellular proteinase of *Serratia marcescens D2* loses its activity rather slowly (D=3,3*10E3 s = 55 min) at 65°C. Also the extracellular proteinase of *Pseudomonas fluorescens 22F* loses its activity very slowly at 70°C (D=1,45*10E5 s = 40.3 h). Similarly the decimal time (D) of *Achromobacter sp. 1-10* proteinase is long at 70°C (7,39*10E4 s = 20.5 h) (29). These or other similar thermostable proteinases may have acted during the storage at 45°C where the CFM formed sediment as precipitated protein already after 2 weeks of storage. At other temperatures this was not noticed.

Other bacteria with thermostable extracellular proteinases are e.g. certain other species of *Pseudomonas, Enterobacter, Alcaligenes, Flavobacterium*, and *Serratia* (30, 31). Reactivation of thermostable proteinases after heat treatment has been found to occur (30).

The highest tyrosine equivalent contents were found in other milks except in CFM at 30°C storage temperature. This slightly differs from α–amino-N values found in the earlier study where highest values were usually found at 45°C (12). The difference is probably due to the differences in analytical methods used. Tyrosine equivalent analysis using Folin reagent detects mainly tyrosine and tryptophan in proteins (33).

The Maillard reaction was best eliminated in CFM where reducing sugars were almost nonexistent and the reaction could not take place. Therefore also the available lysine survived best in CFM. The colour change was negligible in CFM during the storage at all temperatures tested.

5. Conclusions

In order to avoid lysine blockage due to the Maillard reaction and the proteolytic breakdown of milk proteins due to the plasmin enzyme system or the proteolytic enzymes from the contaminating microbes or lactase preparation the best results were achieved with chromatographically separated, carbohydrate free skim milk (CFM). The chromatographic treatment separated reducing sugar lactose from the milk proteins which contributed to avoidance of the Maillard reaction. The treatment temperature 65°C with a duration of 2.5 to 3.5 h was probably able to inactivate the plasmin enzyme system almost completely. During the 12 week storage no significant release of γ-casein was noticed referring that plasmin was inactive. In HM and CRHM high storage temperatures have to be avoided to keep nutritional deterioration and browning of milk as low as possible.

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