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## Evaluation of UHT milk processed by direct steam injection and steam infusion technology

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# **Evaluation of UHT milk processed by steam injection and steam infusion technology**

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**Lund 2007**



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Fakultetsopponent: Dr. ir. J.A. Nieuwenhuijse. Friesland Foods, Deventer, Nederländerna

Academic thesis which by due permission of Faculty of Engineering at Lund University will be publicly defended on Friday 9<sup>th</sup> of February 2006 at 13.15 , the Centre for Chemistry & Chemical Engineering, Lund, for the degree of doctor of Philosophy in Engineering.

Faculty opponent: Dr. ir. J.A. Nieuwenhuijse. Friesland Foods, Deventer, Netherlands

Evaluation of UHT milk processed by steam  
injection and steam infusion technology

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To my mum....in memoriam, to René and Annika for their future....

## LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

- I. Malmgren, B., Dejmek, P., Zakora, M., Paulsson, M. & Ardö, Y.: Evaluation of UHT milk processed by steam injection and steam infusion technology. 1. Enzymatic activity by CE and HPLC, *International Dairy Journal*, manuscript
- II. Malmgren, B., Dejmek, P., Paulsson, M. & Langton, M.: Evaluation of UHT milk processed by steam injection and steam infusion technology. 2. Microstructure during processing steps by TEM. *International Dairy Journal*, manuscript
- III. Malmgren, B., Dejmek, P., Paulsson, M. & Langton, M.: Evaluation of UHT milk processed by steam injection and steam infusion technology. 3. Structure and stability during storage. *International Dairy Journal*, manuscript
- IV. Malmgren, B., Nilsson, M., Altskär A., Dejmek, P., Paulsson, M. & Langton, M.: Evaluation of UHT milk processed by steam injection and steam infusion technology. 4. Microstructure by TEM and immunogold labelling. *International Dairy Journal*, manuscript

## **My contribution to the papers**

Paper I        I did the experimental work and partly analyses together with Aksana Persson, Mona Østergaard and Prof.Ylva Ardö . I wrote the manuscript.

Paper II       I did the experimental work, partly the analysis together with Aksana Persson, Maud Langton and Annika Altskär. I wrote the manuscript.

Paper III      I did the experimental work, partly the analysis together with Aksana Persson, Maud Langton and Annika Altskär. I wrote the manuscript.

Paper IV      I did the experimental work, partly the analysis together with Aksana Persson, Martin Nilsson, Maud Langton and Annika Altskär. I wrote the manuscript.

**Abstract**

UHT direct steam injection and steam infusion are widely used; however there is no comparison of their impact on milk components. This study evaluates the structural changes at different steps during the UHT processing of whole milk in a full-scale UHT plant by transmission electron microscopy (TEM) and particle size measurements, and follows the enzymatic activity.

The results of structural analyses by TEM show the formation of agglomerates during the final heating step using both technologies; however in samples from steam injection, the agglomerates were larger and more frequent. Regardless of the heating system, big agglomerates were still present after the vacuum cooling, but very rarely in the final product. The creation of big protein agglomerates in the milk from steam injection could be a result of the accumulation of proteins on the surface of steam bubbles formed during the introduction of steam into the product and may influence its stability at the early stage of storage (Paper II.).

Sedimentation was the only parameter significantly dependent on the final heating technology and preheating temperature. The amount of sediment was significantly higher in milk processed by steam injection and when preheated to 75°C compared to 80°C. Sediment in all samples contained large protein particles, some of them containing fat globule agglomerates covered by a thick protein layer (Paper III.).

The casein micelles in milk stored at 5°C, 22°C and 30°C had an irregular surface that reduced in size on storage while tendrils became extended. These structural changes led to gelation within four or five

months' storage at 22°C and 30°C. At 40°C the tendrils dissociated and the samples never gelled. The absence of gel formation could be a combined effect of plasmin activity and lactosylation (Paper III.).

The volume-weighted mean diameter of casein micelles in samples produced by steam infusion was larger than in samples from steam injection. This could be explained by the  $\beta$ -lactoglobulin/ $\kappa$ -casein complex being better anchored to the casein micelle surface than in samples from steam injection, where the complex was released to a greater extent or mechanical damage of casein micelles during steam injection leading to micelle disintegration and disaggregation (Paper III.).

The structural changes and localization of the individual proteins in UHT milk kept at 22°C or 40°C for 6 months were followed using transmission electron microscopy combined with immunogold labelling on total caseins,  $\kappa$ -casein and  $\beta$ -lactoglobulins A and B. In the freshly produced UHT milk, the labelling of total casein concentrated mainly on the micelles, while  $\beta$ -lactoglobulin and  $\kappa$ -casein were localized in the serum phase and on the micelle surface. In the stored samples, tendrils protruding from the micelles showed positive labelling against all the analyzed proteins. The heavy particles that sedimented after 6 months storage showed positive labelling for total casein but not for  $\kappa$ -casein suggesting that the sediment composition based on caseins was most probably the hydrophobic parts of the casein micelles or protein fragments from proteolyses by plasmin (Paper IV).

The enzymatic activity was the same in milk produced in both systems; however the sedimentation rate was greater in samples treated



## ***Abstract***

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with steam injection. Based on that, we can say that analyses of results of proteolytic action measured by capillary electrophoresis of supernatant cannot alone be used for predicting the shelf life or stability of UHT milk (Paper I).

Plasmin activity was negligible at 5°C, increased with temperature at 22°C and 30°C, but decreased at 40°C. Gel formation occurred in samples stored at 22°C and 30°C, but not at 5°C and 40°C. Lactosylation started at 22°C and increased with increasing storage temperature. After 6 months storage at 40°C, all  $\beta$ -lactoglobulin molecules contained at least 2 lactose residues, and the proteose-peptones were also lactosylated. The extensive lactosylation at 40°C may play an important role in the absence of gelation. Destabilization of casein micelles and restructuring of proteins have a huge impact on both sediment and gel formation (Paper I).

## **Introduction and Objectives**

The consumption of UHT milk (Ultra High Temperature) is becoming more and more common in many parts of the world. UHT treatment involves heating to temperatures exceeding 135-150° C for a few seconds, which not only destroys the potentially pathogenic microorganisms, but also most of the thermoresistant spores (Fellows, 2000). The UHT treatment leads to products with a shelf life of from six months up to a year without refrigeration and in this way the technology opens the possibility to distribute products even in countries with undeveloped cold chains or over great distances.

The UHT process is time, space and energy saving in relation to conventional pasteurization (Bylund, 1995). One of the disadvantages is the problem of maintaining the quality of the product characterized by flavour changes and instability which may lead to sediment and gel formation during long-term storage.

There are two main types of UHT systems on the market: direct and indirect. The former are characterized by the direct contact of the treated product with the heating medium, for example steam, whereas in indirect systems the heat is always transferred indirectly through the wall of a heat exchanger. There are two different technologies of direct heating used for UHT processing of dairy products - steam injection and steam infusion. In steam injection, steam is injected into the product and in steam infusion the UHT processed product is introduced into a steam filled vessel.

## ***Introduction and Objectives***

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The different ways of mixing the treated product with steam were a subject of discussion for long time and different investigation have been carried out, however, in most cases the comparisons were not made in the same installation, on the same raw product, so only the way of the final heating differs.

**The objective** of this study was to conduct fully comparable tests based on UHT milk production and evaluate the qualities of the milk during processing and under different storage conditions.

## 1. UHT technology

### 1.1 Principles of UHT technology

Heat treatment in the production of long life products is called 'sterilisation'. In such processes the treated product is exposed to such powerful heat treatment that the relevant microorganisms and most of the enzymes are inactivated and the processed product is given excellent keeping qualities and can be stored for several months under ambient conditions.

When high temperatures are applied to the product, two types of effects occur (Burton, 1994). Firstly the bactericidal effect which is the aim of the process, secondly chemical changes like changes in colour, flavour, texture and nutritional value, which are undesirable. All kinds of heat treatments are effects of the combination of the processing temperature and time. The UHT treatment is defined as heat treatment at 135°C for not less than a second (ECC directive, 1992). The aim of the operation is to achieve '**commercial sterility**'. A commercially sterile product is free from microorganisms that can grow under storage conditions. According to the ECC directive (ECC directive, 1992) in random sampling checks, UHT milk must meet the following standards after incubation at 30°C for 15 days:

Plate count (30°C)	≤10 0.1 mL <sup>-1</sup>
Organoleptic quality	normal

To compare the various effects of heat treatments, different values are calculated. For the microbiological effect **F<sub>0</sub> value** is already used in classical canned sterilization technology and is defined as the number of minutes at 121.1°C (250°F) to which the process is equivalent and is calculated according the following formula :

$$F_0 = \frac{t}{60} \cdot 10^{\frac{(T - 121.1)}{z}}$$

Where:

- t is the sterilization time in seconds at temperature T in °C
- T is the sterilization temperature in °C
- z is a value expressing the increase in temperature necessary to obtain the same lethal effect in 1/10 of the time (in the region 10-10.8°C) generally set as 10°C

For commercial applications the required F<sub>0</sub> value is 5 – 6 (Bylund, 1995).

Another criterion introduced by Horak and Kessler (Horak, 1980; Kessler & Horak, 1981a; Kessler, 1981) is **B\* value**. B\* value equals 1 correspondent to 9 decimal reduction of natural thermophilic spores (incubation at 55°C) (Figure 1). The minimum recommended B\* value is 1 (Bylund, 1995).

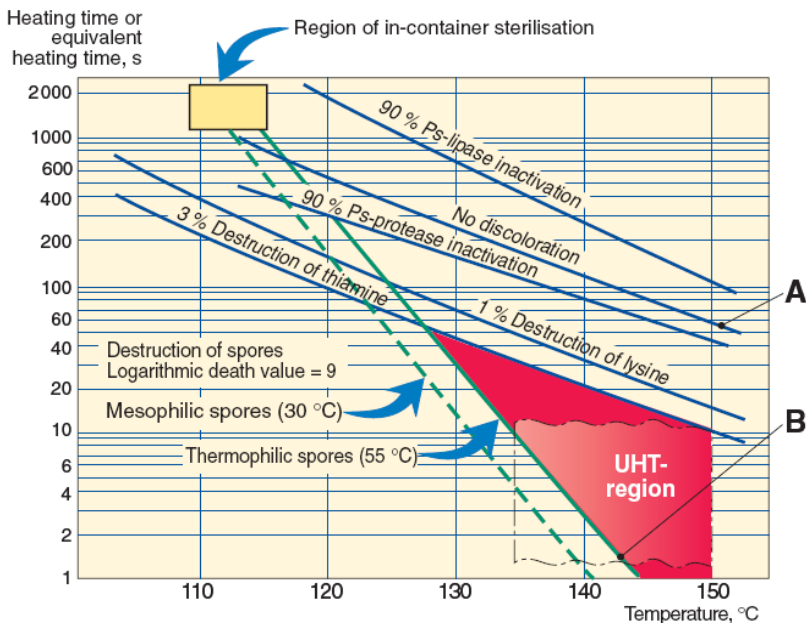


Figure 1: Bacteriological killing effects and chemical changes in heat treated milk (Source: Kessler)

The chemical effects can be assessed in similar ways to those used for the sterilization performance. The same data for the time-temperature performance is used. One of the values used for comparison of chemical changes caused by different time-temperature combinations to the chemical change caused by boiling of the product is C-value and is

expressed in minutes. C-value is equal to 1, when the chemical change corresponds to the boiling of the product for 1 minute.

The C-value can be calculated according the following formula:

$$C = \frac{t}{60} \cdot 10^{\frac{(T - 100)}{z}}$$

Where:

- t is the sterilization time in seconds at temperature T in °C
- T is the sterilization temperature in °C
- z is a value expressing the increase in temperature necessary to obtain the same chemical change in 1/10 of the time (generally set at 30°C).

Similar to B\* values for microbiological effect, Horak and Kessler introduced the **C\* value** for chemical changes (Horak, 1980; Kessler & Horak, 1981a; Kessler, 1981) and it is based on the thermal destruction of the vitamin thiamine. It was assumed that an acceptable limit of chemical changes can be described by time-temperature combinations corresponding to the loss of 3% of thiamine and corresponds to a C\* value equal to 1 (Figure 1). Based on this knowledge it can be

concluded, that a UHT plant is working at optimal conditions when the following is satisfied:

$$\begin{aligned} B^* &> 1 \\ C^* &< 1 \end{aligned}$$

The way in which the rate of reaction is influenced by temperature is most simply described by the **Q<sub>10</sub> value**, the factor by which the rate of reaction changes when the temperature is changed by 10°C (Burton, 1994). The Q<sub>10</sub> values for the microbiological killing effect on thermoresistant spores is in the range 10-20, however the values for most of the chemical changes are much lower, about 3 (Burton, 1994). This means that increasing the temperature gives more efficient killing of spores with less impact on the sensorial and nutritional product quality.

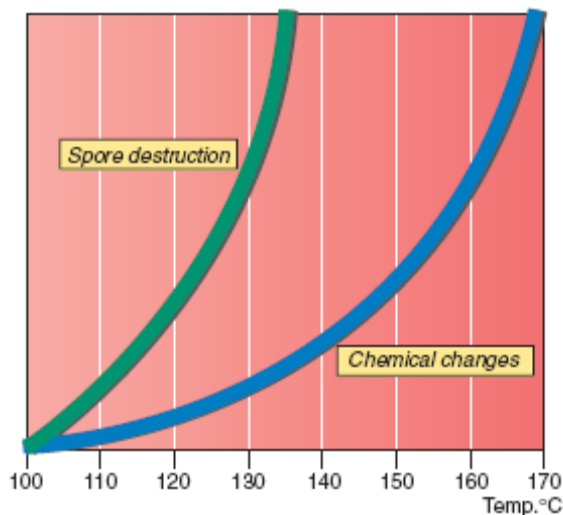


Figure 2: Effect of temperature on spore destruction and chemical changes (Source: Tetra Pak, 2003)



The extensive destruction of spores at elevated sterilisation temperatures requires only extremely short holding time to achieve commercial sterility. The relations are clearly expressed in the comparison of different time and temperature combinations in Figure 3, and clearly motivate the use of UHT treatment for production of long-life products with minimal change of colour, taste and nutritional value. Using an ultra-high sterilization temperature, the required time necessary to achieve the required killing effect is very short and the attendant chemical changes are minimal.

Temperature [°C]	F <sub>0</sub> [minutes]	Time [seconds]	C [minutes]
121	6.0	360	30.0
130	6.0	45	7.5
135	6.0	14	3.5
140	6.0	5	1.7

*Figure 3: Comparison of heat effects caused by different time-temperature combinations*

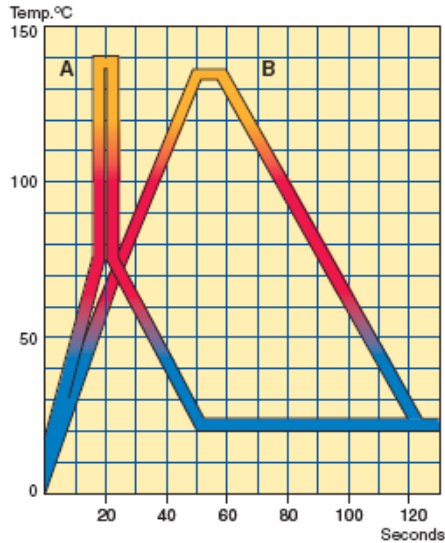
### 1.2 UHT processing systems

UHT plants became commercially available in 1960 when aseptic filling technology, which is a necessity to maintain the commercial sterility of the UHT treated product, was developed.

The purpose of UHT processing plant is to heat the product to the sterilization temperature (in the range 135 - 150°C), hold it there for a few seconds, and then cool it to a suitable filling temperature. As described in the introduction, there are two main technologies distinguished by the medium used for heating to the ultra-high temperature. One is direct systems using steam which is directly mixed with the product under pressure and the vapour is later removed by use of vacuum cooling and indirect systems where the final heating medium is hot water or steam separated from the processed product by a heat-conducting barrier made of stainless steel, preventing their direct contact.

