Impact of sampling month and processing and storage conditions on UHT milk stability

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Abstract

Consumption of ultra-high temperature (UHT) milk has increased dramatically worldwide, driven by the extended shelf-life under ambient distribution and storage conditions. Fat destabilisation is a prominent factor limiting UHT milk quality. This thesis explored the effects of season and of UHT processing, storage time and temperature on fat destabilisation in UHT milk. The aim was to provide information on lipid changes to farmers and dairy industries for use in quality control in raw milk, processing improvement and shelf-life prolongation.

Comparable seasonal variations in lipids were found in raw milk and corresponding UHT milk, with summer milk containing higher proportions of phospholipids (PL) and unsaturated fatty acids (UFAs) than milk from other months. This is the first report of seasonal variations in lipids in processed UHT milk. The seasonal variations originated from raw milk and persisted throughout the storage of UHT milk.

Storage time and temperature affected lipid profiles in UHT milk. Fat aggregation, lipid oxidation and lipolysis from triacylglycerol (TAG) and PL were observed when UHT milk was stored for longer (>6 months) and at higher temperatures (20 °C, 30 °C, 37 °C).

Commercial UHT processing was found to influence the physical structure of fat globules, with smaller globule size and thinner membranes in processed UHT milk. The distribution of TAG, PL and some fatty acids (FA) was altered in the final UHT product compared with the raw milk. To chart the effect of UHT processing on lipid profiles, milk was sampled at four key steps during processing and the structure of fat droplets and their membranes and FA composition in TAG and PL were analysed. The results revealed that homogenisation and UHT treatment affected the microstructural organisation of milk fat globules and membranes, thereby accelerating lipid lipolysis of PL and TAG to form free FA in UHT milk.

This new information can assist in improving UHT milk processing and lipid instability in UHT milk during storage.

Keywords: UHT milk, fat globule, microstructure, lipid class, fatty acids, sampling month, processing, storage

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Dedication

To my little boy – ZhongRui Wang

Living without a goal is like sailing without a compass.
John Ruskin

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Jing Lu, Jana Pickova, José L. Vázquez-Gutiérrez, Maud Langton (2018). Influence of seasonal variation and ultra high temperature processing on lipid profile and fat globule structure of Swedish cow milk. *Food Chemistry* 239, 848-857.

II Jing Lu, Maud Langton, Sabine Sampels, Jana Pickova (2019). Lipolysis and oxidation in ultra-high temperature milk depend on sampling month, storage duration, and temperature. *Journal of Food Science* 85(5), 1045-1053.

III Jing Lu, Jana Pickova, Geoffrey Daniel, Maud Langton. The role of key process steps on microstructural organisation of fat globules and lipid profiles in a UHT pilot plant. (submitted)

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The contribution of Jing Lu to the papers included in this thesis was as follows:

I. Participated in the design of the study together with the supervisors. Performed the laboratory work, data analysis and manuscript writing.

II. Was responsible for the experiments and data evaluation and wrote the manuscript.

III. Participated in sample collection and performed the analytical work, data analysis and preparation of the manuscript.

Abbreviations

CLA	Conjugated linoleic acid
CLSM	Confocal laser scanning microscope
FA	Fatty acid
FAME	Fatty acid methyl ester
FDA	Food and Drug Administration
FDR	False discovery rate
FFA	Free fatty acids
GC-FID	Gas chromatogram-flame ionisation detector
GLM	General linear model
HPTLC	High performance thin layer chromatography
LPL	Lipoprotein lipase
LSM	Least squares mean
MDA	Malondialdehyde
MFG	Milk fat globule
MFGM	Milk fat globule membrane
MUFA	Monounsaturated
PC	Phosphatidylcholine
PCA	Principal component analysis
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipids
PLS-DA	Partial least squares discriminant analysis
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
Rh-DOPE	Phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)
SFA	Saturated fatty acid
SM	Sphingomyelin
TAG	Triacylglycerol

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TEM	Transmission electron microscope
TFA	Total fatty acids
TL	Total lipids
TLC	Thin layer chromatography
UFA	Unsaturated fatty acids
UHT	Ultra high temperature
UI	Unsaturation index
VE	Tocopherol

1 Introduction

Milk has a long tradition in the human diet, as it contains good quality proteins and fat, numerous essential nutrients such as conjugated linoleic acid (CLA) and omega-3 fatty acids (FA), vitamins and minerals, which have positive effects on human health (Haug, Høstmark, & Harstad, 2007). Consumption of fresh milk has become more popular in some regions, due to the current tendency for "natural consumption", its fresh taste and the nutritional benefits. However, the high nutrient content and high water activity in fresh milk provide an excellent environment for the growth of detrimental microorganisms, which can be toxic and have negative impacts on human health (Claeys *et al*., 2013). Hence, heat treatment is widely used to guarantee the safety of consumers and to prolong the shelf-life of milk products. Categories of milk products subjected to different heat treatments, *i.e*. pasteurised milk (63 °C for 30 min or 72 °C for 15 s) and ultra high temperature (UHT) milk (135-140 °C for a few seconds), are commercially available (De Buyser, Dufour, Maire, & Lafarge, 2001). While the demand for UHT milk is increasing worldwide, consumption of this form of milk varies between regions, with *e.g*. lower consumption in the Nordic countries than in other European countries (Elliott, 2007). The effect of UHT processing and storage on the stability of milk proteins has been examined in a number of studies (*e.g*. Datta & Deeth, 2003; Ismail & Nielsen, 2010; Gaucher *et al*., 2011), but information on how the characteristics of fresh milk and UHT processing influence fat stability in stored UHT milk is still lacking.

1.1 Milk fat in raw milk

1.1.1 Milk fat composition

Milk fat is an important source of energy and nutrients (*e.g*. essential FA, vitamins) for consumers in daily life. Raw milk generally contains

approximately 3-5% fat in nature, of which about 98% comprises lipids, a mixture of TAG (triacylglycerols, esters of glycerol and fatty acids) (Kontkanen *et al*., 2011). The chemical structure of TAG is a glycerol backbone, to which three molecules of FA are attached (Figure 1). The patterns and distribution of these FA can greatly affect the physical properties of TAG, particularly crystallisation, and determine the predominant FA released by enzymatic hydrolysis (Walstra, Jenness, & Jennes, 1984).

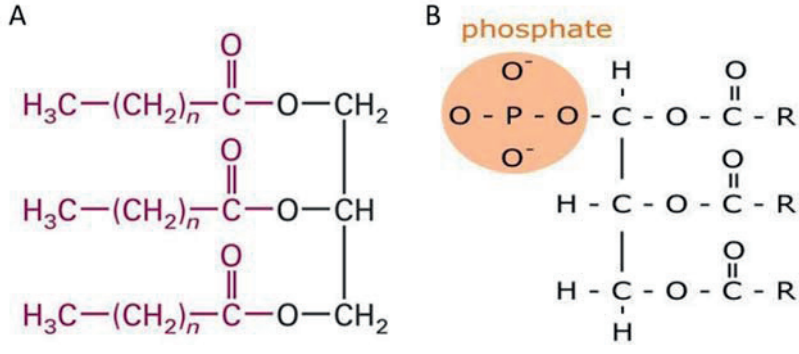

Figure 1. Chemical structure of (A) triacylglycerols (TAG) and (B) polar lipids.