The most important advantage of direct heating technology is very fast heating and in this way minimizing the rate of chemical changes. It takes just a few tenths of a second to reach the sterilizing temperature. This is the main reason why direct systems are used to ensure superior product quality. However, it is very difficult to recover the energy used in direct heating and the operation is becoming very costly. This is why direct heating systems are always a combination of direct and indirect heating. The indirect part, based mainly on plate or tubular heat exchanger, is used at temperatures below 80°C where the chemical changes caused by more slow heating are minimal. The

comparisons of time-temperature curves characteristic for treatment of milk in direct and indirect systems are shown in Figure 4.



*Figure 4 : Time temperature curve for processing of milk in a direct system (A) and indirect system (B) (Source: Tetra Pak, 2003)*

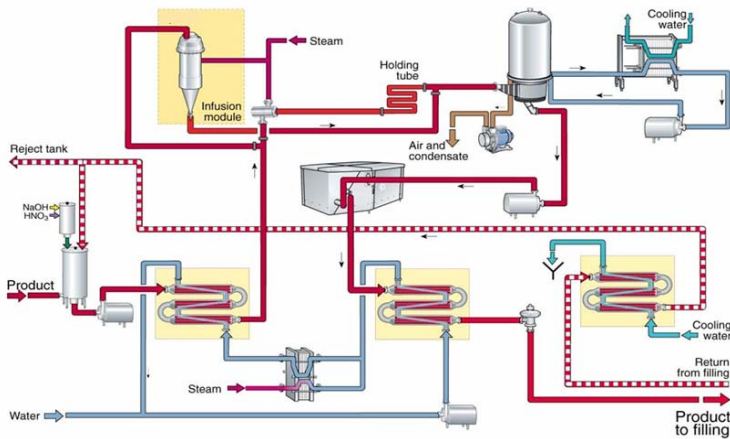
### *1.2.1 UHT direct systems*

As this study deals with the evaluation of products processed in direct heating systems, the working principal of this kind of UHT plant is explained in more in detail.

In a direct heating system (Figure 5), the product is first preheated indirectly in a plate-based or tubular heat exchanger to about 70-80°C. The final heating stage to the sterilization temperature is carried out by mixing with the steam under pressure. The steam

condenses and transfers its latent heat of vaporization to the product followed by an extremely rapid temperature increase. It takes about 0.1-0.2 seconds to heat the milk from the preheating temperature to the UHT temperature in the range of 135 - 150°C. After a few seconds holding in order to achieve the required killing effect and obtain commercial sterility, the product passes through a restrictor to an expansion cooling vessel. In this vessel is a vacuum corresponding to the boiling of the product at the same temperature, as the temperature was after the indirect preheater. In this way the amount of vapour corresponding to the amount of steam used for the heating is boiled off and the product attains the same composition as before the steam based heating. The vacuum cooling is also very rapid and requires about the same time as the heating by steam. In order to improve the storage stability, the milk processed in direct heating systems is homogenised aseptically after the vacuum cooling and cooled in a heat exchanger to the filling temperature. A flow chart of the TetraTherm Aseptic VTIS<sup>®</sup> UHT plant (Tetra Pak, Lund, Sweden) based on both direct heating technologies steam injection and steam infusion is shown in Figure 5.

The steam injection system is also called ‘steam into product’ system. The principal requirement is the rapid condensation of steam achieved by introducing the steam into the processed liquid in the form of well-distributed small bubbles, which collapse rapidly and transfer their energy to the liquid.



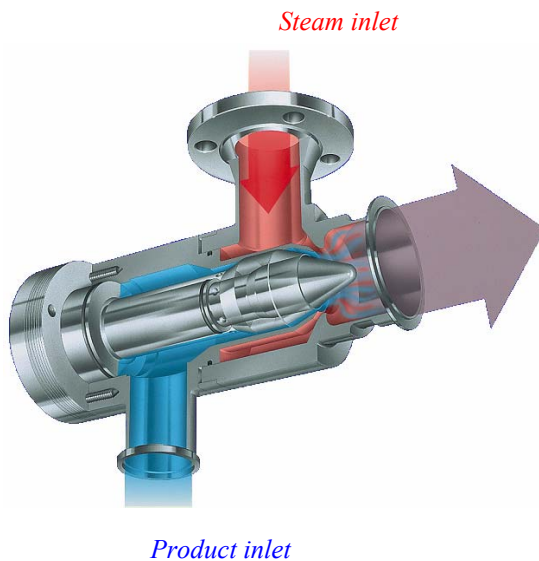
*Figure 5: Flow diagram of TetraTherm Aseptic VTIS<sup>®</sup> UHT direct system plant equipped with direct steam injection and steam infusion technology for the final heating (Source : Tetra Pak, Sweden)*

The rapid collapse of the air bubbles causes sharp local changes in pressure and can make the injector noisy. It has been said that higher steam pressures lead to higher product temperatures at the first contact point between the steam and the treated product and lead to damage of the product through overheating. However Hallström (1981) suggested that as condensation is much more rapid than diffusion, a film of condensate is formed on the surface of the bubbles and separates the hot steam from the processed product and in this way prevents the product from overheating. The condensate is then mixed into the product by turbulence.

## *UHT technology*

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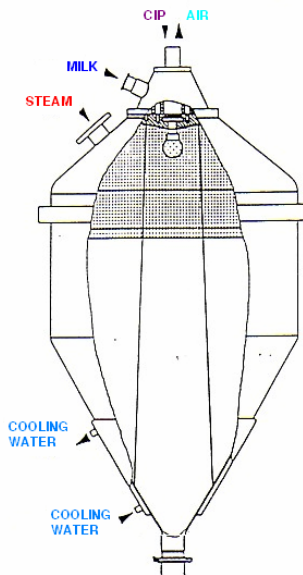
The steam is at a higher temperature than the product and must be thermally separated as far as possible to avoid surface fouling and reducing the effectiveness of the steam injector. In this way injectors are designed to minimize the indirect heat transfer until it reaches the mixing zone. The injector used in our test is called a ‘ring space’ steam injector (Figure 6) design by Tetra Pak, Lund, Sweden.



*Figure 6: The steam injection nozzle design by Tetra Pak, Lund, Sweden*

Steam is injected into the product at a sharp angle across the product flow in the form of an inwardly directed cone. The right angle across the flow minimizes the indirect heat transfer and with it the fouling on the stainless steel surface.

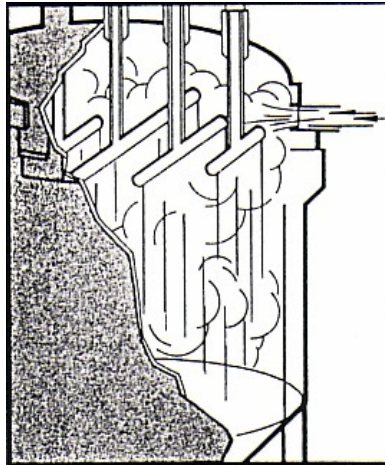
The steam infusion or the ‘product into steam’ system is based on a steam-pressurised vessel with a conical base. The design of the inlet of steam, the product and the proportions of the infusion chamber differ from one manufacture to another. The Direct Steam Infusion systems of APV (former known as Pasilac, now Invensys APV) (Figure 7) has been available on the market since 1960 and is probably the most common infusion system on the market.



*Figure 7: Infusion chamber Direct Steam Infusion (APV Invensys)*

A distribution device of this type of steam infusion is based on bundles of free-falling jets passing downwards through the steam atmosphere and collecting in the bottom of the infusion chamber according to the holding requirements determined by a timing pump sometimes combined with an additional holding cell.

Another infusion system that was once common, especially in the Americas which was developed about 60 years ago, is the DASI FreeFallingFilm<sup>®</sup> system with the infusion chamber design shown in Figure 8.

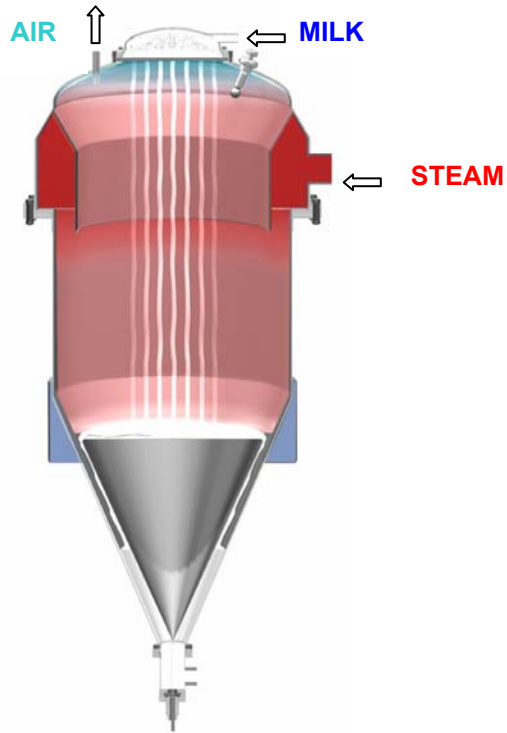


*Figure 8: Infusion chamber from DASI FreeFallingFilm<sup>®</sup> system*

Using distribution in this type of chamber, the milk flows into a series of parallel horizontal distribution tubes with thin slits along their bottom, from the product falls in a laminar free falling film. The chamber is kept at the sterilization temperature by saturated culinary steam and the temperature of the milk rises to this temperature in a fraction of second as it falls down through the chamber.

The latest infusion system was developed by Tetra Pak, Lund, Sweden and introduced on the market in 2003. This type of infusion was used in our study and the design is shown in Figure 9.





*Figure 9: Infusion vessel from TetraTherm Aseptic VTIS<sup>®</sup> designed by Tetra Pak, Lund, Sweden*

In this kind of infusion vessel, the treated milk is distributed to stream jets on a stainless steel disc with nozzles. The jets streams are split into droplets that fall through the steam atmosphere and within 0.1-0.2 seconds reach the bottom having obtained the sterilization temperature. The holding time required to achieve commercial sterility is created by the channel in between the wall of the conical bottom of the infusion chamber and the inverted stainless steel conical body. The holding time is controlled by the level of the product in this channel and can be adjusted continuously, giving the advantage of allowing variable plant capacity without any shutdowns.

All types of the infusion chambers mentioned with conical bottoms and double walls allow continuous cooling so the contact temperature obtained is a few degrees less than the temperature of the sterilized product. This gives the advantage of reducing the fouling of the high temperature section.

After the product has been sterilised it is forced to the vacuum chamber for cooling to about the same temperature as that after the preheating, followed by high pressure homogenization and indirect cooling to the outlet temperature.

### *1.2.2 Comparison of UHT direct systems*

Since very little is known about what exactly happens directly heating takes place, the comparisons are to a great extent based on theory.

The following is claimed about the two direct UHT systems:

#### *1.2.2.1 Heat treatment*

- The steam injection is the fastest method of heating. The product reaches design temperature within tenths of a second. However, product in the direct line of steam injection can due to the rapid heating be over-heated before the temperature equilibrates throughout the remaining product (Mans,1988).

- When the incoming product is rapidly heated in the steam infusion vessel, some of this air leaves the product and enters the steam and increases the total pressure in the infuser above the steam pressure needed to give a correct processing temperature (Burton, 1977). Therefore there is considerable risk of overheating for non-deaerated product.
- To avoid temperature and pressure instability problems during the processing, high pressure on the processed product must be maintained. To enable the injection of steam into the product, the supplied steam pressure must be higher than the product pressure; considerably the steam temperature will be above the desired processing temperature. This might cause overheating of the product at the point of first contact with the steam (Burton, 1977).
- Injection is regarded as more turbulent and stressful than infusion (TCI, 1985).
- There are two reasons to why the infusion heaters are claimed to be better than other heaters. Firstly, the gentle heating of the product with minimum temperature difference between heating medium and heated product, and secondly, the entire product receives the same heat treatment (Perkin, 1985).

- The use of a thin, free falling film provides a very high ratio of heat transfer surface area to volume in the DASI heater. On account of this the heating is very fast, efficient and uniform (TCI, 1985).

### *1.2.2.2 Fouling*

- If metal parts of the injection in contact with the product are heated to a high temperature by the steam, deposits may build up (Burton, 1977).
- More deposits remain in the infuser than in the head of the injector (Steffen, 1992).
- Since the product does not come in contact with a metal surface hotter than itself, the burn-on and the development of cooked flavour is minimised (TCI, 1985).
- Fouling of the infusion vessel can be avoided if the initial Reynolds number of the milk jets was over 10 000 (De Jong, Waalewijn & Van der Linden, 1994)

*1.2.2.3 Structure and stability*

- Cavitations during heating must be avoided, because it destabilises the fat emulsion and results in a homogenising effect, detrimental to a wide range of products, for instance whipping cream (Moberg & Hegg, 1986).
- The high-pressure of the injected steam may cause violent turbulence and severe shearing of the product (Mans, 1988). This is believed to be contributory to product instability during storage (Woods, 1976).
- The milk from steam infusion plant does not contain as much sediment as the milk from a steam injection plant (APV Pasilac AS).
- Gelation was observed in infusion milk stored at 22-25°C for 84-89 days. Similar temperature effects have been reported for milk processed by the steam injection system (Manji, Kakuda & Arnott (1986).
- Albumin denaturation was similar to or lower than that obtained with other direct systems (Nahra, 1980).

- Denaturation measurement of whey proteins indicated that the heat treatment of the infuser was gentler and gave less denaturation of whey proteins than steam injection plant operating at similar temperatures and residence times (De Jong et al. 1994).

### *1.2.2.4 Organoleptic properties*

- In the infusion chamber during the heating process both condensable and non condensable gases are released from the product (APV Pasilac AS).
- The DASI-method eliminates the cooked flavour (Nhara, 1984)
- Since the product does not come in contact with a metal surface hotter than itself, the burn-on and the development of cooked flavour is minimised (TCI, 1985).
- Milk treated in an injection UHT system was compared to milk treated in an infusion UHT system (DASI). The taste panellists were unable to distinguish between the injection milk (VTIS) and the infusion milk (Farmer, 1988).

- The choice of direct steam infusion or steam injection sterilisation method had no impact on the sensorial quality (Farmer, 1988; Steffen, 1992 ).
- The infusion-heated milk is typical of other UHT milks and is completely different from pasteurised milk (Perkin, 1985; Farmer, 1988).
- The DASI-steam infusion sterilized milk, aseptically packaged in paper or glass containers stored at room temperature (20°C) for periods up to 10 weeks, had no significant taste difference when compared with fresh, pasteurized milk.( The taste test were performed using a trained panel of milk testers) (DASI Industries).

### 1.3 Properties and changes of UHT processed milk

UHT milk properties are influenced by two types of changes: changes during processing and changes during storage. Changes during processing are mainly the result of the type of and severity of heat treatment; the changes taking place during storage are strongly dependent on storage temperature, exposure to the light and oxygen. Results of both kinds of changes lead to alteration in the colour, flavour, texture and product stability, and may reduce the nutritional value (Burton, 1991).

#### *1.3.1 Changes of fat*

During UHT production, the fat in milk does not change physically or chemically, so it cannot have adverse nutritional consequences (Burton, 1991). Only the structure is changed. The fat in raw milk is organized in fat globules covered by a natural fat globule membrane composed mainly of lipoproteins. The fat globules in raw cow's milk vary considerably in diameter from  $< 0.2 \mu\text{m}$  to about  $30 \mu\text{m}$  with a mean diameter of about  $3 \mu\text{m}$ . Globules of  $2\text{-}6 \mu\text{m}$  contain over 90% of the fat and those  $< 1 \mu\text{m}$  represent about 80% of the total number of globules, but only 1-2% of the fat content.

Heat treatment alone does not affect the size of fat globules (Garcia-Risco, Ramos & López-Fandiño, 2002), but all technologies used in the production of UHT milk include a high pressure



homogenization step, when the fat globule size is dramatically reduced and the composition of the surface is changed. Homogenization results in globules that are not uniform in diameter, but their size variation is less than in untreated milk. Most of them are less than one  $\mu\text{m}$  in size and the homogenized fat globule surface consists to a large extent of casein micelles and their components that link the fat globules together (Henstra & Schmidt, 1970; Darling & Butcher, 1978).

Hillbrick, McMahon & McManus et al. (1999) suggested that fat separation is not caused by clusters, but by large fat globules, as a relatively small number of large fat globules contain a large amount of fat. The clusters contain a relatively high amount of proteins which increases their density and limits their tendency to separate. This theory is also strengthened by the fact that in high pressure homogenized products, where many more aggregates were found, the fat separation layer was thinner.

Direct heating after homogenization appears to cause re-agglomeration of the small fat globules with the formation of a solid fat layer during storage. To prevent this fat separation, homogenization in direct UHT plants takes place in the downstream position (after the final heating step and vacuum cooling).

During storage at elevated temperatures, there can be an increase in the content of free fatty acids. The production of free fatty acid is more noticeable in milks with higher fat content and is greater in milk produced in direct rather than indirect systems (Schmidt & Renner, 1978) as a consequence of insufficient inactivation of heat resistant lipases either of natural origin or produced by psychrotrophic bacteria

during cold storage of raw milk. This free fatty acid production eventually leads to off flavours and may exceed the level at which it is detectable and negatively influence the UHT milk flavour.

### *1.3.2 Changes of proteins*

#### *1.3.2.1 Protein chemistry*

Milk proteins can be divided into caseins (80%) and whey proteins (20%). On acidification to pH 4.6 at around 30°C, the isoelectric point of bovine milk, the caseins precipitate out of solution and whey proteins remain in solution. This fraction is sometimes called serum proteins or non-casein nitrogen. According to the decrease in the electrophoretic mobility, caseins were divided to  $\alpha$ -,  $\beta$ - and  $\gamma$ -caseins (Figure 10) and represent 75%, 22% and 3% of the total casein content.  $\alpha$ -caseins were further separated into the two  $\alpha_{s1}$  and  $\alpha_{s2}$  fraction, where the 's' indicates the sensitivity to  $\text{Ca}^{2+}$  as they are precipitated by low a concentration of these ions while  $\kappa$ -casein is insensitive to  $\text{Ca}^{2+}$  (Fox & McSweeney, 1998).

Currently, the complete primary structures are known and this has revealed to recognizing of four different peptide chains:  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -caseins (Figure 10), which differentiate in phosphorylation and glycosylation (Walstra, Geurts, Noomen, Jellema & van Boekel, 1999).

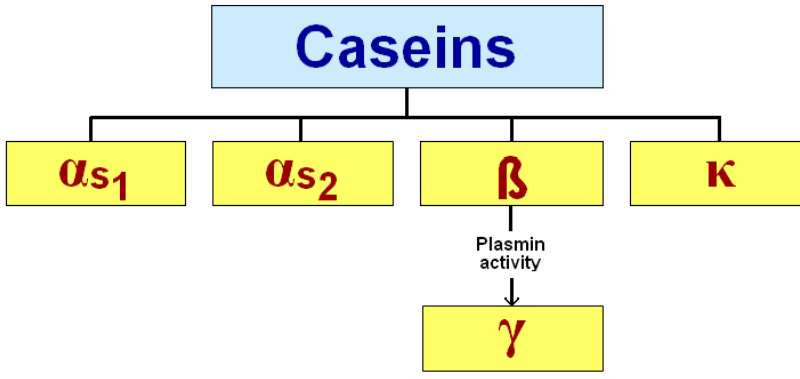


Figure 10: Classification of caseins in bovine milk

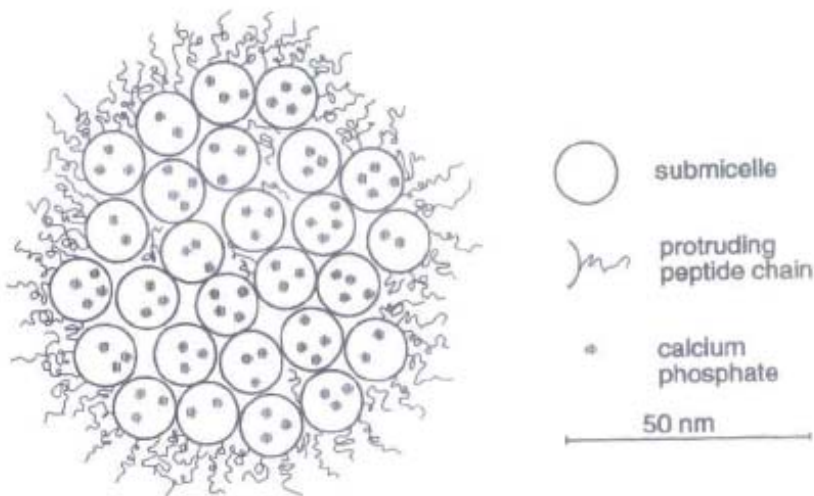
$\alpha_{s1}$ -casein has the highest charge and highest phosphate content.  $\alpha_{s2}$ -caseins exist in indifferent variants which differ by number of ester phosphate groups and contain two cysteine residues forming –S–S– bridges.  $\beta$ -caseins are the most hydrophobic casein and its molecule can be divided into two parts: hydrophilic charged ‘head’ and a long apolar ‘tail’. After storage for some hours at low temperature, part of  $\beta$ -caseins goes reversibly into solution. Enzymatic degradation of  $\beta$ -caseins by plasmin (alkaline milk proteinase) leads to the production of  $\gamma$ -caseins and proteoso-peptones. The last casein,  $\kappa$ -casein greatly differs from other caseins. It has two cysteine residues which make possible to create disulphide bonds. The peptide bond between residues 105 – 106 is rapidly hydrolyzed by enzymes like rennet and results in the formation of para- $\kappa$ -casein and glycomacropeptide (Walstra et al. 1999).

Caseins are organized in casein micelles, colloidal particles, large enough to flocculate as a result of van der Waals forces. However, under

## ***UHT technology***

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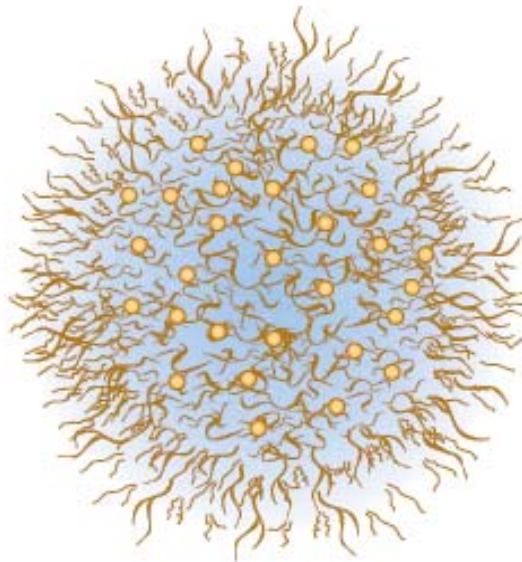
physiological condition in the milk they do not flocculate because of some counteracting forces. When the conditions in the milk are altered the micelles may flocculate. Such a change in the milk can be an increase in acidity,  $\text{Ca}^{2+}$  ions concentration, the addition of ethanol or the application of very high heat (Walstra et al.1999). Various models of casein micelle structure have been proposed and are progressively refined. Although there are some disagreements between all the models, there is a strong support for composition based on submicelles of 10 – 15 nm in diameter linked together by colloidal calcium phosphate (CCP), giving the micelles open porous structure. The first submicellar model was suggested by Morr (1967) but the most known is the model of Paynes (1979 and 1982) modified and published latest by Walstra et al. (1999).



*Figure 11: Modified model of cross-section throughout casein micelle from Walstra et al.1999*

On removal of CCP the micelles disintegrate, however disintegration can also be achieved by addition of reagents, which do not solubilise CCP, suggesting that other forces e.g., hydrophobic or hydrogen bonds contribute to the stability of the micelles. Schmidt (1980, 1982) suggested the  $\kappa$ -casein content on the submicelles is not uniformly distributed. The  $\kappa$ -casein-deficient submicelles are mainly located in the interior and the  $\kappa$ -casein-rich submicelles more oriented to the surface of the micelles creating the protective layer with hairy structure and about 5-10 nm thickness.(Walstra et al 1999).If the hairy layer collapses e.g., hydrolysis by rennet or by ethanol the colloidal stability of the micelles is reduced and they precipitate (Holt & Horne, 1996).

Holt (1992) depicted the micelle as a tangled web of flexible casein molecules forming a gel-like structure integrated by CCP microgranules with C-terminal region of  $\kappa$ -casein forming the hairy layer (Figure 12).



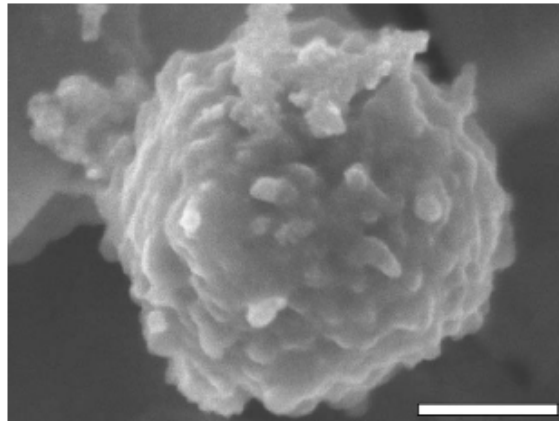
*Figure 12: Modified model of casein micelle from Holt, 1994*

The ‘submicellar’ structure described above is also questioned by McMahon & McManus (1998) who used cryofixation for studying the casein micelle structure. They concluded that the submicellar structure of the micelle in electron micrographs is an artefact and suggested that the non-homogeneities detected by neutron scattering are in fact micro-domains of calcium phosphate. According to Holt, De Kruif, Tuinier & Timmins, 2003, these micro-domains act as structure-forming centres attaching the more phosphorylated  $\alpha_s$ - and  $\beta$ -caseins. Based on that, Holt et al., 2003 suggested that most of the caseins in the micelles are homogeneously distributed and not organized in submicelles and the hairy layer is not a well-defined structure but rather a layer with reduced protein density.

Nowadays the ‘hairy’ casein micelle model is generally the most accepted. The common view is that casein micelles up to 200 nm in size are regarded as a colloidal particle consisting of about a thousand small nanoclusters of about 10-20 nm. The casein micelles are characterized by the presence of  $\kappa$ -caseins as a brush of grafted polymer on the surface, which protrude into the solvent and provide the steric stabilization of the casein micelles (De Kruif, 1999; De Kruif & Holt, 2001). The micelles are linked together by Ca bridges, the formation of CCP linkages or at high temperature by the formation of covalent bonds between amino acid residues. These kinds of aggregation are mostly irreversible (Walstra et al.1999).

The newest findings suggest that the surface of the micelles is much more complex and composed of subunits (submicelles) which are not spherical but have a tubular structure with tubes about 20 nm in

diameter which correspond to the size of calcium phosphate/casein nanoclusters (Holt, Timmins, Errington & Leaver, 1998). The surface is not smooth and contains gaps between these substructures like entrances to pores. The tubules are presumably based on caseins with ends protruding from the bulk which may be based on  $\kappa$ -casein and  $\beta$ -caseins, and which protect the micelle from close approach by large particles such as other casein micelles (Dalgleish, Spagnuolo & Goff, 2004). In this way, this ‘hairy layer’ is impenetrably tight. But seen from a perspective of serum proteins or enzymes the layer has gaps large enough to permit penetration or attachment (Figure 13). The distribution of  $\kappa$ -casein on the surface can be strengthened by the fact that normally the amount of  $\kappa$ -casein would be insufficient to cover the micellar surface even if it was smooth. It is also easily attacked by rennet as well as whey proteins and it also explains why natural protein aggregates are not larger than 30-40 protein molecules. This is about what is necessary to cover all the protruding ends (Dalgleish et al., 2004).



*Figure 13: Electron micrograph of single casein micelle by Dalgleish et al. (2004) (bar 100 nm)*

A model, where  $\kappa$ -casein is positioned at the ends in ‘brushlets’, would replace the model originally suggested by Paynes (1979 and 1982) modified and published latest by Walstra et al (1999), based on a hard surface surrounded by a continuous hair layer (Dalgleish et al., 2004). A casein micelle model up to date was presented by Dalgleish (2006), when even water molecules have been incorporated in the structure.

The steric stabilization generated by a relatively scarce brush is the most important stabilizing factor of the micelle (Tuinier & De Kruif, 2002). Only the extended brush is capable of sterically stabilizing. If the brush collapses, van der Waals interactions induce flocculation. The stability of the brush, whether it is extended or collapsed depends on brush density, pH, divalent salt concentration and ethanol level. Brush stability is reduced by lowering brush density, adding divalent cations, lowering the pH (charge density) and changing the solvent by for example the addition of ethanol. Combination of all these effects has an additive function (De Kruif, 1999). The  $\kappa$ -casein brush is not homogeneously distributed on the micelle surface and because of that provides steric stabilization against the approach of large particles such as other micelles. But for small particles with the dimensions of individual proteins or smaller, the gaps provide a relatively easy access and agglomeration will take place.

### *1.3.2.2 Influence of heating, homogenisation and cooling*

Heating of milk (Figure 14) leads to the formation of a heterogeneous  $\beta$ -lactoglobulin/ $\kappa$ -casein complex (Zittle, Thompson, Custer & Cerbulis,



1962; Sawyer, 1969; Smits & van Brouwershaven, 1980; Hill, 1989; Noh & Richardson, 1989; Jang & Swaisgood, 1990). The major interaction appears to involve thioldisulfide exchange between  $\beta$ -lactoglobulin and  $\kappa$ -casein at the micellar surface (Sawyer, 1969; Smits & van Brouwershaven, 1980; Jang & Swaisgood, 1990; Walstra et al, 1999). However, some studies suggest that ionic and/or hydrophobic interactions may play a significant role in the aggregation, particularly at the early stage (Mulvihill & Donovan, 1987; Hill, 1989). Most of these associations are irreversible on cooling (Walstra et al., 1999). Holt & Horne(1996) suggested, that the stiff rod-like aggregates of unfolded  $\beta$ -lactoglobulin must first find their way through the  $\kappa$ -casein hairy layer to react with the disulfide bond of  $\kappa$ -casein and when attached they will protrude from the micellar surface (Mohammad & Fox,1987) and by steric effects could limit further  $\beta$ -lactoglobulin becoming attached. If the unfolded  $\beta$ -lactoglobulin is oriented by the SH-group from the surface or is in an aggregate, it will be unable to react; in contrast, unfolded monomeric  $\beta$ -lactoglobulin will penetrate very easily.

The level of association of the whey proteins to the casein micelles is influenced by the heating conditions. The amount of  $\beta$ -lactoglobulin associated with the casein micelle increases with the heating time. The trend is similar for  $\alpha$ -lactalbumin but to a lesser extent (Elfagm & Wheelock, 1978). The association is much stronger when the temperature is increased slowly (Smits et al.1980; Corredig & Dalglish, 1996b). The non-associated unfolded whey proteins remain in milk serum as aggregates (Singh & Creamer, 1991a; Corredig et al.