Besides TAG, minor amounts of diacyglycerol, monoacyglycerol, polar lipids, free fatty acids (FFA) and sterols and trace amounts of vitamins can be found in milk lipids (Rodríguez-Alcalá & Fontecha, 2010). Polar lipids generally account for around 0.2-1% of total lipids in milk fat, possibly depending on the surface area of milk fat globule membrane (MFGM) and the size of fat globules. Lopez (2011) reported that smaller fat globules with larger total surface area can have a higher MFGM/TAG ratio than milk with larger fat globules. Glycerophospholipids and sphingolipids (mainly sphingomyelin, SM) are the typical polar lipids for biological membranes. The structure of glycerophospholipids comprises a glycerol backbone with two molecules of FA esterified in the sn-1 and sn-2 positions and a phosphate residue with different organic groups in the sn-3 position (Figure 1). Compared with the TAG core of fat globules, MFGM polar lipids contain higher amounts of unsaturated FA, especially n-6 and n-3 FA, which could lead to higher sensitivity to lipid oxidation compared with TAG (MacGibbon & Taylor, 2009; Sánchez-Juanes, Alonso, Zancada, & Hueso, 2009). A small percentage (<1% of total lipids) of FA is freely dispersed in the milk fat phase. These FFA are the result of catabolic and anabolic processes. Compared with esterified FA, the FFA are more prone to lipid autoxidation, resulting in

deterioration of milk quality (Barrefors, Granelli, Appelqvist, & Bjoerck, 2010).

1.1.2 Microstructure of fat globules

The natural lipid entities assembled and released by mammary epithelial cells have a spherical shape and are usually called milk fat globules (MFG). With the development of microscopy techniques, the structure of fat globules has been widely investigated. In general, the average diameter of these biological droplets is around 3.5-4 µm, with variations caused by factors such as species, physiology and dietary changes (Ward, German, & Corredig, 2006).

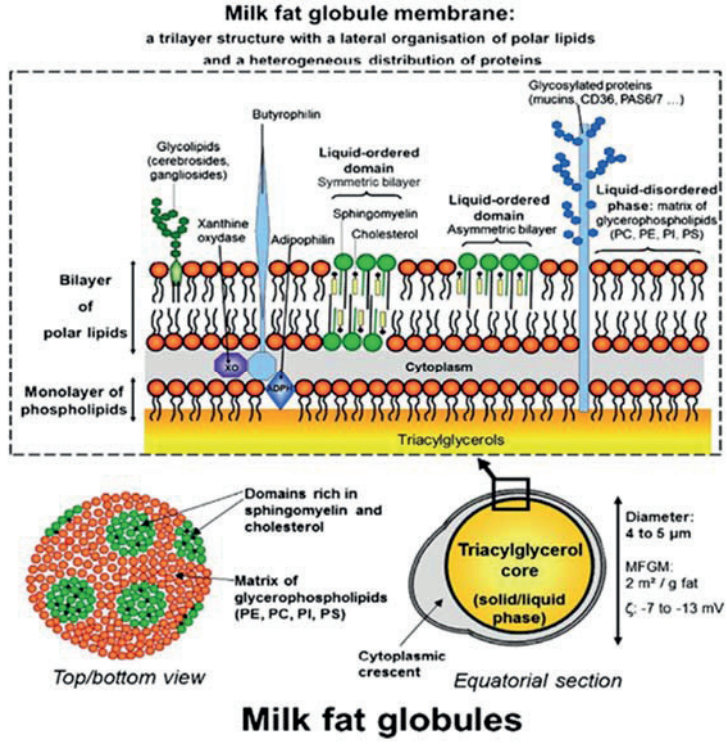

Figure 2. Structure of raw milk fat globules, with a schematic representation of the membrane. Adapted from Lopez (2011).

Milk fat globules consist of a core of TAG, covered by a tri-layer membrane structure (MFGM, thickness 10-50 µm). The inner layer consists of proteins and a monolayer of phospholipids, and originates from endoplasmic reticulum. The phospholipids located in the outer bilayer originate from the apical plasma

membrane. The MFGM is subjected to interaction with fat globules and proteins. The interfacial area and surface composition may influence its stability and activity (Jukkola & Rojas, 2017), particularly in preventing flocculation and coalescence of fat globules and enzymatic activity (Singh & Gallier, 2017). According to the new structure model of MFGM (Figure 2), two lipid phases coexist in the membrane: (1) A fluid matrix made up of glycerophospholipids and proteins; and (2) micro-domains rich in SM, originating from the lipid raft of the apical plasma membrane of the lactating cell, and cholesterol (Lopez, 2011). Cytoplasm can sometimes be entrained between the core of fat globules and membranes, leading to different sizes of cytoplasmic crescents (Evers *et al*., 2008), which may vary with species and individual.

1.1.3 Factors influencing lipid composition

With the increasing demand for bovine milk due to increasing consumption, the chemical properties of milk components that could affect quality have attracted great research interest. In particular, lipid changes depending on intrinsic and extrinsic factors, *e.g*. animal breed, lactation stage, feeding regime and season, have been studied (Månsson, 2008). Within dairy cow breeds (*e.g*. Holstein-Friesian, Jersey, Montbeliarde), there are significant differences in FA associated with breed or genotype (Stoop, van Arendonk, Heck, van Valenberg, & Bovenhuis, 2007; Soyeurt *et al*., 2010; Liu, Zhang, Kang, Meng, & Ao, 2016). Lactation stage, especially early stage, is reported to have a pronounced effect on many FA (Stoop, Bovenhuis, Heck, & van Arendonk, 2009). According to Craninx *et al*. (2008), odd- and branched-chain FA with 14 and 15 carbon atoms increase in milk from early lactation, while FA with 17 carbon atoms decrease. Different lipid classes are also reported to be affected by lactation stage, with *e.g*. TAG remaining constant and phospholipids declining with advancing lactation (Bitman & Wood, 2010).

Significant effects of feeding patterns and season on milk FA composition have also been reported (Kliem, Shingfield, Livingstone, & Givens, 2013; Larsen, Andersen, Kaufmann, & Wiking, 2014; Liu *et al*., 2016). Conventionally, there are two main feeding regimes, outdoor and indoor feeding, for dairy cows, in summer and winter, respectively. However, the outdoor feeding period differs between the Nordic countries and other European countries, due to *e.g*. the shorter summer (May to July in Sweden) and longer winter in the Nordic countries (Thorsdottir, Hill, & Ramel, 2004). In general, grass silage, maize silage and concentrates are fed to cows in winter, while cows are pastured on fresh grass in the grazing season (Heck, van Valenberg, Dijkstra, & van Hooijdonk, 2009). This difference in feeding regime results in a lower

amount of saturated FA (SFA) and higher amount of monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) in summer milk compared with winter milk, even when other management conditions are similar (Collomb *et al*., 2008; Ferlay, Martin, Pradel, Coulon, & Chilliard, 2010; Revello *et al*., 2010). The greater supply of unsaturated fatty acids (UFA) (in particular C18:2(n-6), C18:3(n-3), in the pasture-based diet is suggested to cause incomplete UFA bio-hydrogenation, which leads to high concentrations of UFA in milk (Leiber, Kreuzer, Nigg, Wettstein, & Scheeder, 2005). A higher content of CLA has also been observed when cows graze pasture in summer, which may raise the nutritive and therapeutic value of the milk due to the anticarcinogenic and antioxidative functions of CLA (Chen, Chan, Kwan, & Zhang, 1997; Gill & Cross, 2000; Cichosz, Czeczot, Ambroziak, & Bielecka Marika, 2017).

1.2 Characteristics of UHT milk

Nowadays, UHT milk is widely produced, consumed and accepted by producers and consumers, owing to the advantages it provides of safe and long shelf-life under ambient distribution and storage conditions (Rysstad & Kolstad, 2006).