1996b; Oldfield, Singh & Taylor, 1998). On heating,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin initially aggregate in the serum phase and these complexes subsequently associate with casein micelles (Corredig & Dalgleish, 1996a and 1999) and with the fat globule membrane (Corredig & Dalgleish, 1996a; Kim & Jimenez-Flores, 1995; Houlihan, Goddard, Nottingham, Kitchen & Masters, 1992).  $\alpha$ -lactalbumin can interact with  $\kappa$ -casein only in the presence of  $\beta$ -lactoglobulin, possibly through the initial formation of  $\alpha$ -lactalbumin/ $\beta$ -lactoglobulin aggregates, which then interact with  $\kappa$ -casein (Elfagm et al., 1978). If milk is heat treated instantaneously (by direct heating) all whey protein begin unfold at the same time and this gives a greater opportunity for the unfolded monomers to aggregate and consequently the attachment to the casein micelles will be less efficient (Oldfield, et al., 1998). These facts explain why association is greater in indirect systems than in direct systems and there may also be a difference between a pilot plant and a full-scale installation. The rate of denaturation is increasing dramatically at 90°C for  $\beta$ -lactoglobulin and at 80°C for  $\alpha$ -lactalbumin (Dannaberg & Kessler, 1988; Oldfield, Singh, Taylor & Pearce, 1998). The association with casein micelles is pH dependent and decreases as the pH increases in the range pH 6.3-7.3. The micellar size shows a similar dependence in range pH 6.5-6.7 (Skelte & Yuming, 2003).

On increasing temperature the micelles shrink somewhat and the amount of colloidal phosphate slowly increases. The additional colloidal phosphate may not have the same properties, as the natural one. At temperatures above 70°C the casein molecules become more flexible as

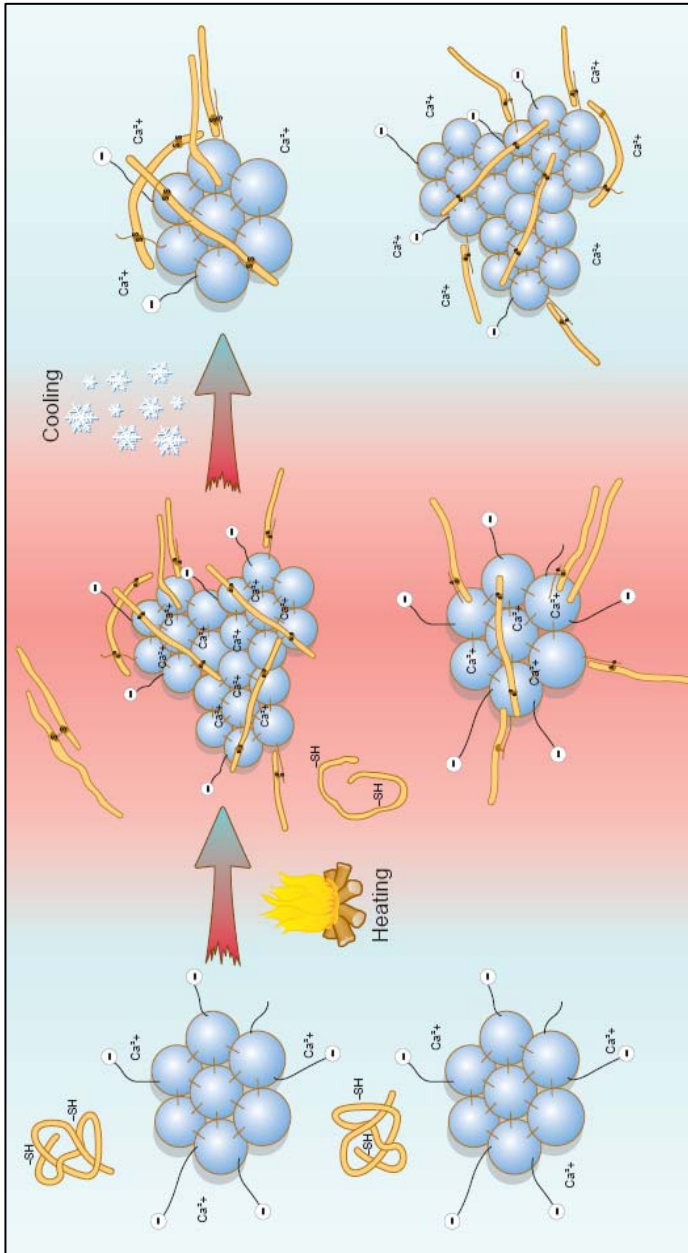


Figure 14: Effects of heat treatment and cooling on milk proteins

if part of the micelle has melted. Below this temperature they are not flexible. At even higher temperatures, above 100°C, a dissolution of part of the  $\kappa$ -casein occurs.

The extent of this dissolution is pH dependent. Below pH 6.2, no dissolution takes place but at pH 7.2 it is almost complete (Walstra et al. 1999).

Heat treatment alone did not affect the size of fat globules but increased the number of big micelles and led to a wider size distribution (Creamer & Matheson, 1980; McMahon & Yousif, 1993; Corredig & Dalgleish, 1996a; Garcia-Risco et al, 2002). The effect is greater at higher temperatures and pH (Creamer & Matheson, 1980).

Preheating an infusion system to 65.6°C and 82.2°C for 150 seconds did not alter the mean size as compared to raw milk although there was a narrow size distribution with fewer micelles in the lower size class and increased tendril formation. Samples of UHT milk treated for 3.4 seconds exhibited larger mean micelle diameter and greater tendril formation than those treated for 1.5 or 9.0 seconds. An increased numbers of small diffuse particles were present in samples treated for 9.0 seconds. Initial heat-induced micellar growth was followed by micellar disintegration or disaggregation (Freeman & Mangino, 1981). The binding of the  $\beta$ -lactoglobulin to the casein micelles and the release of  $\kappa$ -casein from the micelles surface on heating was also confirmed by Garcia-Risco, 2002).

Homogenization tends to reduce the average micelle size. When samples are first heated and after homogenized, the micellar size is smaller than

in after the opposite processing flow (Garcia-Risco *et al.*, 2002).

The lower the temperature, the better is the colloidal stability of the casein micelles. Dissolution of considerable part of  $\beta$ -casein occurs mainly due to weakening of hydrophobic bonds. Even other caseins dissolve but to a lesser extent with  $\alpha_{s1}$ -casein dissolving the least. The voluminosities of casein micelles increase due to the formation of another category of hairs on the surface. As  $\beta$ -caseins dissolve and other caseins can also leave the surface, the  $\beta$ -caseins may protrude from the surface of the micelles. A limited disintegration of casein micelles to smaller ones may also occur, most probably due to the dissolution of colloidal calcium phosphate (CCP) as the association of  $\text{Ca}^{2+}$  ions with caseins is reduced as the temperature decreases. These changes take some 24 hours at 4°C to be completed and are reversible. On subsequent heating,  $\beta$ -casein returns to the micelles and the amount of CCP increases again (Walstra *et al.* 1999). Weakening of bonds can be caused by the addition of calcium binding agents like citrates, EDTA and oxalate or dodecyl sulphate, or large quantities of urea, which breaks hydrogen bonds (Walstra *et al.* 1999).

### *1.3.3 Changes of minerals*

On temperature, calcium and magnesium citrate and phosphate ions change their form reversibly between the colloidal form in the casein micelle and the soluble form in the milk serum (Burton, 1991). Their form is also dependent on pH and milk concentration (Fox &

McSweeney, 1998). Acidification is accompanied by a progressive solubilization of the colloidal calcium phosphate from the casein micelle. The solubilization is completed at pH 4.9. Alternatively, the addition of divalent cations causes precipitation of the soluble phosphate as colloidal calcium phosphate, decreases the concentration of soluble phosphates and decreases the pH. Colloidal calcium phosphate is also precipitated by the addition of sodium and potassium phosphates and also decreases the concentration of soluble calcium and increases the pH (Fox & McSweeney, 1998).

The solubility of calcium phosphate decreases with increasing temperature. Due to the milk being heated, the soluble calcium phosphate precipitates as colloidal calcium phosphate (CCP) and the change is reversible on cooling (Fox & McSweeney, 1998).

Changes in the mineral balance influence the heat stability of the milk as well as stability during storage.

### *1.3.4 Changes of lactose*

Several reactions involve milk sugar during UHT processing and subsequent storage. The most important are the epimerization of lactose to lactulose, where glucose from lactose is isomerized to fructose. Lactulose is not present naturally; it is only produced during the sterilization of dairy products. As lactulose is neither present in raw milk nor in pasteurized milk, it is used as a heat load indicator for high temperature processed milk and was even proposed by the IDF and

Milchforschung in Kiel, Germany (Schlimme, Buchheim & Heeschen, 1993) to be used for distinguishing between in-container sterilized milk and UHT milk.

Lactose participates in non-enzymatic browning reactions, Maillard reactions, which involve carbonyl groups from lactose and  $\epsilon$ -NH<sub>2</sub> of lysine from proteins forming glycosamine. Maillard reaction products lead to a brown colour and off flavours (Fox & McSweeney, 1998).

### *1.3.5 Changes of structure*

In the study of UHT milk structure by Hillbrick et al., (1999), he found that casein micelles, their fragments and even individual  $\kappa$ -casein and whey proteins were attached to the fat globule membrane and are its most important proteins. Their complex is a result of their interaction at elevated temperatures as described by Jang & Swaisgood (1990). As single casein, only  $\kappa$ -casein was present in the fat globule membrane without whole or a part of a casein micelle attached as a result of the dissociation of  $\kappa$ -casein from the casein micelles on heating (Aoki, Suzuki & Imamura, 1975; Singh & Fox, 1985 and 1987; Hillbrick, et al., 1999).

Casein micelles were occasionally found linking the fat globules together, were round but with an irregular surface, well separated and

wide size distribution. Linkage of fat globules was more apparent at the high homogenization pressures. In the samples processed without any homogenization pressure, UHT milk produced by direct steam injection also showed an immunolabelling for casein on the fat globule membrane related to the homogenization effect caused by steam injection (Zadow, 1969; Corredig & Dalgleish, 1996a; Hillbrick et al.1999). However, the homogenization effect caused by the steam injector did not contribute significantly to the homogenizing effect as the size reduction by the steam injector is very small, compared to that created by the homogenizer. Labelling of  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin revealed their presence, especially on the fat globule membrane, but less so on casein micelles and in the interstitial space. The presence in the free space could be due to the loss of  $\beta$ -lactoglobulin during the sample preparation.  $\alpha_{S1}$  caseins and  $\beta$ -caseins were present on casein micelles and on the fat globules in the place where the casein micelle or its part was attached.

### *1.3.6 Enzymatic activity*

Proteolysis of UHT milk during storage is a problem since is limiting the shelf life through changes in stability, flavours and texture. The texture changes may start with sedimentation, increase of viscosity or bottom gel formation leading in some cases to gel formation.

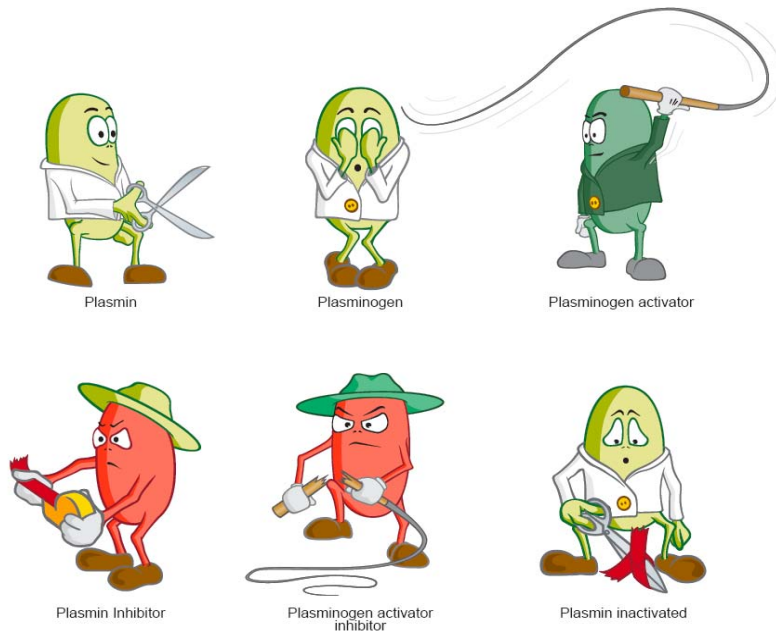


The enzymes responsible for the proteolysis are mainly of two different origins: native milk alkaline proteinase (plasmin), and heat stable extracellular bacterial proteinases produced by bacteria in raw milk or milk prior final UHT treatment. *Bacillus* species have higher extracellular and intracellular proteolytic activity than other bacteria. However another species – *Pseudomonas* bacteria producing also very heat resistant enzymes are the mainly species found as contaminants of milk. These two kinds of proteinases react differently and produce different peptides in the heat-treated milk.

Plasmin is sufficiently heat stable to play a part in “age gelation” of UHT milk. It is an indigenous enzyme - exists in milk both in its active form – “plasmin” or as its enzymatically inactive precursor form – “plasminogen”. The origin of plasmin is the cow’s blood, where it has the physiological role to solubilise the fibrin clots.

The total plasmin system (Figure 15) has five elements: plasmin, plasmin inhibitors, the inactive plasminogen, plasminogen activators and plasminogen activators inhibitors (Grufferty & Fox, 1988).

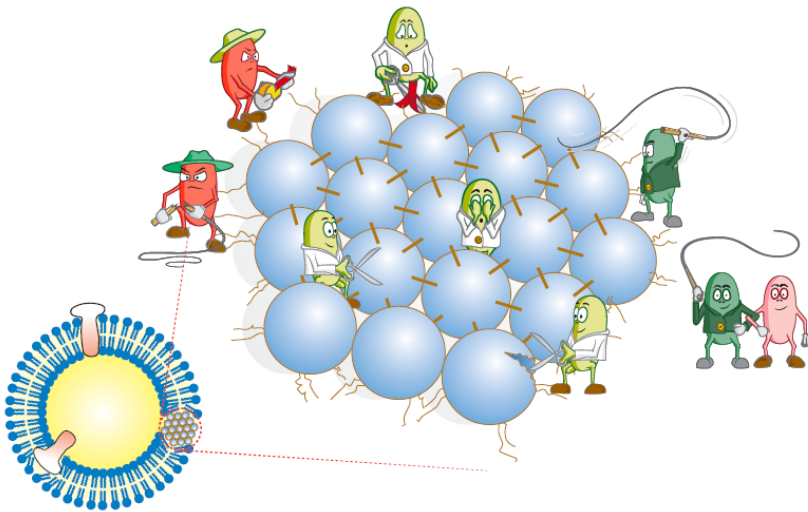
Plasmin in milk occurs mainly as inactive precursor plasminogen, activated through proteolysis by plasminogen activators (Fang & Sandholm, 1995) which are known to be also very heat stable (Richardsen, 1983; Lu & Nielsen, 1993). Activation of plasminogen is slower or does not happen at all at 4 °C. At 24 or 37°C the conversion is higher (Manji, Kakuda & Arnott (1986).



*Figure 15: Schematic picture of plasmin system*

Milk plasmin is associated with casein micelles and milk fat globule membrane (Figure 16). The presence in the fat globule membrane is actually due to presence of casein in the membrane (Politis, 1992, Benfeldt et al.1995). Plasminogen is also associated with casein micelles which also play a roll as an immobilisation matrix for activation of plasminogen (Baer, Ryba & Collin, 1994; Politis et al. 1995), particularly  $\alpha_{s2}$ -casein (Johnson, Ravn, Berglund, Petersen, Rasmussen, Heegard, Rasussen, Benfeldt & Fedosov (1998).The total plasmin activity is, as mentioned above, controlled in milk by a system of activators and inhibitors (Richardsen,1983). Activators have been

detected in serum and casein fraction of milk and in association with somatic cells, (Verdi & Barbano, 1991), while plasmin inhibitors and plasmin activator inhibitors are found predominantly in serum phase of milk (Grufferty & Fox, 1988a; Bastian & Brown, 1996) and may appear in several different forms, possibly due to formation of complex with different proteins (Preceti et al.1997).



*Figure 16: Schematic picture of distribution of the plasmin system*

Plasmin acts mainly on  $\beta$ - and  $\alpha_{s2}$ -casein and hydrolyse  $\beta$ -casein to three C-terminal fragments -  $\gamma_1$ -,  $\gamma_2$ - and  $\gamma_3$ - caseins (Figure 17)(according to the new nomenclature of Farrell, Jimenez-Flores, Bleck, Brown, Butler, Creamer, Hicks, Hollar, Ng-Kwai-Hang & Swaisgood, 2004,  $\beta$ -CN X - IP(f29 - 209),  $\beta$ -CN X - IP(f106 - 209) and  $\beta$ -CN X - IP(f108 - 209).

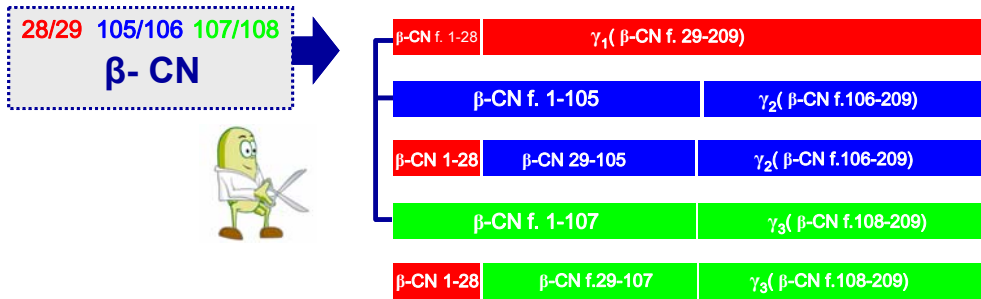


Figure 17: Schematic picture of plasmin activity on  $\beta$ -casein

Plasmin cleaves  $\alpha_{s2}$ -casein at 8 sites (Figure 18) (Visser et al. 1989) producing 14 peptides, three of which are potential bitter ( $\alpha_{s2}$ - CN f198-207,  $\alpha_{s2}$ - CN f182-207 and  $\alpha_{s2}$ - CN f189-207)(LeBars & Gripon,1989).



Figure 18: Schematic picture of plasmin activity on  $\alpha_{s2}$ -casein

Plasmin is much less active on  $\alpha_{s1}$ - casein, however several cleavage sites were reported by Le Bars and Gripon (1993) and McSweeney (1993) leading to formation of different fragments of the protein.

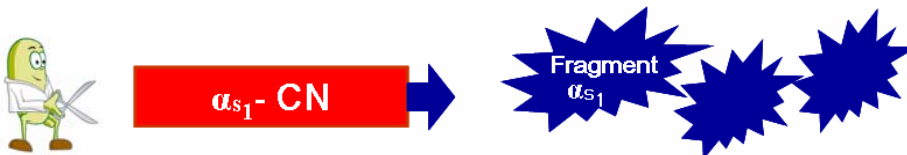


Figure 19: Schematic picture of plasmin activity on  $\alpha_{s1}$ -casein

$\kappa$  - casein is not or very little affected by plasmin. Eigel (1977) find no hydrolysis of  $\kappa$ -casein at conditions leading to full hydrolysis of  $\alpha_{s1}$ -casein, however Andrews and Alichanidis, Wrathall & Andrews (1986) as well as Pihlanto-Leppaelae, Pahkala, & Antila (1993) also reported release of some peptides from  $\kappa$ -casein by plasmin. Trujillo, Guamis & Carretero (1998) claimed that para- $\kappa$ -casein is susceptible to hydrolyses by plasmin.

Plasmin is not as active on whey proteins as on caseins because of their tight globular structure. However Caessens, Visser, Gruppen, & Voragen (1999a, b) observed considerable hydrolysis of  $\beta$ -lactoglobulin by plasmin, noting also that significant disulphide-sulphydryl exchange had taken place at the same time.

Plasmin is highly heat resistant (Visser, 1981) and is identical to the enzyme of the same name found in blood serum (Kaminowaga et al. 1972). This enzyme displays optimal activity at  $\text{pH} \approx 7.5$  and at  $37^\circ\text{C}$ . Both native and denatured  $\beta$ -lactoglobulin may act as potential inhibitor (Bastian et al. 1993), probably by interaction with casein micelles throughout steric hindrance of the approach of the enzyme to the cleavage sites (Benfeld et al. 1997; Enright, Bland, Needs & Kelly, 1999) or thiol-disulphide interchange reactions (Alichanidis et al. 1986, Grufferty & Fox, 1988 b; Kennedy & Kelly, 1997) .

The role of somatic cells plasmin activators was highlighted by Ravn, Berglund & Petersen (1995) and Kelly & Foley (1997). Kelly et al. (1997) reported that plasminogen injected to UHT milk immediately after the UHT treatment it was rapidly transferred to plasmin. This

activation occurred more rapidly in milk from raw material with high somatic cell count and this milk shown gelation after 150 days storage at 20°C. Milk without plasminogen added shown only sedimentation - not gelation.

Mastitis can lead to an increase in plasmin activity in milk due to an increase in somatic cell count (Saeman, Verdi, Galton & Barbano, 1988); Auldist, Coats, Rogers & Dowell, 1995).

The activity of plasminogen activator in UHT milk after heat treatment can increase due to inactivation of their inhibitors, as plasminogen activators are more heat stable as their inhibitors (Richardsen, 1983). Plasminogen activators are normally only slightly inhibited by UHT treatment. Treatment of milk at 72°C for 15 s inactivates plasmin by about 10-17%, 80°C for 10 minutes shown full inactivation (Kaminowaga et al.1972). Heat treatment of 142°C for 18 s is necessary to completely prevents proteolysis during subsequent storage (Grufferty & Fox , 1988a and b).

Bacterial extracellular proteinases produced by psychrotropic bacteria have much higher heat stability than plasmin and may stay at least partly active even after retort sterilisation. D-values for inactivation of some bacterial proteinases of *Achromobacter* and *P.fluorescens* at 130°C were by Driessen (1983) determined to 8 and 11 minutes respectively. Consequently, production of the enzyme before the heat treatment is very important factor influencing the activity in the final product, more than the heat treatment. A special heat treatment - LTI (Low Temperature Inactivation) method taking carried out e.g. at 50°C

for 30 minutes result in inactivation of these enzymes by autoproteolysis, however in presence of other proteins, the inactivation rate is limited (Schokker & van Boekel, 1998 a and b). They attack predominantly  $\kappa$ -casein with formation of material similar to para- $\kappa$ -casein (Snoeren, van der Spek, Dekker & Both (1979) followed by extensive non specific hydrolysis (Law, Andrew & Sharpe, 1977) of  $\beta$ -casein over whey proteins and  $\alpha_s$ -caseins (Gebre-Egziabher, Humbert & Blankenagel, 1980; Fairbairn & Law, 1986; Tryantafyllidou & Roussis (1999). Pseudomonas proteinases cause the formation of hard gel, similar to gel cause by rennet. They preferable hydrolyse the glycomacropeptide from  $\kappa$ -casein on the surface of the micelle and leaves the casein micelle mainly intact allowing the formation of more compact gel, compare to plasmin (Snoeren et al.1979; Datta & Deeth, 2003).

Proteolytic destabilisation of UHT milk leads to increase of viscosity with eventual formation of sediment or gel and eventually bitter off flavours (McMahon, 1996).

#### *1.3.6.1 Theories of gel formation in UHT milk*

The cause is not clearly identified, but there are three theories all involving proteins:

- the **enzymatic modification of caseins** by proteinases attack and subsequent rearrangement of the milk proteins

- based on the **physical chemical effects involving non enzymatic rearrangement of casein micelles and perhaps whey proteins** ( $\beta$ -lactoglobulin)
- **combination of both theories** when proteinases are needed to hydrolyse the caseins, which then leads to physico-chemical effect of gelation.

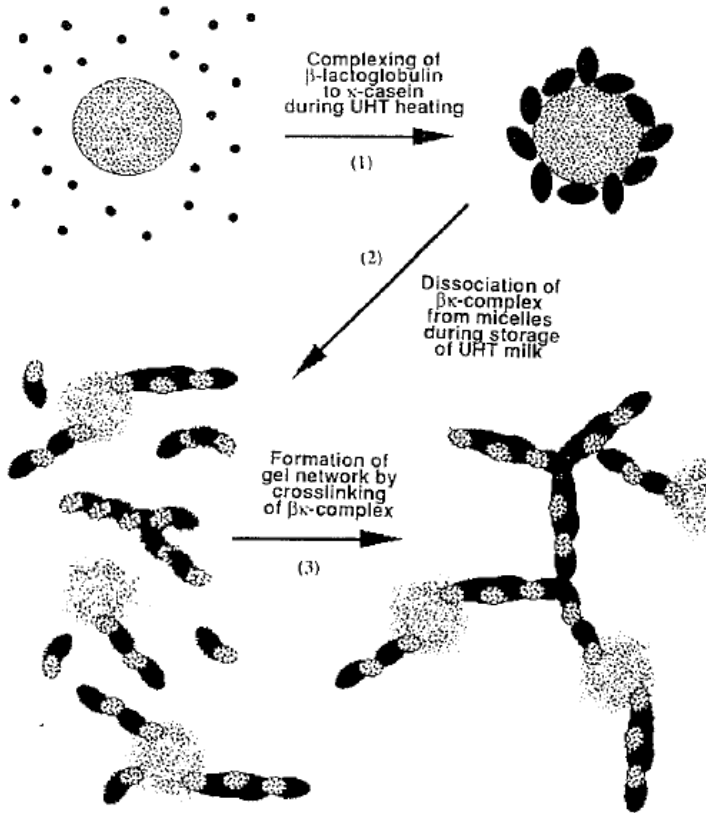
The gelation on non-enzymatic basis was explained by:

- chemical modification of casein micelles by Maillard reactions (Samel, Weaver & Gammack (1971), but this theory is not consistent at temperatures exceeding 35°C
- reaction between lactose and  $\epsilon$ -NH<sub>2</sub> groups of lysine, cross linking protein chains into very large complexes. This reaction occurs most frequently with caseins (most  $\kappa$ -casein, than with  $\alpha$ - and  $\beta$ - caseins), than with whey proteins (Turner, Swaisgood & Hansen (1978). This theory is not fully consistent as UHT casein micelle dispersions with lactose or sorbitol gelled at the same time and was also by Venkatachalam, Mc Mahon & Savello (1993).



Proteolytic destabilisation of UHT milk are caused, or at least accelerated, by hydrolyses of caseins and releasing the  $\beta$ -lactoglobulin/ $\kappa$ -casein complex, formed during heat treatment. The released complex subsequently aggregates and forms three-dimensional network of cross-linked proteins, which cause the milk gel (McMahon, 1996). Any conditions, which accelerate or delay release of the  $\beta$ -lactoglobulin/ $\kappa$ -casein complex from the casein micelles will accelerate or delay the age gelation. Both plasmin and bacterial proteinases can accelerate age gelation. They do not hydrolyse the  $\beta$ -lactoglobulin/ $\kappa$ -casein complex, but they hydrolyse the proteins that attach this complex to the casein micelles and in this way allow the release of the complex from the micelle (McMahon, 1996). If it is assumed that the movement of  $\beta$ -lactoglobulin/ $\kappa$ -casein complex to serum initiate gelation, it is likely that higher proportion of denaturated whey proteins not attached to a surface, which is the case in skim milk, provide less resistance to aggregation.

Extensive and fast protein degradation does not correlate with gelation which was explained by missing ability of the physical association of casein micelles needed to gelation during the fast degradation process (Kocak & Zadow (1985); Manji et al.(1986), Kohlmann, Nielsen & Ladisch,1988). Whereas slow proteolyses, when plasmin activity it self is even not measurable and when plasminogen is converted slowly to plasmin and cause slow protein degradation is leading to gel formation (Kohlmann et al.1988).



*Figure 20: Proposed mechanisms of age gelation by Mc Mahon (1996)*

Milk UHT processed by direct steam injection is more affected by gelation than the one produced by indirect method (Harwalkar, 1982). Directly and indirectly processed milk differs in gelation for two reasons. First due to the larger portion of enzymes is inactivated by the indirect heating, secondly because of more denatured  $\beta$ -lactoglobulin in the indirectly produced milk, which inhibits the proteolytic enzymes activity of native proteinases.

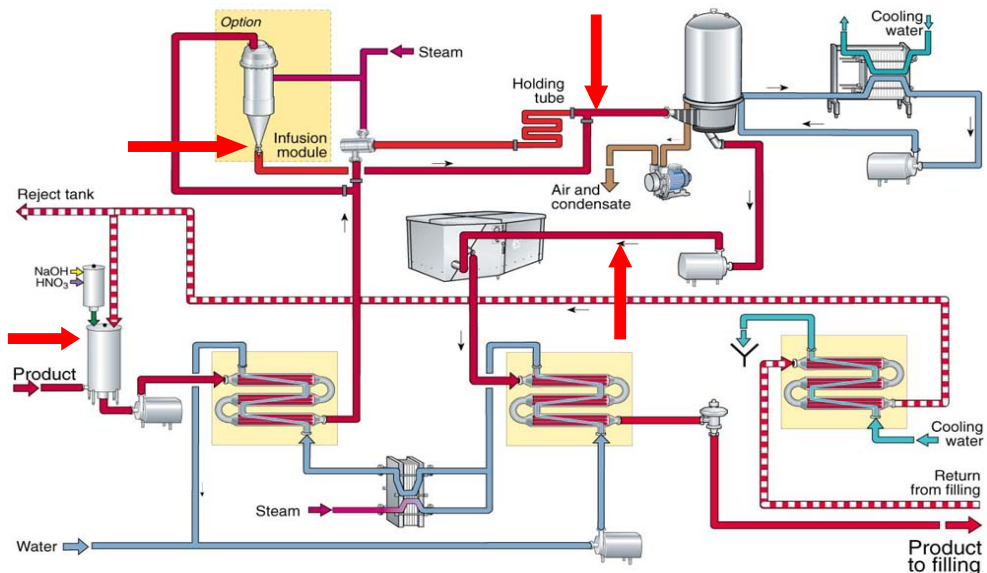
There are three mechanisms by which heat causes loss of activity of proteinases (Daniel, Toogood & Berqvist, 1995). First conformational unfolding (denaturation), second self-digestion – autoproteolysis and third irreversible non-enzymatic covalent modification - deamidation. Presence of large amount of proteins prevents autolysis (Schokker et al. 1998a and b). Low temperature inactivation technology showed much more inactivation at 55-60°C for 30 minutes than at temperatures above 60°C (Barach, Adams & Speck, 1976 and 1978), due to formation of casein-enzyme aggregates.

Severe heat treatments such as UHT results in a structural alteration via interaction with whey proteins and hence reduced acceptability of plasmin to milk casein and lost the activity. Oxidation of the thiol groups of  $\beta$ -lactoglobulin disrupter the interaction of the whey protein with casein and allows higher enzyme activity (Enright et al. 1999).

## 2. Materials and Methods

### 2.1 Sample preparation

The same standardized pasteurized (75°C for 15 seconds) whole milk with a fat content of 3.5% prepared the previous day from high quality raw material, was used for the production of three batches of UHT treated milk in a commercial scale UHT plant, TetraTherm Aseptic VTIS<sup>®</sup> (Tetra Pak, Lund, Sweden) (Flow Chart 1).



*Flow Chart 1 : Direct UHT plant TetraTherm Aseptic VTIS<sup>®</sup>, Tetra Pak, Lund, Sweden*

The unit was equipped with both steam injection and steam infusion units for the final heating. After indirect preheating to either 75 or 80°C followed by 20 seconds holding time, the milk was heated by steam injection or steam infusion to the final UHT temperature of 140°C for 4

seconds, followed by vacuum cooling to the same temperature as that used for preheating. Thereafter, the product was homogenized at aseptic conditions and finally cooled indirectly to 20°C and collected in an aseptic storage tank. Sample for structural studies during the UHT processing were taken out from the incoming milk as well as aseptically inline after final heating, vacuum cooling, homogenization (arrows in Flow Chart 1) Aseptic inline sampling was done using a pre-sterilized capillary tube (diameter 0.13 mm, length 4 m) immersed in ice water where the sampled milk was cooled below 40°C within 2 seconds.