1.2.1 Development and market status of UHT milk

Raw milk is a good medium for microbial growth, as it contains high amounts of nutrients. Therefore, a heat treatment or sterilisation is necessary to kill microorganisms and inactivate some enzymes, in order to ensure a substantial shelf-life (Pak, 2016). The first heat treatment systems, namely indirect heating with continuous flow (125 °C, 6 min) and direct heating with steam (130-140 °C) were established in 1893 and 1912, respectively. Development of the UHT process was hindered at that time by lack of a commercial aseptic system to prevent possible re-contamination after processing. The main development of UHT milk production started around 1960, when aseptic processing and aseptic filling of cartons with sterilised milk became possible. Between 1960 and 1980, work concentrated on using paper-foil-plastic laminated packages instead of metal cans. The real turning point was in January 1981, when the Brik Pak filler was approved by the Food and Drug Administration (FDA) of the United States and used commercially for the first time (Chavan, Chavan, Khedkar, & Jana, 2011; David *et al*., 2012). After the invention of aseptic packaging of milk by Tetra Pak, storage and distribution at ambient temperatures became a reality worldwide. This was an especially important development for tropical countries with high ambient temperatures and limited refrigerated distribution (Douglas Goff, 2008). According to an industry review of global aseptic packaging, global

consumption of UHT milk then increased dramatically in terms of packs and volume, but with variations between countries (Chavan *et al*., 2011).

1.2.2 UHT processing

Ultra high temperature treatment is a technique that involves exposing a milk product to intensely high temperature (within the range 130-140°C) for a few seconds (normally 4 s) to kill detrimental microorganisms and inactivate endogenous enzymes (Jelen, 2013).

In general, UHT treatment takes place in a closed system where the milk product quickly passes through the heating and cooling stages. This airtight continuous process can efficiently lower the risk of contamination of the milk by airborne microorganisms. Two alternative strategies for UHT treatment are available commercially: (1) Direct heating by steam injection or milk infusion to steam, with cooling by expansion under vacuum; and (2) indirect heating and cooling in a heat exchanger (Pak, 2016). Direct heating is based on the product being in contact with the heating medium, while indirect heating is based on heat transfer from media to product through partition. The main advantage of direct heating is the fast heating rate from preheating to high heat (<0.5 s), which can minimise the level of chemical changes and development of a cooked flavour. However, the energy and water requirements and associated costs are high and thus a combination of direct and indirect heating is used in commercial UHT milk production.

Due to the different heating principles in the two systems, time-temperature characteristics with higher heat load for equivalent bactericidal effectiveness have to be applied in the indirect heating system (Malmgren, 2007). Moreover, the higher oxygen level may result in a high level of stale or oxidised flavour in milk sterilised by indirect heating (Lewis & Deeth, 2008). These two different types of UHT heating can both lead to some degree of adverse chemical changes, *e.g*. fat separation, protein gelation and sedimentation and formation of off-flavour compounds, which could reduce milk quality and limit its shelf-life during storage (Datta, Perkins, & Deeth, 2002; Tran Le, Saveyn, Hoa, & Van der Meeren, 2008; Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015).

1.2.3 Commercial workflow for UHT milk

The commercial production of UHT milk from raw milk generally includes the following process steps: fat standardisation, pre-heating (pasteurisation), upstream or downstream homogenisation, UHT treatment, cooling and aseptic packaging (Figure 3).

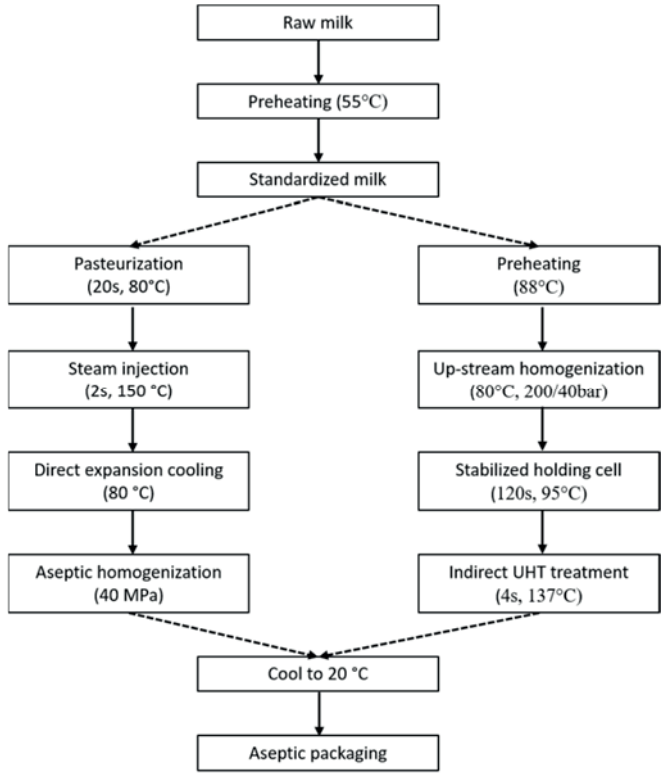

Figure 3. Typical steps involved in commercial production of UHT milk.

Fat standardisation is performed with a separator to separate the cream and skim milk, following which appropriate amounts of cream and skim milk are mixed to reach a standardised target fat content in the milk (Jelen, 2011). The preheating and final cooling stages are generally performed using an indirect heat exchanger, while the UHT treatment can involve either a direct or indirect system followed by different cooling methods, as discussed above. Plate or tubular heat exchangers are usually applied in the indirect heating system. Plate exchangers may have some disadvantages compared with tubular exchangers, such as being more susceptible to burn-on or fouling and a high risk of leaking and contamination (Datta *et al*., 2002).

Homogenisation is a standard commercial process for disrupting existing fat globules into smaller globules, in order to minimise the tendency for coalescence. The disruption of fat globules is achieved by physical forces, *i.e*. turbulence and cavitation, to break large globules into globules approximately 1 μm in diameter, accompanied by a dramatic increase in total surface area.

Single-stage and two-stage homogenisation can be applied as the homogenisation process. The latter gives optimal homogenisation and is primarily used with a high fat content or products with desired low viscosity (Pak, 2016).

1.3 Instability of fat in UHT milk during storage

1.3.1 Structural changes in fat globules

Native fat globules are present as spherical shapes with diameters ranging from 0.1 to 10 µm, covered with an intact tri-layer MFGM structure. However, harsh treatment (*e.g*. pumping, agitation and homogenisation) during UHT processing can alter the natural structure of fat droplets and membranes. The smaller fat globules created by homogenisation may not be of uniform diameter, but the size range (0.1-3.0 µm) is narrower than in raw milk (Malmgren, 2007). The decrease in fat globule size and the concurrent increase in total surface area in UHT milk leads to the adsorption of proteins attaching to the newly formed surface (Michalski, Briard, & Michel, 2001). Three main types of particles are found in homogenised milk (Figure 4).

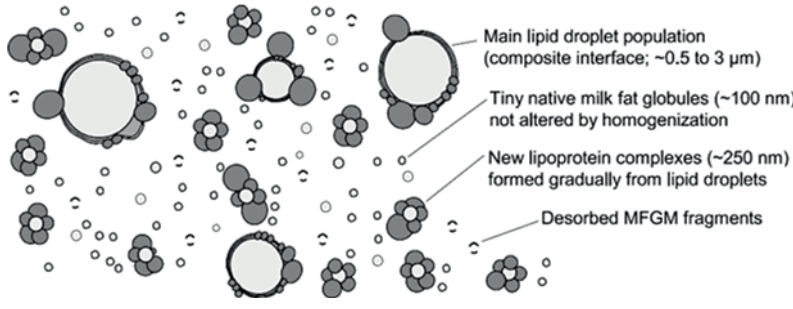

Figure 4. Proposed general organisation of lipid particles in homogenised milk. Adapted from Michalski & Januel (2006).