The UHT processed milk from the aseptic tank was packaged in aseptically into 200 mL Tetra Brik Aseptic Slim packages (Tetra Pak, Lund, Sweden). Samples were stored at 5, 22-23 (ambient conditions), 30°C and 40°C and analysed at approximately 1 month intervals during 6 months storage.

## 2.2 Analyses

### *2.2.1 Measurement of heat load effects*

The heat effect of the processing caused by the direct UHT systems was measured by determination of the amount of native whey proteins and by measuring of transformation of lactose to lactulose in order to confirm the comparability of samples.

#### *2.2.1.1 Amount of native whey proteins*

The amount of non-denatured  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin was measured according to IDF standard method 178:2005, IDF(2005). After

## ***Materials and Methods***

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precipitation of casein at the isoelectric point (pH 4.6) and centrifugation the supernatant was analysed using RP-HPLC. The analyses were performed one week after production.

### *2.2.1.2 Lactulose content*

The lactulose content of the UHT samples was analyzed by means of an enzymatic method according to the IDF standard 175:2004/ISO 11285 Milk - determination of lactulose, IDF (2004). The analyses were performed one week after production.

### *2.2.2 Analyses of microstructural changes during processing and storage*

#### *2.2.2.1 Transmission Electron Microscopy (TEM) - standard methods*

The samples for TEM analyses were prepared according to the method used by Hillbrick et al. (1999). About 12 hours after production, the samples which were stored at ambient conditions were fixed in formaldehyde by the addition of 1 mL of 16% paraformaldehyde to 3 mL of milk and then encapsulated in small cubes made of agarose gel as described by Alleyne, McMahon, Youssef and Hekmat (1993). The samples were further fixed by placing the microcubes into the remaining milk-paraformaldehyde mixture where they were kept overnight in a refrigerator. The next day the cubes were dehydrated by a graded ethanol series of 50, 70, 95 and 99.5% concentration, infiltrated with LR White resin (Electron Microscopy Sciences) and polymerized at 55°C overnight. Before analyses, ultra-thin sections (~70-80 nm) were cut on

a diamond knife using an ultramicrotome (Reichert-Jung Ultracut E) and collected on 400 mesh copper grids. Sections were double stained with uranyl acetate and lead citrate. The sections were examined by using a transmission electron microscope LEO 906e (LEO Electron Microscopy Ltd, Oberkochen, Germany) at 80 kV.

*2.2.2.2 Transmission Electron Microscopy (TEM) combined with immunogold labelling*

This method was used in order to determine and follow the distribution and movements of the different protein fractions both during the processing as well as storage.

*Preparations for immunogold labelling*

The PBS (phosphate buffer saline) buffers were prepared the day before the immunogold labelling. The buffers contained 20 mM phosphate and 150 mM NaCl. The pH was then adjusted to 7.2 and 8.5 with 0.1 M HCl or 0.1 M NaOH to meet the pH optimum for the different antibodies used. Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, USA) was then dissolved in the PBS buffer with pH 7.2, to create a 2% BSA solution (w/v).

*Immunogold labelling*

The immunogold labelling method was based on that of Alleyne (1994), Hillbrink, et al. (1999) and Zhang, Z. & Goff, H. D. (2004). In order to achieve satisfactory results, several immuno-labelling experiments were conducted to establish suitable blocking conditions, incubation times,

## ***Materials and Methods***

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dilutions for the antibodies and staining conditions. Alterations of the primary antibody concentration resulted in significant changes in labelling intensity. Elongation of the incubation time for the secondary antibody showed to some extent positive effects on the labelling intensity, mostly apparent when the milk was labeled against  $\beta$ -lactoglobulin A and B.

The grids were placed on drops containing the different solutions, with the thin section facing down and positioned on Para film inside Petri dishes. The thin sections were firstly treated with a blocking solution containing 2% BSA (Sigma-Aldrich, St.Luis, USA) diluted with pH 7.2 PBS buffer. This step was followed by incubating at room temperature for one hour incubation on one of the primary polyclonal antibodies, sheep anti-bovine casein (Immunology Consultants Laboratory, Inc., Newberg, USA) diluted 1:140 in PBS (pH 7.2), a mixture of sheep anti-bovine  $\beta$ -lactoglobulin A (Biogenesis, Poole, UK) diluted 1:140 in PBS (pH 7.2) and sheep anti-bovine  $\beta$ -lactoglobulin B (Biogenesis Poole, UK) diluted 1:280 in PBS (pH 7.2) or mouse monoclonal anti-bovine  $\kappa$ -casein antibodies (RIKILT-Institute of Food Safety, Wageningen, The Netherlands, earlier used in study Haasnoot, Smits, Kemmers-Voncken & Bremmer, 2004). The negative controls were incubated in PBS (pH 7.0) instead of primary antibody. The grids were then washed, 3x5 min with drops of PBS pH 7.2 and 3x5 min with drops of PBS pH 8.5, to remove excessive primary antibodies. The pH change during washing was preformed to create the optimum pH for the secondary antibodies. The next step was labelling during incubation with the secondary antibodies labelled with 12 nm gold particles for 2 hours



against casein or for 3 hours against  $\beta$ -lactoglobulin A and B (donkey anti-sheep IgG (Jackson Immuno Research) diluted 1:25 in PBS (pH 8.5). Finally the thin sections were washed 3x5 min with drops of PBS pH 8.5 and 3x5 min with drops of distilled water.

The negative controls were the primary antibodies were omitted, showed no or very slight labelling indicating no non-specific binding of the secondary antibodies to the samples. In the cases where gold particles could be localized they were random with no correlation to the protein structures.

#### *Examination by TEM*

Sections were double stained with uranyl acetate and lead citrate (Alleyne, 1994). The sections were examined by using a transmission electron microscope LEO 906e (LEO Electron Microscopy Ltd, Oberkochen, Germany) at 80 /100kV.

### *2.2.3 Determination of the particle sizes*

#### *2.2.3.1 Determination of the mean volume of particles*

Image analysis of 20-30 TEM micrographs captured at 7 750x magnification. Connecting micelles were separated by a watershed function and the size was estimated by determining the volume weighted mean volume of protein particles -  $v^*$ . The star volume  $v^*$  is defined as

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‘the mean volume of all parts of an object that can be seen unobscured in all directions from a particular point’.

This is calculated using the formula,

$$v^* = \frac{\pi}{3} l_0^3$$

where  $l_0$  is the intercept length, and therefore the length from one boundary through the random point to the other boundary.  $v^*$  was estimated unbiased where  $l_0$  is the line intercept length and the average is  $l_0^3$  (Cruz-Orive, 1976; Russ, 1990; Gundersen, Bendtsen, Korbo, Marcussen, Møller, Nielsen, Nyengaard, Paakenberg, Sørensen, Vesterby & West, 1988; Langton & Hermansson, 1996).

A coefficient of error ( $CE$ ) is calculated as:

$$CE = \sqrt{\frac{\sum (l^3)^2}{(\sum l^3)^2} - \frac{1}{N}}$$

where  $l$  is the individual intercept length and  $N$  is the number of intercepts (Gundersen & Jensen, 1985). Coefficient of error below 0.2 is giving satisfactory accuracy. For easier visualization, the diameter of the mean size particle was calculated assuming spherical micelles i.e.

$$V = \frac{1}{3}4\pi r^3$$

### *2.2.3.2 Particle size measurement by Coulter Counter*

A thin silicon layer was applied on the side of the Tetra Brik Aseptic from the top to the bottom and the package was divided to 8 layers of equal height. Samples (1 mL) were taken out from centre of each layer, by penetration of the wall of the package and the silicon layer by syringe, starting from layer 1 (the top layer). The sample (a few droplets) was analyzed by Laser diffraction using an LS-230 (Beckman Coulter, Fullerton, CA, USA) with a measuring range of 0.04-2000 microns.

### *2.2.4 Measurement of sedimentation*

Evaluation of the sediment in the stored samples was performed by weighing of the sediment at the bottom of the package. The measurement of the amount of sediment was conducted in the samples until gel formation was noticed.

#### *2.2.4.1 Gravimetric analyses*

The amount of wet sediment was determined by transferring the firm sediment from the bottom of the carton with the help of 70 mL distilled water to a centrifuge tube, which was then centrifuged for 30 minutes at the 2500 rpm, after which the water was decanted and the amount of settled substance was determined by weighing the tube containing the sediment. The wet sediment was then transferred from the tube into an aluminum dish with the help of 2 mL distilled water, and the dry mass of

## ***Materials and Methods***

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sediment was determined after drying for 3 hours at 115°C. Results are based on the analysis of triplicate samples.

### *2.2.5 Measurement of enzymatic protein changes*

#### *2.2.5.1 Capillary electrophoresis of milk proteins*

Duplicate milk samples (10 mL) were centrifuged at 3000 x *g* for 30 minutes to enable the fat to be removed from the top (in most cases the samples were centrifuged twice). A sample (1800 µL) of the ultra-centrifuged skimmed milk supernatant was mixed with 4.2 mL sample buffer (Ardö & Polychroniadou, 1999) under vigorous agitation and incubated for 2 h at ambient temperature. The solution was then deep frozen to -18°C and stored at this temperature until analysis.

Casein components in milk were analysed using capillary electrophoresis (CE) (Ardö, Lindblad & Qvist, 1999). CE was conducted at 45°C using a HP3DCE System (Hewlett Packard A/S, Birkerød, Denmark) with a hydrophilic-coated, fused silica capillary column (Supelco Celect P1, Bellafonte, PA, USA or G1600-61219, Agilent Technologies).

#### *2.2.5.2 High-performance liquid chromatography of pH 4.6-soluble fractions*

Milk samples for high-performance liquid chromatography (HPLC) analysis were prepared by precipitating casein at pH 4.4 - 4.6 using 1M

HCl, adjusting the volume to 10 mL by adding distilled water and incubation for 30 minutes at 30°C. Thereafter, the samples were cooled to 4°C and centrifuged at 3000 x g for 30 minutes at this temperature. The supernatant was obtained by filtration (0.2 µm) and then injected into the column. The pH 4.6-soluble fraction of milk was analysed regarding peptides using reverse phase (RP) HPLC by injecting 50 µL into a C18 reversed phase RP-HPLC column (Nucleosil 5 mm C18, Macherey-Nagel GmbH, Düren, Germany). The flow rate was 1.0 mL minutes<sup>-1</sup> and the eluents used were: 0.1% trifluoroacetic acid (TFA) (denoted A) and 0.1% TFA in 60/40 mixture of acetonitrile and water (denoted B). The elution gradient was 100% A for 10 minutes, followed by linear increase of B from 0 to 80% over 80 minutes, 100% B for 10 minutes and finally 100% A for 20 minutes (Ardö & Gripon, 1995). HPLC was controlled using an Alliance workstation with the software Millennium (Waters, Hedehusene, Denmark), and the separated peptides were detected at 210 and 280 nm with a Waters Programmable Multiwavelength detector (Waters, Hedehusene, Denmark).

### *2.2.5.3 Liquid chromatography mass spectrometry of pH 4.6-soluble peptides*

Milk samples were prepared for liquid chromatography-mass spectrometry (LC-MS) analysis as for HPLC, as described above. LC-MSD analysis was performed using Agilent 1100 LC-MSD Trap an (Agilent Technologies A/S, Nærum, Denmark) operated with LS/MSD Trap Control data analysis software version 4.01. A Zorbax 300 SB-C18 column (2.1 x 150 mm, 5 µm) was used (Agilent Technologies), and operated at 40°C. Hydrolysate samples (10 µL) from HPLC were

## ***Materials and Methods***

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injected and components were eluted using a flow rate of 0.25 mL minutes<sup>-1</sup>, and a gradient consisting of 8-55% B for 40 minutes (ending at 49.5% acetonitrile as RP-HPLC gradient). The column was rinsed with 100% B for 5 minutes and re-equilibrated with 8% B for 10 minutes before the next injection. Buffer A was 0.1% TFA in water and buffer B was 0.1% TFA in 90% acetonitrile.

The trap was operated in the positive mode with a nebulizer pressure of 50 psi, a flow of nitrogen of 9 mL minutes<sup>-1</sup> and drying temperature of 300°C. Mass spectra were recorded using either a standard range from 200 to 2 200 m/z<sup>-1</sup> at the normal scan resolution, or a target mass set of 1521 m/z<sup>-1</sup>. The skimmer 1 voltage was set to 55.6 V and the capillary exit offset was 84.4 V. The ICC was in and adjusted to the target of 20 000 and maximum accumulation time of 50 ms. Data were processed by Bruker Daltonics DataAnalysis, version 2.1. Peptide masses were assigned to particular sequences of the casein components using results obtained earlier (Ardö, Lilbæk, Kristiansen, Zakora & Otte, 2006; Larsson, Ardö, Paulsson & Dejmeek, 2006) together with the software GPMAW, version 3.04 (Lighthouse Data, Odense, Denmark).

### *2.2.6 Analyses of composition*

Samples of UHT milk in 200 mL TBA packages were stored at temperatures 5°C, 22–23°C, 30°C and 40°C. After every month, 2 packages from each sample were deep frozen in a fast-freezing tunnel. Before analysis, samples were cut into 8 equal layers from top to

bottom. The layers were melted at ambient temperature and brought to 20°C before determining the composition using a MilkoScan™ FT120 (Foss, Hillerød, Denmark). The MilkoScan™ FT120 works on the principle of infrared spectrophotometry. The infrared energy produced by a broad band source is filtered through IR filters of specific wavelengths and focused through the samples under test. This energy passes through the samples and strikes a detector which converts it into a corresponding electrical signal. This signal, after amplification and certain corrections, is processed by a microprocessor and then displayed. The results presented are based on two duplicates.

#### *2.2.7 Statistical evaluation of data*

Statistical evaluations of data were conducted using t-test (Excel, Microsoft Office XP, Microsoft, USA), one way analysis of variance and general linear model (ANOVA) using of Minitab statistical software version 14, Minitab Inc. USA at the 95% significance level.

### **3. Main results & discussions**

#### **3.1 Unfolding of whey proteins and lactulose content**

Samples preheated to 75°C and UHT processed by both direct heating methods contained approximately the same lactulose levels and amounts of native  $\alpha$ -lactalbumin. No significant correlation was found between lactulose content neither degree of  $\beta$ -lactoglobulin unfolding and the heating technique applied, indicating that the heat load and the heating methods were fully comparable. The results of  $\beta$ -lactoglobulin unfolding are in disagreement with the findings of de Jong, Waalewijn. & Van der Linden (1994), who claimed a higher degree of  $\beta$ -lactoglobulin unfolding in steam injection system.

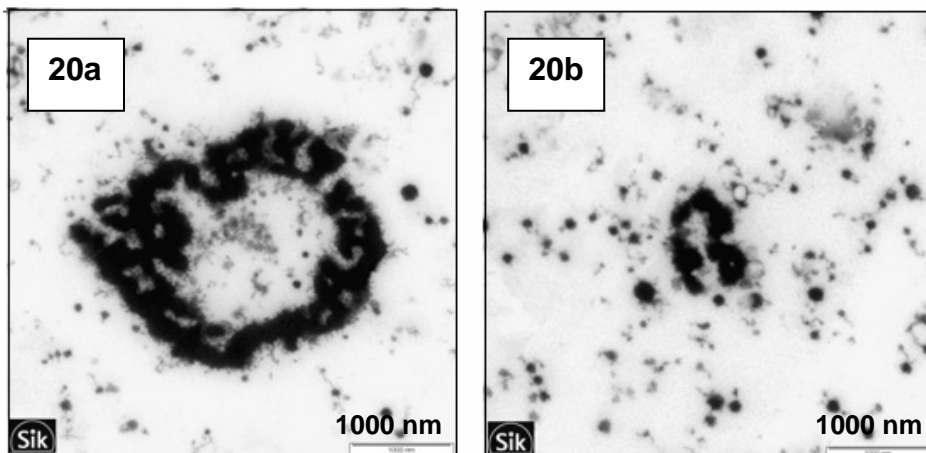
#### **3.2 Microstructure during processing**

The microstructure of whole milk was analyzed at different processing steps during the production of UHT milk produced by direct steam injection as well as infusion technology.