These are: (1) homogenised fat globules disrupted from raw milk, partly covered by proteins and native MFGM as described above; (2) tiny native fat globules, which may not be affected by homogenisation; and (3) small lipid-protein complexes with new membranes, mainly covered with caseins (Michalski, Michel, & Geneste, 2002). Heat treatment alone has no influence on fat globule size. However, when it is combined with homogenisation, regardless of order,

fat droplets tend to be smaller, leading to a larger surface area. This can promote interactions between native constituents of MFGM and proteins, leading to changes in MFGM (Sharma & Dalgleish, 1993; Kim & Jimenez-Flores, 2010). These changes induced by processing steps could alter the composition of the interface and thereby cause some chemical reactions, such as lipolysis of TAG and PL, affecting the stability of fat globules and intrinsic quality of UHT milk during storage (Lopez, 2005; Michalski & Januel, 2006).

1.3.2 Lipid lipolysis and oxidation

Lipid lipolysis and oxidation are associated with some main reactions in the development of off-flavour in milk, which can influence consumer acceptance and reduce shelf-life and quality of milk during storage (Santos, Ma, Caplan, & Barbano, 2010). In stored UHT milk, the total surface area of fat globules is high due to the homogenisation step. Coalescence of fat takes place as the casein is prone to sedimentation and becomes limiting, leading to insufficient coverage on fat globules. These changes caused by processing can increase the susceptibility to lipolysis and oxidation during storage (Serra, Trujillo, Pereda, Guamis, & Ferragut, 2008).

Lipolysis can be mediated by either lipoprotein lipase (LPL), which accounts for most lipolytic activities, or by microbial activities initiated by psychrotrophic bacteria such as Pseudomonas, which catalyse the release of FFA hydrolysed from TAG and some PL in bovine milk (Serra *et al*., 2008). In general, lipolysis in milk can be characterised as spontaneous or induced. The molecules susceptible to spontaneous lipolysis in milk are strongly affected by lipase activity level, the integrity of MFGM and the balance of lipolysis-activating and lipolysis-inhibiting factors. Physical actions such as agitation, pumping, homogenisation and activation by temperature changes during processing, transport and storage can initiate induced lipolysis by disrupting the microstructure of membranes, leading to exposure of the lipid substrate to lipase in milk (Park, 2010). Both spontaneous and induced lipolysis can occur when milk is stored under refrigeration (Deeth, 2006). The oxidative changes are the major cause of quality deterioration in milk, resulting in undesired flavour and potential toxic components unsuitable for human consumption. The general process of lipid oxidation is a series of complex chemical reactions between lipids and intermediates of oxygen-active species in oil-in-water emulsions (McClements & Decker, 2000). The initiators of oxidation reactions include light, heat, metals, enzymes and microorganisms, catalysing complex progresses of autoxidation, photo-oxidation and thermal or enzymatic oxidation. In the presence of these initiators, UFA are the major reactants targeted to produce one

hydrogen atom and free lipid radicals, which are prone to form peroxyl radicals by further reacting with oxygen. The peroxyl radicals act as the chain carrier to accelerate the oxidative progress by continuously attacking new lipid molecules until all available oxygen is used or the chain reaction is disrupted by some antioxidants during the propagation stage (Shahidi & Zhong, 2010). Lipid hydroperoxides are the primary products during the propagation process, but they are unstable and easily break down to wide-ranging secondary oxidation products, such as aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, epoxy compounds *etc*. The most common method for evaluating oxidation is to estimate the amount of aldehyde products present, as they can react with thiobarbituric acid (TBA) to produce thiobarbituric acid reactive substances (TBARS), which can be measured by spectrophotometry. This method has the advantages of sensitive, simple operation and low cost, and the disadvantage of non-specific TBA possibly reacting with other compounds during analysis (Devasagayam, Boloor, & Ramasarma, 2003).

2 Objectives

The overall aim of this thesis was to study the lipid properties, including the microstructure, of UHT milk as affected by seasonal variations in corresponding raw milk and UHT processing and storage conditions.

Specific objectives were to:

- Investigate seasonal variations in lipid class and FA composition in raw milk and the corresponding UHT milk (Paper I).

- Explore the effects of UHT processing on the composition of the final commercial UHT milk product, compared with that of the raw milk, by analysing the microstructure of fat globules and lipids (Paper I).

- Determine the impact of sampling month, storage time and temperature on lipid lipolysis and oxidation in stored UHT milk (Paper II)

- Evaluate the effects of key steps in UHT processing, in a pilot plant, on the microstructural organisation of fat globules and FA composition, using confocal microscopy, transmission electron microscopy and gas chromatography (Paper III).

3 Materials and methods

A brief description of the materials and methods used in the work presented in this thesis is provided in this chapter. An outline of the entire work and the major methodology involved is presented in Table 1. More details of each method can be found in the respective papers.

Table 1. *Summary of study plan in Papers I-III*

	Paper I	Paper II	Paper III
Milk sample	Raw milk	UHT milk	Pasteurised milk
	UHT milk		Homogenised milk
			Stabilised milk
			UHT milk
Treatment	Sampling month	Sampling month	Processing
	Processing	Storage time	
		Storage temperature	
Statistics	General linear model	General linear model	Tukey's post-hoc test
Analysis	CLSM[a] analysis	CLSM analysis	CLSM analysis
	Lipid class	Lipid class	TEM[b] analysis
	Fatty acids profile	Fatty acids profile	Fatty acids profile
		TBARS[c] analysis	

[a]Confocal laser scanning microscopy. [b]Transmission electron microscopy. [c]Thiobarbituric acid reactive substances.

3.1 Milk collection (Papers I-III)

3.1.1 Raw milk sampling and commercial UHT process (Paper I)

Pooled (80 farms) raw milk samples were collected at the Norrmejerier dairy plant in Luleå, northern Sweden, in March, May, June, July, September and

November 2015 and transported to the Swedish University of Agricultural Sciences (SLU, Uppsala) at chill temperature (4 °C) for further analysis within three days.

The corresponding UHT milk was processed as follows (Figure 5): preheating of unprocessed raw milk at 55 °C; standardisation to a fat content of 1.5% in UHT milk by re-blending some cream (about 40% fat) into the skimmed milk; UHT treatment (indirect tubular mode at 137 °C for 4 seconds); homogenisation at a pressure of (150 + 30) bar; and cooling to 20°C. The final UHT milk was packed in Tetra Brik Aseptic cartons (1 L) and transported to the laboratory at ambient temperature. All samples were analysed in triplicate.

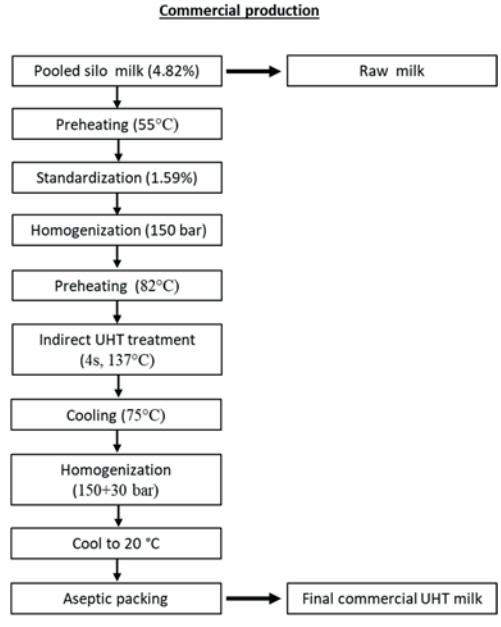

Figure 5. Scheme of commercial UHT production.

3.1.2 Storage conditions of commercial UHT milk (Paper II)

Cartons of processed UHT milk made from unprocessed raw milk collected in May, July and November 2015 (same UHT milk samples as described in Paper I) were stored in the upright position in the dark in temperature-controlled rooms at 4 °C, 20 °C, 30 °C and 37 °C. At fixed times (3, 6, 9 and 12 months) for each sampling month, the fat layer in a newly opened Tetra Brik Aseptic package was directly separated off and subsamples were used for further analysis.

3.1.3 UHT process in pilot plant (Paper III)

Whole milk from Skånemejerier was pasteurised and homogenised (test sample 1, TS1), preheated at 88 °C and upstream homogenised at 80 °C and 200/40 bar (test sample 2, TS2). The milk was then heated to 95 °C and stabilised in a holding cell at 95 °C for 120 s (test sample 3, TS3). Finally, the milk was heated to 143 °C for 4 s in the indirect tubular mode of Unit Pilot 300 L/h size, cooled to 20 °C and packed aseptically in 250 mL Tetra Brik Aseptic cartons (test sample 4, TS4). A flowchart of the processing unit is shown in Figure 6. All test milk samples were transported to the Swedish University of Agricultural Sciences (SLU) at 4 °C and analysed within three days of production.

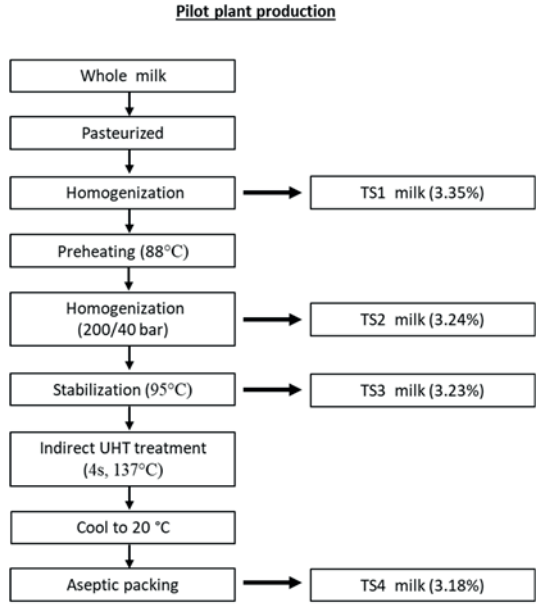

Figure 6. Scheme of UHT process in pilot plant.

3.2 Confocal laser scanning microscopy analysis (Paper I-III)

3.2.1 Sample preparation

Neutral lipids were stained with the lipid-soluble fluorescent dye Nile Red (5H-benzo aphenoxazine-5-one, 9-diethylamino; Sigma-Aldrich, St Louis, USA) by mixing 0.5 mL of milk with 5 µL of Nile Red stock solution (0.1% Nile Red in acetone) for 15 min at room temperature. The protein network (Paper III) was

stained by adding 10 µL of Fast Green (Sigma-Aldrich, St Louis, USA) to the neutral lipid-stained milk samples. Rh-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), Avanti Polar Lipids, USA) was used to reveal phospholipids distributed on the surface of the MFGM (Papers I-III). Rh-DOPE solution (8 µL, 1 mg/mL in chloroform) was added to 2 mL milk samples in Eppendorf tubes for 30 min. Low melting point agarose at 10 g/L (100 µL, Sigma, St Quentin Fallavier, France) was prepared and mixed with 100 µL stained samples in an Eppendorf tube, and 20 µL of the mixture were placed on a concave microscope slide for analysis. During all staining procedures, the Eppendorf tubes were wrapped in aluminium foil to minimise loss of fluorescence signal.

3.2.2 CLSM analysis

A Zeiss LSM 780 confocal laser scanning microscope (Jena, Germany), comprising an inverted Zeiss Axio Observer and supersensitive GaASp detector, was used for microstructural analysis. A 488 nm argon laser and 633 nm HeNe laser were used as excitation sources and the fluorescence emissions of wavelengths between 500-530 nm and 655-755 nm were detected for Nile Red (Papers I-III) and Fast Green (Paper III), respectively. The HeNe laser was operated at 543 nm excitation wavelength and emission wavelengths from 565 nm to 615 nm were used to detect Rh-DOPE (Paper I-III). Images were acquired using a C-Apochromat 63x oil immersion objective (NA 1.32) with resolution of 1024x1024 pixels.

3.3 Transmission electronic microscopy analysis (Paper III)

3.3.1 Sample preparation

Samples of milk (1 mL) were mixed with 50 µL formaldehyde (2.5%) in Eppendorf tubes and left for one hour to fix the protein network. Agar solution (40 g/L, Agar Noble Difco Laboratories, Detroit Michigan, USA) was prepared by heating to 85 °C under continuous stirring and cooling to 50 °C before being added to the samples. The mixture was shaken to allow homogenisation and transferred to a plastic plate. The plate was placed in the refrigerator for four hours to allow hardening of the samples. Triplicate 1-mm^3 pieces were taken from each plate, placed in plastic baskets and introduced into a Leica FM tissue

processor for plastic embedding. The samples were dehydrated in grade ethanol series (30, 50, 70, 95 and 99.5 %), followed by embedding in LR White resin. The samples were embedded with fresh resin in small plastic moulds and the blocks were left to harden for 24 hours at 60 °C in an oven. Ultra-thin sections (~70-80 nm) were cut with a diamond knife under a Leica ultra microtome (Reichert-Jung Ultracut E, Leica 2 Microsystems, Switzerland) and collected on 400-mesh copper grids. The sections were double-stained with uranyl acetate and lead citrate (Langton & Hermansson, 1992).

3.3.2 TEM analysis

Samples were examined by transmission electron microscopy (TEM) using a Philips CM12 TEM device (Philips, Eindhoven, Netherlands) at 80 or 100 kV. Negative TEM films were scanned using an Epson Perfection Pro 750 film scanner.

3.4 Lipid class composition analysis (Papers I-II)

Lipid class composition analysis was performed according to the method of Mraz and Pickova (2009) with minor modification, using high performance thin layer chromatography (HPTLC). Samples with a concentration of 4 µg/µL extracted lipids were applied on pre-coated HPTLC silica gel 60 plates (20 cm x 10 cm, 0.20 mm layer; Merck, Germany) using a Camag ATS4 automatic TLC sampler (Kovalent AB, Västra Frölunda, Sweden). Lipid classes were separated by a Camag ADC2 automatic developing chamber (Kovalent AB, Västra Frölunda, Sweden) with mobile phase hexane:diethyl ether:acetic acid (85:15:2, v/v/v). Phosphomolybdic acid in ethanol was used for derivatisation and a Camag TLC scanner 3 (Kovalent AB, Västra Frölunda, Sweden) was used to scan the dried plates at 650 nm. Peak identification for lipid class was conducted by comparing with an external standard (TLC 18-5A; Nu-check prep Inc. MN, USA) and expressed as the percentage of total area. Each sample was displayed on three plates to minimise the variation.

3.5 Fatty acid analysis (Papers I-III)

Lipid extraction (Papers I-III) from milk samples was based on the methods of Hara & Radin (1978), using a mixture of hexane and isopropanol (3:2, v/v). Total lipid content was extracted, dried under a stream of nitrogen and weighed. Fatty acid methyl esters (FAME) were obtained from methylation according to Appelqvist (1968) and analysed by gas chromatography using a CP 3800

instrument (Varian AB, Stockholm, Sweden), fitted with a flame-ionisation detector (GC-FID). Chromatographic separation was carried out using a fused silica capillary column BPX 70 (50 m x 0.25 mm inner diameter, 0.25 µm film thickness, SGE, Austin, TX, USA). Peak identification was performed by comparing retention time with that of available FA methyl ester standard mixture GLC-461 (Nu-chek Prep, Elysian, MN, USA). Peak areas were integrated using Galaxie chromatography software (Version 1.9, Varian AB, Stockholm, Sweden). Total fatty acids were calculated as percentage of the sum area of total identified FA (g/100g FAME in Papers I-II) and quantified as µg FA/mL milk (Paper III). Phospholipids (PL), free fatty acids (FFA) and triacylglycerol (TAG) were separated by thin-layer chromatography (TLC) (Pickova *et al*., 1997) and methylated using the same methods as applied for total FA (Appelqvist, 1968). Individual FA concentrations from PL, FFA and TAG (Paper III) were expressed as g/100g FAME (Paper II) and quantified gravimetrically on microbalance (Mettler Toledo, UMT 2, Switzerland) as µg FA/mL milk using a known amount of internal standard methyl-15-methylheptadecanoate (Larodan Fine Chemicals AB, Malmö, Sweden). All samples were analysed in triplicate.