TEM micrograph of the raw material – pasteurized homogenized whole milk - was typical for the material used. The structure earlier identified as caseins and whey proteins (McMahon, Yousif, & Kaláb, 1993; Hillbrick et al.1999) were attached to the newly homogenized fat globule surface. Agglomerates of proteins with fat globules and proteinsin between were very common. Casein micelles were smooth, round and compact.



Large protein agglomerates were observed immediately after the final heating by both direct heating technologies. In the milk processed by direct steam injection, some of the aggregates exceeded 2  $\mu\text{m}$  in diameter (Figures 20a) composed to mainly of caseins with some  $\beta$ -lactoglobulin concentrated on the surface. The size of aggregates in the steam infusion samples was smaller and their presence more sporadic (Figure 20b).



*Figure 20.: TEM micrographs of a large agglomerate in UHT milk samples taken out immediately after the steam injection*

These aggregates may be a result of bubble formation caused by introduction of steam into the product when using the steam injection technology. The proteins may agglomerate on the hot surface of the steam bubbles and remain as large size agglomerates after the steam inside condenses. Similar structures are described by Walstra, Geurts, Noomen, Jellema & van Boekel (1999).

High numbers of long and curly protein strands, previously identified with heterogeneous complex  $\beta$ -lactoglobulin/ $\kappa$ -casein (Hillbrick et al.1999), were often attached to the surface of casein micelles. The

sample from steam injection seemed to contain more of the loose tendrils of  $\beta$ -lactoglobulin/ $\kappa$ -casein complex attached than the ones from steam infusion although the amount of denatured  $\beta$ -lactoglobulin was equal in both samples. The volume weighted mean diameter of casein micelles in samples produced by steam infusion was larger than in samples from steam injection, which could be explained by the  $\beta$ -lactoglobulin/ $\kappa$ -casein complex being better anchored to the casein micelle surface than in samples from steam injection, where the complex was released to a higher extent. Another explanation could be mechanical damage of casein micelles during steam injection leading to micelle disintegration and disaggregation.

The big agglomerates observed after the final heating were still present in both samples after vacuum cooling step. Some of them are in the form of very dense protein rings.

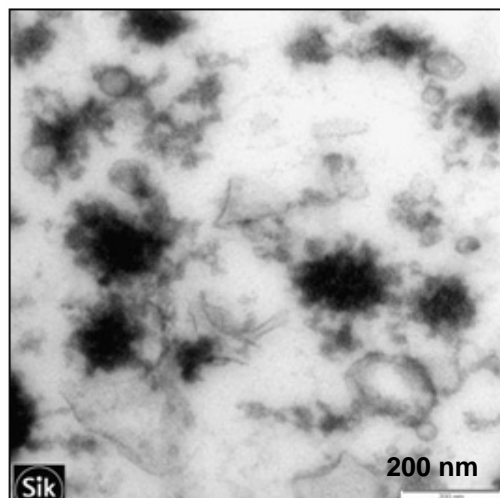
Though the microstructures of both samples after the whole UHT process were more equal and homogenous but the sample from steam injection contained still more of larger agglomerates.

The structural changes during the final heating step may lead to instability problems during subsequent storage.

### 3.3 Microstructure during storage

The visual structure did not change dramatically when samples were stored at 5°C. Small amounts of soft, gelled and glossy sediment appeared after 3 months storage in samples from steam injection and at 5 months for the samples from steam infusion. The hairy tendrils on the

casein micelles of irregular surface extended upon storage and a high number of short protuberances were present on the surface of the micelles (Figure 21).



*Figure 21: TEM micrographs of UHT milk samples stored at 5°C for 1 month*

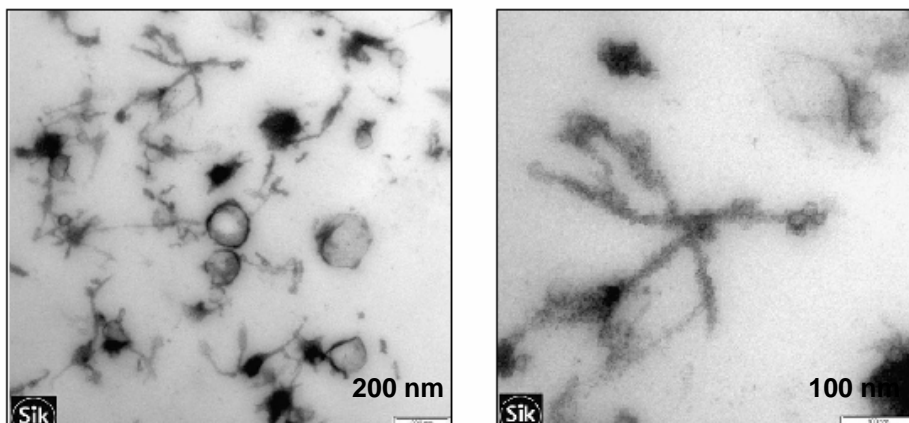
The development of the protuberances was attributed by Walstra, Geurts, Noomen, Jellem & van Boekel (1999) to dissolution of a part of primarily  $\beta$ -caseins due to weakening of the hydrophobic bonds responsible for their binding. Even other caseins could dissolve but to a lesser extent. As  $\beta$ -caseins were dissolving and other caseins also left the surface, the  $\beta$ -caseins protrude from the surface and the formation of another category of hairs led to the voluminosities of the casein micelles increasing. As the studies of the microstructure of these samples by TEM showed, the size of the casein micelles in the milk phase after storage for 6 months was reduced compared to the freshly produced product. The size reduction of the casein micelles can be caused by

## ***Main results & discussions***

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sedimentation of the large and heavy micelles with a large diameter as well as by the decomposition of the casein micelles due to the calcium phosphate bridges dissolving because of improved solubility of  $\text{Ca}^{2+}$  salts at the low storage temperature (Walstra et al.1999).

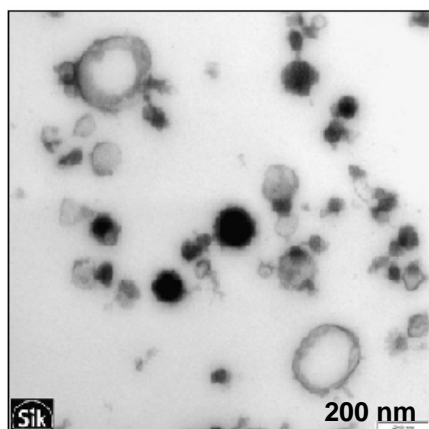
In milk samples stored at 22°C, sediment formations started after 2 months in samples processed by steam injection technology and after 4 months in the samples produced in the steam infusion system (Chart 1). The samples started to gel after 5 months, independently of the sterilization system used. The gel formation begun from the bottom and one month later was distributed throughout the whole content of the package. The structural changes are similar to the changes described earlier for samples stored at 5°C - the reduction of size of the casein micelles in the milk phase, increasing number of the tendrils connected to the casein micelles surface, their extension and changing of their shape from curly to more spiky at the time of gelation (Figure 22). The formation of a similar three dimensional network was also described by McMahon (1996).



*Figure 22: TEM micrographs of gelled UHT milk samples stored at 22°C for 6 months*

The immunogold labelling for  $\kappa$ -casein of samples after 1 month storage at 22°C gave a positive reaction not only in the micelles but also in the protruding strands, indicated the possible migration of this protein from the micelles. The results of immunogold labelling of gelled samples showed that total casein,  $\kappa$ -casein and  $\beta$ -lactoglobulin were present in the tendrils protruding from the micelles and connected them at the time of gelling. However, due to the fact that the epitop for immunogold labelling for  $\kappa$ -casein is located on glycomacropeptide the presence of specifically the whole  $\kappa$ -casein could not be verified.

The structure of the UHT milk samples stored at 40°C was composed of very small agglomerates or single particles and never gelled. The structural changes at the high storage temperature developed completely differently. The casein micelles lost their rough surface, became more round, the hairy tendrils became shorter and often disappeared from the micelle surface (Figure 23).



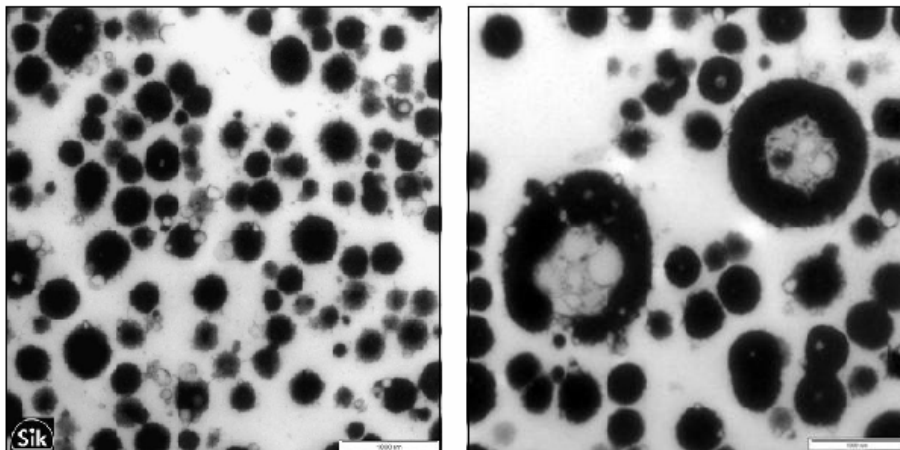
*Figure 23: TEM micrographs of UHT milk samples stored at 40°C for 5 months*

## ***Main results & discussions***

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The reason for these changes could be lactosylation, and because of that, increased solubility of  $\beta$ -lactoglobulin molecules connected to the surface of the micelles as well as blocking of the sulphhydryl group for attachment of other proteins. According to Singh & Fox (1987a, 1987b), electrostatic repulsion may be responsible for the dissociation of  $\kappa$ -casein. The reason for the loss of  $\kappa$ -casein could be the increased storage temperature, which decreases the solubility of  $\text{Ca}^{2+}$  and in this way supports the level of dissociation due to the enhanced electrostatic repulsion. The interaction of amino groups and lactose may also contribute to charge modification and in this way dissociation of the  $\kappa$ -casein from the micellar surface (Singh & Creamer, 1991a and b).

The sediment in the bottom in samples stored at 22°C was based on protein particles of mixed sizes, some of them up to 500-600 nm in diameter, connected by stiff strands causing the gel formation. Many large protein particles in the sediment were in a form of dense protein rings. Sediment particles in samples stored at 40°C were completely round (Figure 24), with lots of micro fat globules attached to the protein surface and without any tendrils attached, which explains the absence of gel formation at this storage temperature. While the casein micelles were very predominant, up to 800 nm in diameter, the interstitial spaces were very small. The sedimented particles showed strongly positive reaction on immunogold labelling against the total casein but no immunolabelling reaction for  $\kappa$ -casein and  $\beta$ -lactoglobulin. Based on that, it can be suggested that the sediment formation involves  $\alpha$ - or  $\beta$ -caseins dissociated from the micelles during storage or even more



*Figure 24: TEM micrographs of sediment in UHT milk samples stored at 40°C for 5 months (bar 1000 nm)*

probably the peptides formed due to their proteolysis. This hypothesis is derived from the large amount of  $\gamma$ -caseins found in these samples by use of capillary electrophoresis in our work published earlier (Malmgren et. al. submitted a). However even para- $\kappa$ -casein can be present in the sediment, as the epitope used for immunolabelling on  $\kappa$ -casein is situated on glycomacropeptide, it means the immunolabelling does not give a positive response for presence of para- $\kappa$ -casein only.

### 3.4 Stability defined by composition and particle size

The influence of the final heating technology on the fat separation is not of practical importance. The fat content in the top and bottom layers at all storage temperatures was independent of the final heating technology and preheating temperature, and was only dependent on the time of

## ***Main results & discussions***

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storage. The diagrams show the storage time and temperature-dependent increase in the fat content of the top layers and at the same time the reduction in the fat content of the lower layers, showing that the fat globules and light fat containing clusters rise to the surface.

Even the protein content in different layer in the package was significantly independent of the final heating technology.

The shapes of the curves displaying the mean size of particle distribution measured by Coulter Counter are reminiscent of the fat content changes in different layers and most probably show the rising of the fat globules at the same time as the sinking of the small protein particles to the bottom layers. This theory is supported by the reduction in the fat content and the increase in the protein content in the downwards direction in the package. The increase in the mean particle size in samples stored for 5 months at 22°C shows that agglomeration has taken place in the first phase of gel formation.

Generally, the calculated volume weighted volumes and diameters of casein micelles based on image analysis of 20-30 TEM micrographs in the milk stored at 22°C are lower after 1 month storage compared to the freshly produced final product and start to increase in size again after 3 months storage; they do not differ significantly between samples from steam injection and steam infusion (Table 1).



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	Calculated volume weighted			
	Volume size [ $\mu\text{m}^3$ ]		Diameter [ $\mu\text{m}$ ]	
	Injection	Infusion	Injection	Infusion
Final product	0.022	0.030	0.16	0.18
1 month at 22 °C	0.011	0.012	0.13	0.13
3 months at 22 °C	0.016	0.016	0.15	0.15

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*Table 1: Calculated volume weighted volumes and diameters of casein micelles in UHT whole milk*

### 3.5 Sediment formation

The amount of sediment in the samples produced by steam injection was significantly higher ( $p < 0.001$ ), independent of storage temperature and time, and sedimentation was seen earlier, than in those produced by steam infusion (Chart 1). Further sediment reduction was seen in our samples when increasing the preheating temperature from 75°C to 80°C ( $p < 0.001$ ). The extent of sedimentation was found to depend strongly on the storage temperature, and increased with increasing with the storage temperature and time.