3.6 Thiobarbituric acid reactive substances analysis (Paper II)

Analysis of TBARS was performed in triplicate as described by Ulu (2004), with minor modifications. Milk samples (1 mL) were mixed with 0.5 mL 0.2% butylated hydroxytoluene (Sigma-Aldrich, USA) and 5.0 mL of 10% trichloroacetic acid (Sigma-Aldrich, USA) in 0.2M H_3PO_4 (Merck, Germany), vortexed and centrifuged at 4000 x g for 5 minutes. The supernatant (1 mL) was transferred to a test-tube with 1 mL 0.02 M 2-thiobarbituric acid (TBA; Sigma, USA). The solution was heated at 85 °C for 45 min in a thermostatic bath and then cooled to room temperature. The absorbance at a wavelength of 532 nm was recorded against a blank containing 1.0 mL deionised water (Millipore Synergy 185, Germany) and 1.0 mL of TBA solution, using a spectrophotometer. Malondialdehyde (MDA) concentration was calculated based on a standard curve prepared using 1,1,3,3-tetramethxypropane within a range from 0 to 2.5 µM and TBARS values were expressed as µg of MDA equivalents per mL of milk.

3.7 Statistical analysis

The General Linear Model (GLM) statement in SAS (SAS 9.3, SAS Institute, Cary, NC, USA) was used to investigate the effects of season and processing on

lipid profiles including lipid classes, FA composition (Papers I and II) and TBARS (Paper II). Before GLM modelling analysis, non-normally distributed data, assessed by the Anderson-Darling test, were log-transformed and re-tested (Paper I). The difference between each value and baseline within sampling months was calculated, to eliminate the effect of fresh UHT milk on measured parameters (Paper II). False discovery rate (FDR, q value) was used to offset the problems of multiple comparisons, with $q<0.5$ set as significant. Least squares means (LSM) obtained from fitted models are presented in Papers I-II. Means and standard deviations for triplicate milk samples affected by different processing steps in Paper III were compared by Tukey's post-hoc test using Minitab software (Minitab Inc., State College, PA, USA). Tukey's post-hoc test was used for pair-wise comparisons when a significant effect of interest ($P<0.05$) was observed (Papers I-III). Spearman correlation analysis was used to calculate correlation coefficients between investigated variables with significance level $P<0.05$ (Papers II and III).

For multivariate statistics, principal component analysis (PCA) models in SIMCA-P software 13.0 (Umetrics, Umeå, Sweden) were applied to get an overview of data and screen distinct groupings for lipid profiles (Papers I-II). Partial least squares discriminant analysis (PLS-DA) was used to further investigate the effects of season and processing on lipid profile, while partial least squares regression (PLS) was used to evaluate the relationship between FA composition (X variable) and lipid classes (Y variable). The validity of PLS or PLS-DA, shown as R^2X and R^2Y, indicated the level of X and Y variations explained by the model and Q2 values showed the goodness of prediction in the model (Hoydal *et al*., 2016). Model significance was examined by seven-fold cross-validation ($P<0.05$). Auto-scaling was performed for all variables prior to the multivariate analyses (Paper I).

4 Results and discussion

In this chapter, important results are briefly presented and discussed, divided into seasonal effects, UHT processing effects and storage condition effects on milk lipids. More detailed information and comprehensive results can be found in Papers I-III.

4.1 Seasonal effects on milk lipids

In Papers I and II, the effects of sampling month on lipid profiles, including lipid class and FA composition, were investigated in both fresh milk and stored UHT milk.

4.1.1 Seasonal variations in lipids in fresh milk (Paper I)

There were obvious groupings of milk samples in summer months (June and July) in the score plots of PLS-DA (Figure 7). These differed from the groupings in other months, which indicates that seasonal variations may have an important impact on milk lipid profiles. Higher proportions of phospholipids and some FA (C18:0, C18:1 t11, C18:1 c9, C18:2(n-6), C18:3(n-3) and ∑CLA) were found in milk samples from June and July than in other milks (Table 2).

The significant seasonal variations in lipids (lower percentage of *de novo*-synthesised FA and higher percentage of blood-derived FA in summer milk) are in line with previous findings (Chilliard, Ferlay, & Doreau, 2001; Schroeder *et al*., 2003; Lock & Bauman, 2004; Buccioni *et al*., 2015). These seasonal patterns can be attributed to three possible causes: (1) dietary FA supplement; (2) metabolism by rumen microorganisms; and (3) desaturase enzyme activities (Heck *et al*., 2009). Based on the diet composition in 2015, lactating cows in this thesis were kept on grazing in summer months (June and July) and ate fresh

grass, which is different from the conserved forage and silage fed in other

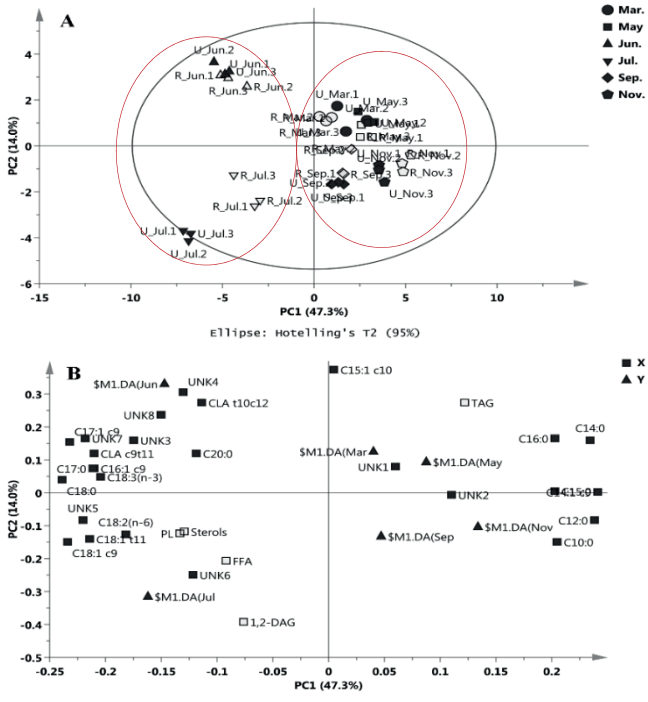

Figure 7. Results of partial least squares-discriminant analysis (PLS-DA) based on seasonal variations in lipid profile in all milk samples. In the PLS-DA model, all fatty acids (FA) and lipid classes are X variables and different months are Y (R2X 0.867, R2Y 0.891 and Q2 0.825, two components). (A) Score plot of all observations. Different form indicates samples collected from different month, with raw milk (R) and UHT milk (U) in the same month shown as white and black. (B) Loading plot describing the scattering of five lipid classes and 26 fatty acids.

months. Generally, the diet of fresh grass in summer provides high levels of C18:2(n-6) and C18:3(n-3), which can result in increased levels of C18:0, C18:1c9, trans-C18:1 and CLA through bio-hydrogenation and desaturation reactions catalysed by microbial activities and desaturase enzymes (Figure 8). High concentrations of plasma lipids (C18 FA) from the pasture diet, caused by a bacterial population shift related to incomplete bio-hydrogenation of PUFA, can also reduce *de novo* synthesis of C10:0-C14:0 and some C16:0 in the mammary glands (Chilliard *et al*., 2001; Schroeder *et al*., 2003). On the other hand, conserved forage and silage may suffer problems with extensive lipolysis and oxidation, which may lead to large losses of some UFA during the process of wilting and ensiling of grass for indoor feeding (Kliem et al., 2013).

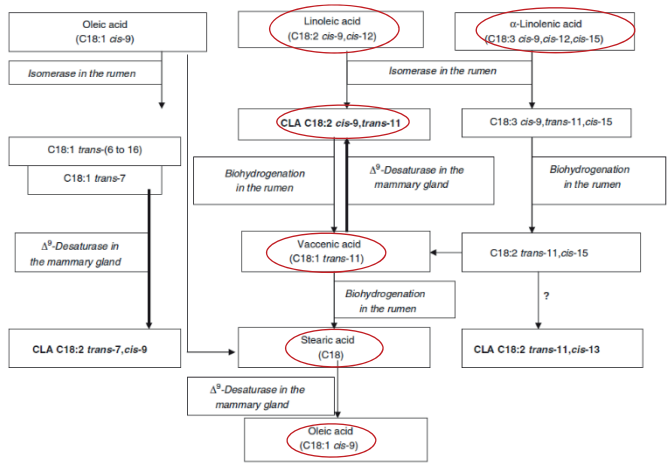

Figure 8. Known metabolic pathways for the formation of conjugated linoleic acid (CLA) isomers. Adapted from Collomb, Schmid, Sieber, Wechsler, & Ryha (2006).

Table 2. *Seasonal variations in lipid class and fatty acid composition in selected months, shown as least square means (Tukey test for group comparisons, P≤0.05)*

	March	May	June	July	September	November
Lipid class:						
Phospholipids	3.34^{c}	3.30^{c}	3.74^{b}	4.06^{a}	3.41^{c}	3.55^{c}
Free fatty acids	3.60^{ab}	2.63^{c}	3.21^{b}	3.85^{a}	3.59^{ab}	3.22^{b}
Triacylglycerols	80.3^{c}	81.6^{d}	79.0^{b}	76.9^{a}	78.0^{b}	78.8^{b}
FA:						
C14:0	12.4^{c}	12.8^{d}	12.0^{b}	11.2^{a}	12.4^{c}	12.9^{d}
C14:1 c9	0.98^{c}	1.03^{d}	0.91^{b}	0.84^{a}	1.06^{e}	1.16^{f}
C16:0	36.9^{d}	35.8^{c}	34.4^{b}	33.4^{a}	34.7^{b}	36.5^{d}
C16:1 c9	1.79^{b}	1.75^{b}	1.87^{a}	1.90^{a}	1.64^{c}	1.66^{c}
C18:0	12.6^{c}	12.1^{bc}	13.1^{b}	13.4^{a}	12.1^{d}	11.0^{e}
C18:1 t11	1.54^{cd}	1.51^{d}	1.85^{b}	2.07^{a}	1.59^{c}	1.57^{cd}
C18:1 c9	22.7^{d}	22.5^{d}	24.7^{b}	25.7^{a}	24.3^{c}	22.3^{d}
C18:2(n-6)	1.57^{c}	1.59^{c}	1.88^{b}	2.19^{a}	1.57^{c}	1.59^{c}
C18:3(n-3)	0.53^{c}	0.49^{d}	0.56^{b}	0.60^{a}	0.43^{e}	0.38^{f}
∑SFA	66.8^{c}	67.0^{c}	63.4^{b}	62.2^{a}	65.2^{d}	67.2^{c}
∑MUFA	27.4^{d}	27.2^{d}	29.8^{b}	30.9^{a}	29.0^{c}	27.1^{d}
∑PUFA	2.68^{c}	2.65^{c}	3.18^{b}	3.43^{a}	2.59^{c}	2.53^{c}
∑CLA	0.58^{c}	0.56^{c}	0.73^{b}	0.63^{a}	0.59^{c}	0.56^{c}
∑Identified FA	96.9^{d}	96.8^{cd}	96.4^{a}	96.6^{b}	96.8^{cd}	96.7^{c}

$^{a–f}$Means within rows with different letters are significantly different. Standard error of the mean (S.E.M) for n = 36 measurements.

4.1.2 Seasonal variations in lipids in stored UHT milk (Paper II)

The PCA plots showed that the changes in some lipid classes and FA composition in UHT milk samples stored for one year depended on sampling month, and that milk sampled in July differed from that sampled in May and November (Figure 9).

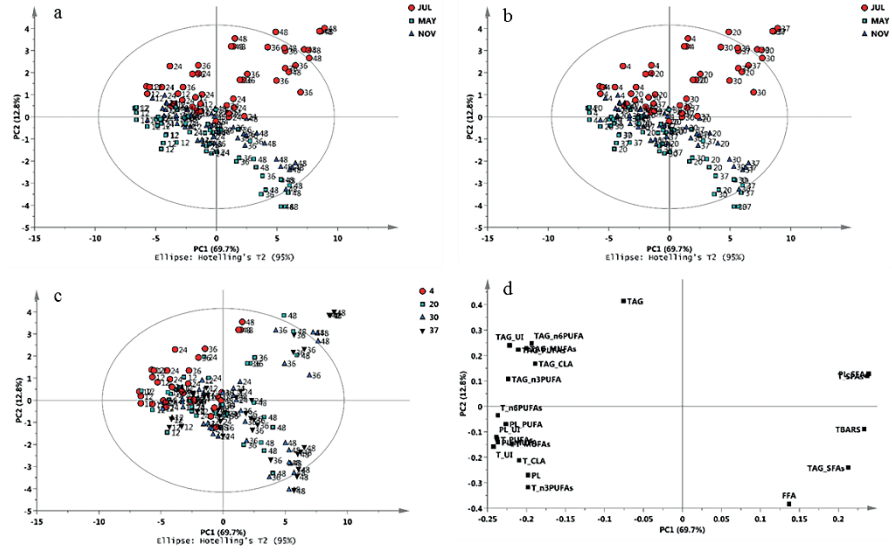

Figure 9. Results of principal component analysis (PCA). The first principal component (PC1) describes 69.7% of the total variation and PC2 explains 12.8. Explained variation (R2X) = 0.987 and predicted variation (Q2) = 0.884 for the two components, indicating acceptable model fit. (a) Score plot for different storage periods (3, 6, 9 and 12 months) and (b) score plot for different storage temperatures (4 °C, 20 °C, 30 °C, 37 °C), both coloured by sampling month (May = turquoise, July = red, November = blue. (c) Score plot for storage time, coloured by storage temperature. Loading plot describes the scattering of variables consisting of three lipid classes, thiobarbituric acid reactive substances (TBARS), total lipids (*e.g.* T_FA) and special fatty acids (FA) in phospholipids (*e.g.* p_FA) and TAG (*e.g.* t_FA).

In general, the percentage of TAG and PL decreased significantly during storage, while the percentage of FFA increased (Table 3). In particular, stored UHT milk from July showed the smallest increase in FFA, which mainly originated from the greater decrease in PL proportion. The greater increases in FFA in UHT milk from May and November were mainly the result of greater decreases in TAG proportions. During storage, the greatest decreases in ∑MUFA and ∑PUFA (-5.27% and -1.15%) were found in PL composition, while non-significant and minor differences in ∑MUFA and ∑PUFA, respectively, were found in TAG in UHT milk sampled in July. The differences in unsaturation index (UI) reflected changes in double bonds that were related to lipid oxidation during storage. The

greatest decrease in UI (-7.57%) was found for PL composition in UHT milk sampled in July, whereas the changes in UI for TAG composition were not significantly different from that in milk sampled in May and November (Table 3).