## Main results & discussions

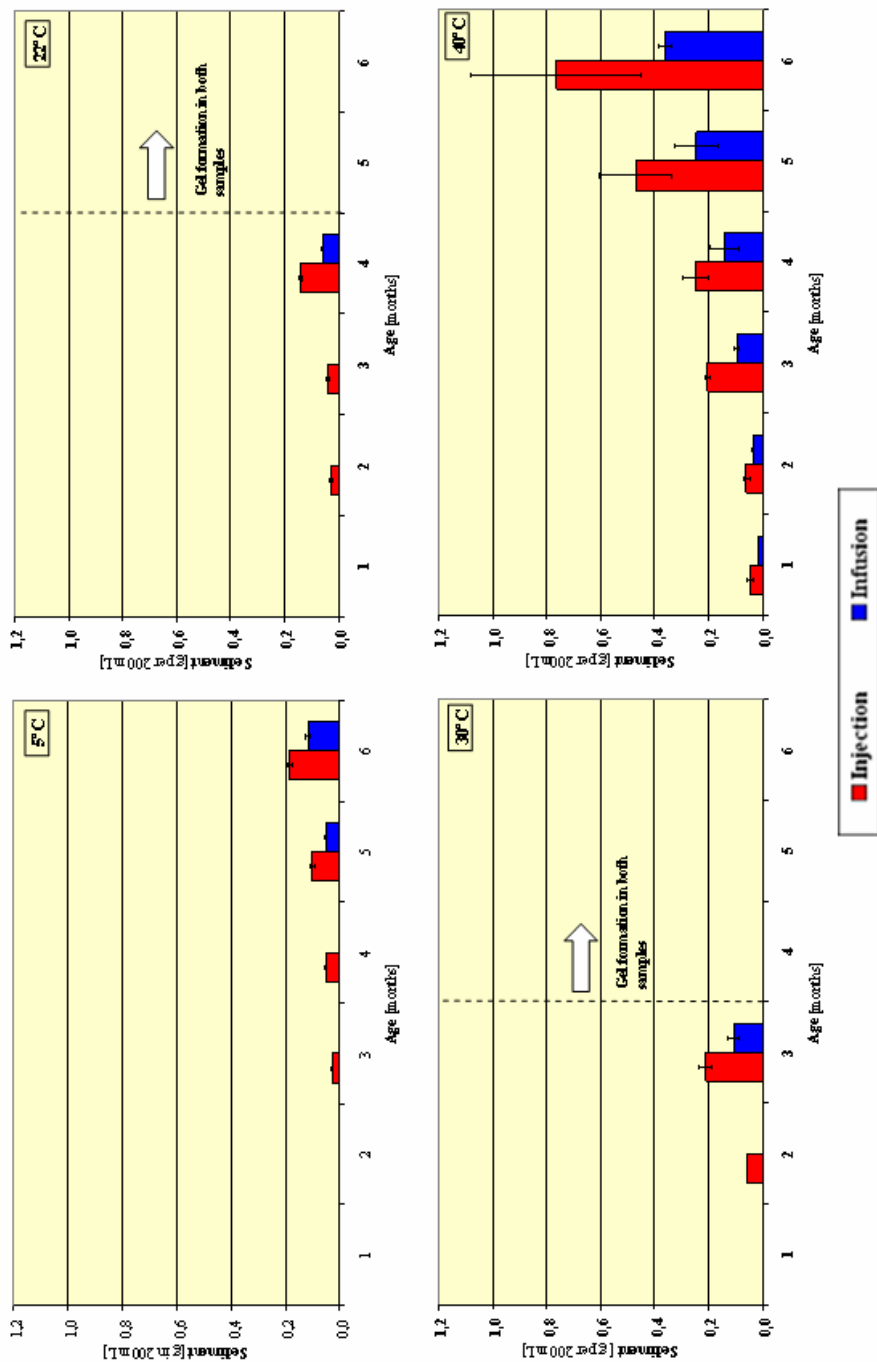
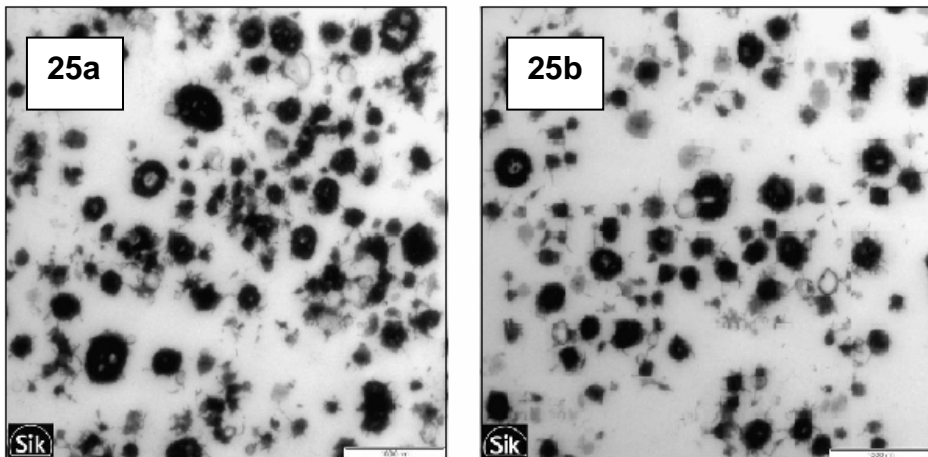


Chart 1 : Sediment formation in UHT milk samples processed by steam injection and steam infusion technology with preheating 75°C stored at 5, 22, 30 respectively 40 °C for 6 months

There is a tendency, based both on measured data (Table 2) and visual inspection of the TEM micrographs (Figure 25a and b) that the sediment particles in the samples from steam infusion are smaller in size than the ones from steam injection, however, this supposition was not statistically confirmed due to an insufficient number of measurements made.

	Calculated volume weighted			
	Volume size [ $\mu\text{m}^3$ ]		Diameter [ $\mu\text{m}$ ]	
	Injection	Infusion	Injection	Infusion
4 months at 22 °C	0.087	0.085	0.26	0.25
6 months at 22 °C	0.035	0.027	0.19	0.17
6 months at 40 °C	0.408	0.075	0.43	0.24

*Table 2 : Calculated volume weighted volumes and diameters of casein micelles in sediment of UHT whole milk*



*Figure 25: TEM micrographs of sediment in UHT milk samples stored at 22°C for 4 months (bar 1000 nm)*

- a) steam injection*
- b) steam infusion*

### 3.6 Enzymatic changes of proteins

Capillary electrophoresis of the defatted supernatant of milk stored at 5°C, 22 °C, 30 °C and 40 °C identical electrophoregrams independent of the heat treatment and heating technology. Storage at 5°C showed a negligible reduction of  $\alpha_{s1}$ 8P and  $\alpha_{s2}$  caseins, and slightly more pronounced reduction of  $\beta$ -caseins . The fact that proteolysis at this low temperature occurs very slowly is that, in principle, no activation of plasminogen occurred (Kang & Frank, 1988) but can even be reduced over time due to autolysation (self-hydrolyzation) and at the same time only minor activation of plasminogen (Guinot-Thomas, Al Ammoury, Le Roux, Laurent, 1995; Crudden, Fox & Kelly, 2005).

Samples treated by steam injection and steam infusion, preheated to 75°C and stored at 22°C and 30°C showed a reduction in  $\beta$ -caseins and  $\alpha_{s1}$ -caseins, and increasing levels of  $\gamma_2$ - and  $\gamma_3$ -caseins, which are products of  $\beta$ -caseins hydrolysis by plasmin. Modification of the shapes of the peaks corresponding to the whey proteins was observed in centrifuged supernatants upon storage. The height of the  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin peaks was reduced, and several additional peaks appeared closer to the main proteins which may be results of proteolysis (Recio, De Frutos, Olano & Ramos,1996) or modification of the protein spectrum by the presence of rest products of proteolysis as well as lactosylation (Siciliano, Rega, Amoresano & Pucci, 2000).

Capillary electrophoregrams of the milk preheated to 80°C are similar to those observed from the samples preheated to 75°C, but with a one-month delay. This delay was closely correlated to the delay of the

sediment gelation and even with the gelation of the whole package contents. Both phenomena occurred in milk preheated to 80°C first 1 month later than in samples preheated to 75°C. The reason for this could be either greater inactivation of the plasmin itself, or unfolded  $\beta$ -lactoglobulin acting as a potential inhibitor of the plasmin system (Bastian, Hansen & Brown, 1993) through steric hindrance during the approach of the enzyme to the cleavage sites and thiol-disulphide interchange reactions (Kennedy & Kelly, 1997; Benfeldt et al. 1997; Metwalli, De Jongh & Van Boekel, 1998; Enright et al.1999; Scollard, Beresford, Murphy & Kelly, 2000).

A reduction in height and broadening of the peaks corresponding to  $\beta$ -caseins and  $\alpha_{s1}$ - caseins, with the formation of new peaks similar to the samples stored at 22°C, as well as simultaneous formation of  $\gamma_2$ - and  $\gamma_3$ -caseins, were revealed by CE at 30°C storage already after 1 month but with no measurable amount of sediment at the bottom of the package.

This means that the protein changes measurable in the supernatant by CE alone do not correspond to the degree of sedimentation.

Despite the extensive modification of the protein pattern at 40 °C storage temperature, no gelation, but only heavy sedimentation, was observed. The lack of gelation in samples stored at high temperatures has been suggested to be due to very fast and extensive protein degradation, which does not allow the physical association of casein micelles needed for gelation (Kocak et al.1985; Mc Kellar, 1981; Manji et al.1986; Mitchell & Ewings, 1985; Kohlmann et al.1988).

## ***Main results & discussions***

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The absence of larger changes in  $\kappa$ -casein in our samples may be explained by the absence of enzymes of bacterial origin in our samples. The proteolytic changes were just a result of plasmin activity.

Applying HPLC and LC-MS on pH 4.6-soluble fraction after 6 months storage, it was confirmed that the skim milk supernatant contained high levels of lactosylated whey proteins and plasmin-derived peptides from  $\beta$ -casein with different numbers of lactose molecules attached. The higher the storage temperature, the larger the number of attached lactose molecules. After storage for 6 months at 40°C all  $\beta$ -lactoglobulin molecules contained at least two lactose residues. Also, the proteose-peptones from plasmin activity on  $\beta$ -casein were lactosylated. Garcia-Risco et al. (1999) observed, that height of the  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin peaks was also reduced, and several additional peaks appeared close to the main proteins, which they attributed to the formation of polymers involving casein-polypeptides and whey proteins, covalently or disulphide linked. In order to elucidate whether  $\kappa$ -casein was involved, they treated serum fractions with rennet, but the peak in the  $\beta$ -lactoglobulin region did not change. This suggests that some of the observed changes in their patterns were the result of lactosylation or products of enzymatic breakdown but not agglomerates of  $\beta$ -lactoglobulin and  $\kappa$ -casein.

The higher degree of lactosylation in UHT milk stored at 40°C may partly explain the lack of gelation at high storage temperatures.

The extent of protein alteration observed in the milk supernatant by the aid of CE alone does not correspond to the actual quality of the stored UHT milk, and can not be used to predict its storage stability.

### 3.7 Gelation

Gelation occurred in samples stored at 22°C and 30°C. Soft gel formation started at the bottom of the package and one month later had spread to the entire contents of the aseptic container. The gelation process was also found to be storage temperature dependent and occurred in samples preheated to 75°C and stored at 30°C already after 3 months storage. Lowering the storage temperature to 22°C postponed gelation by one month, as did increasing the preheating temperature from 75°C to 80°C ( $p < 0.001$ ). The samples started to gel independently of the sterilization system used. The gel formation began from the bottom and was distributed throughout the whole content of the package. Samples stored at 5°C and 40°C did not gel during the evaluation period of 6 months.

Question of gel formation are also discussed in the in the chapter 3.3.

### **4. Conclusions**

#### **4.1 Comparison of UHT milk produced by direct steam injection and steam infusion technology**

We found no significant differences in lactulose content, amount of native  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin, nor enzymatic inactivation and gelation in UHT milk samples processed under the same conditions by steam injection or steam infusion.

The only characteristics, which were significantly different in samples produced by steam injection compared to the steam infusion were:

- Formation of large protein agglomerates during steam injection
- Smaller casein micelle size after the steam injection
- Significantly higher sediment formation

The large protein aggregates may be a result of bubble formation caused by introduction of steam into the product when using the steam injection technology. The proteins may agglomerate on the hot surface of the steam bubbles and remain as large size agglomerates after the steam inside condenses. Similar structures are described by Walstra, Geurts, Noomen, Jellema & van Boekel (1999). The very stable formations may be caused the micelles reacting by the sites devoid of hairs leading to fusion (Walstra et al.1999). Presence of these stable aggregates, even if they are partly disrupted during homogenization, may influence the sediment formation especially in the early stage of storage.



Two kinds of changes occur in the milk proteins in our samples during storage at 5, 22, 30 and 40°C for 6 months: proteolysis caused by the plasmin system and lactosylation. The influence of storage temperature on both reactions was enormous. Plasmin activity was found at all temperatures, but was only very limited at 5°C, increasing at 22 and 30°C, and then decreasing again at 40°C. Lactosylation did not occur at the lowest temperature, but became evident and increased with increasing temperature. After storage for 6 months at 40°C all  $\beta$ -lactoglobulin molecules contained at least 2 lactose residues, and even the proteose-peptones from plasmin activity on  $\beta$ -casein were lactosylated.

#### 4.2 The proposed mechanisms of sediment formation and plasmin induced gelation in UHT milk:

During high temperature treatment of milk the heat sensitive whey proteins unfold and react preferably with  $\kappa$ -casein on the casein micelle surface or with each other and after that, eventually, with the micelle surface (Figure 14). Unfolded  $\beta$ -lactoglobulin can reduce the plasmin activity by steric hindrance as well as by direct inactivation of the plasmin system. During storage, the plasmin system is by plasmin activators transforming the plasminogen to active plasmin, capable the break down milk proteins, preferable  $\beta$ -caseins and split them to  $\gamma$ -caseins. Later even  $\alpha_{s1}$  and  $\alpha_{s2}$ -caseins can be attacked leading to production peptides and protein fragments. In this way the casein micelles are getting more porous and even  $\kappa$ -casein/ $\beta$ -lactoglobulin is

## Conclusions

released from the micellar surface. The  $\kappa$ -casein/ $\beta$ -lactoglobulin complexes react and form tendrils together with the proteins which stayed attached to the casein micelle surface and gelation occurs (Figure 26).

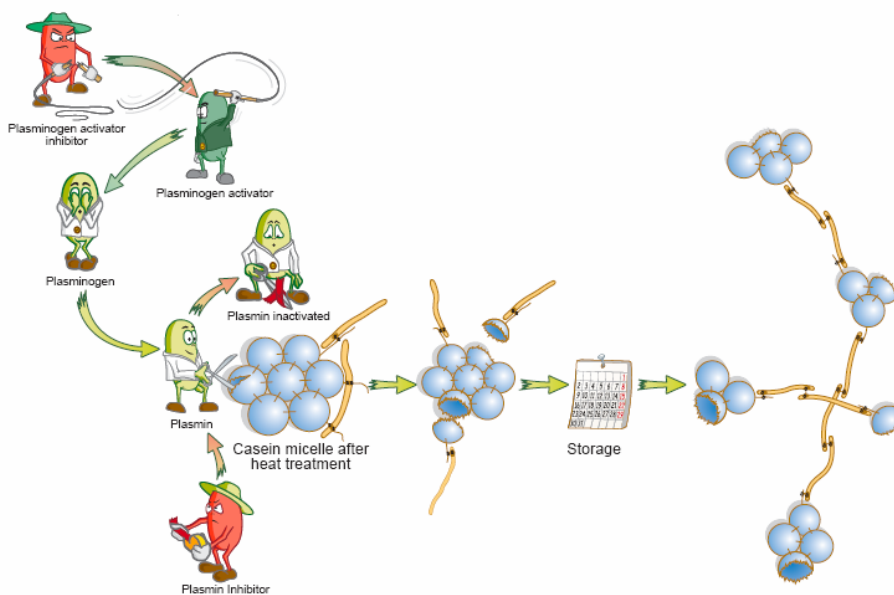


Figure 26 : Schematic picture of plasmin induced gel formation

The hydrophobic parts of the micelles interact to form dense caseins particles and sediment.

Absence of gelation at high storage temperature (above 35°C) can be effect of lactosylation leading to higher solubilisation of  $\beta$ -lactoglobulin and at the same time lactose molecule may also block possibility of other sulphhydryl groups to attach. These two effect lead to reduction of tendrils on the micelle surface and gel formation fails. The hydrophobic parts of the casein micelles react together and sediment in form of casein aggregates.

### 5. Acknowledgements

This was a long journey..... and I met lots of wonderful people on the way. Now it is almost over...and I have new ideas about what to do with my free time.

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## *List of Symbols and Abbreviations*

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### 6. List of Symbols and Abbreviations

#### *Latin symbols :*

t	holding time	[sec]
T	temperature	[°C]
z	decimal reduction temperature	[°C]
w/v	weight per volume	[%]
$l_0$	intercept length	[ $\mu\text{m}$ ]
$l$	intercept length	[ $\mu\text{m}$ ]
$N$	number of intercepts	
$V$	volume of casein particle	[ $\mu\text{m}^3$ ]
r	diameter of casein particle	[ $\mu\text{m}$ ]

#### *Greek symbols :*

$v^*$	volume weighted mean	[ $\mu\text{m}^3$ ]
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#### *Abbreviations :*

UHT	Ultra High Temperature
ECC	Europien Community Council
CCP	Colloidal Calcium Phosphate
LTI	Low Temperature Inactivation
-CN	casein
IDF	International Dairy Federation
RP- HPLC	Reverse Phase-High Performance Liquid Chromatography
TEM	Transmission Electron Microscopy
BSA	Bovine serum albumin
PBS	Phosphate Buffer Saline
IgG	Immunoglobulin

## *List of Symbols and Abbreviations*

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<i>CE</i>	Coefficient of error
CE	Capillary Electrophoresis
TFA	Trifluoroacetic acid
LC-MS	Liquid Chromatography-Mass Spectrometry