Table 3. *Variations between milk sampling months, shown as least square means of differences in lipid class (triacylglycerols (TAG), phospholipids (PL), free fatty acids (FFA)) and fatty acid composition (% of total lipids) in UHT milk stored for one year at all storage temperatures*

	May	July	November
Lipid class:			
TAG	-3.86[c]	-0.49[a]	-1.33[b]
PL	-0.97[a]	-1.58[c]	-1.09[b]
FFA	2.32[a]	1.33[c]	2.09[b]
Fatty acids:			
[d]TAG:			
∑SFA	1.75[a]	1.67[a]	1.68[a]
∑MUFA	-1.31[a]	-1.22[a]	-1.15[a]
∑PUFA	-0.45[a]	-0.45[a]	-0.53[b]
∑CLA	-0.08[a]	-0.09[b]	-0.10[c]
[e]UI	-2.36a	-2.30[a]	-2.37[a]
[f]PL:			
∑SFA	4.31[b]	6.42[a]	3.27[c]
∑MUFA	-3.32[b]	-5.27[a]	-2.62[c]
∑PUFA	-0.99[b]	-1.15[a]	-0.65[c]
[f] UI	-5.30[b]	-7.57[a]	-3.92[c]

All data transformed and shown as the difference between fresh sample and treatment. [a–c]Means for the same parameter with different letters are significantly different ($P<0.05$). [d]TAG: ∑SFA: C10:0, C12:0, C14:0 C15:0, C16:0, C17:0, C18:0 and C20:0; ∑MUFA: C14:1, C15:1, C16:1, C17:1, C18:1 t11 and C18:1 c9; ∑PUFA: C18:2n-6, C18:3n-3, CLA c9t11, CLA t10c12, C20:3n-6 and C20:3n-3. [e]PL: ∑SFA: C10:0, C12:0, C14:0 C15:0, C16:0, C17:0 and C18:0; ∑MUFA: C14:1, C16:1, C18:1 t11 and C18:1 c9; ∑PUFA: C18:2n-6. [f]UI (unsaturation index) = sum of percentage of each unsaturated fatty acid multiplied by number of double bonds, divided by percentage of total fatty acids in each sample.

The higher amount of FFA released from both PL and TAG in stored UHT milk was expected, due to the spontaneous and/or induced lipolysis initiated by lipases, physical damage to the MFGM and some lipolytic bacteria (Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2008; Toušová, Stádník, & Ducháček, 2013). The significant seasonal variations, with a higher degree of lipolysis and decreases in ∑MUFA and ∑PUFA in PL in stored UHT milk sampled in summer (July), can possibly be attributed to the composition of the diet of dairy cows in different sampling months. In Paper I, higher amounts of PL and UFA were

found in both raw and corresponding UHT milk sampled in summer months (June and July), when the cows fed on the fresh grass diet, compared with milk from other months, when the cows were fed conserved forage. These higher amounts of PL and UFA in fresh UHT milk may lead to large decreases initiated by lipolysis and oxidation during storage. In addition, the PL are mainly distributed in the multi-layer of MFGM, which may play a key role in protection of TAG hydrolysis (Shahidi & Zhong, 2010b). On the other hand, poor quality of feed in other months may enhance the activity of spontaneous lipolysis and psychotropic lipolytic bacteria, which could survive high temperature treatment and hydrolyse more TAG with no barrier in MFGM (Deeth, 2006). The large decreases in levels of UFA (∑MUFA and ∑PUFA) in PL were also revealed by the changes in UI value. These changes are associated with degree of lipid oxidation, because free radicals (formed by a few pathways, *e.g*. light, metals, degradation of hydroperoxides) can initiate lipid oxidation by rapidly reacting with double bonds to yield hydroperoxides (Medina-Meza, Barnaba, & Barbosa-Cánovas, 2014). The rate of oxidation is especially dependent on the degree of unsaturation. For example, the proportions of reaction rates for C18 acids with one, two, and three double bonds are roughly 1:30:80 (Ramos & Science, 2008). The stored UHT milk produced in summer contained higher amounts of UFA residues and, consequently, was more rapidly oxidised, starting with PL on the fat globule membranes.

4.2 UHT processing effects on milk lipids

4.2.1 Commercial UHT processing (Paper I)

In Paper I, commercial UHT milk was produced from pooled raw milk in a series of processing steps, *i.e*. fat standardisation, homogenisation and ultra-high temperature heating (Figure 5). The structural characteristics and lipid composition of the different samples are shown in Figure 10 and Table 4.

The neutral lipids (TAG), stained by Nile Red, were evident as spherical shapes mainly located in the core of fat globules in both raw and UHT milk. There was a difference in size, with the average diameter of fat globules being approximately 4 μm in raw milk (excluding aggregated globules), while the diameter of most fat droplets in processed UHT milk was less than 1 μm (excluding coalesced drops shown as arrows in Figure 10B).

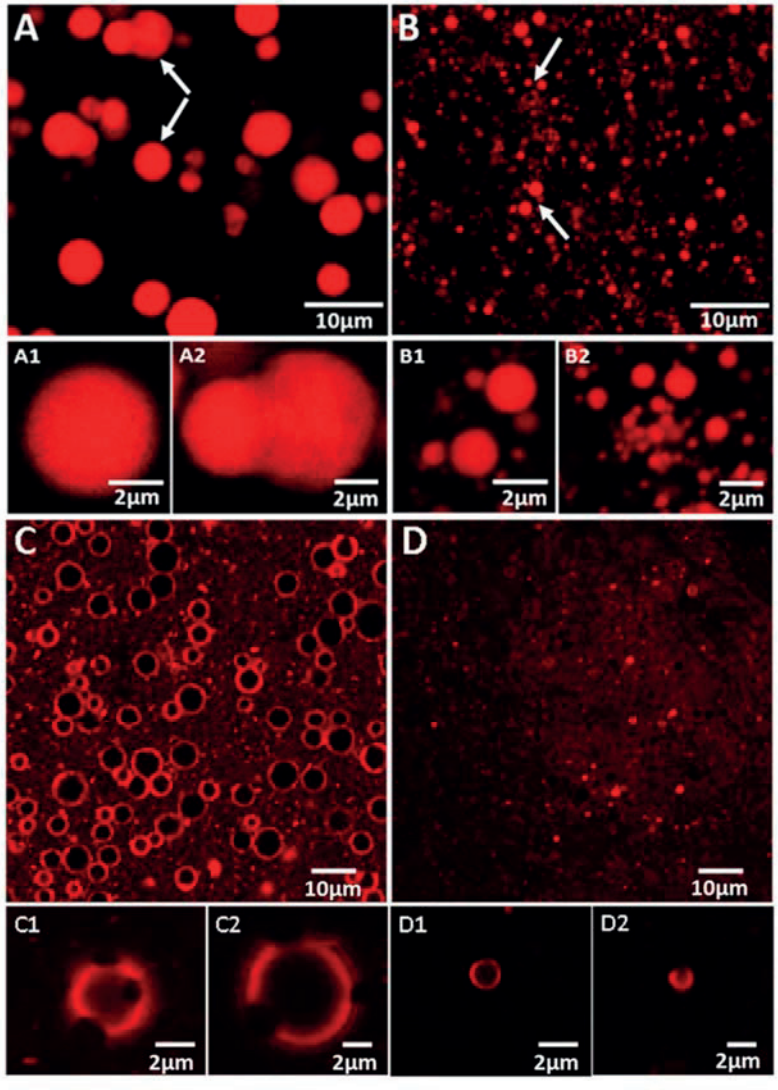

Figure 10. Confocal laser scanning micrographs showing the microstructure of fat globules from raw and UHT-processed milk stained by Nile Red fluorescent dye (red colour in A and B): (A) Fat globules in raw milk, where A1 and A2 are the selected globules indicated by arrows in A. (B) Fat globules in UHT milk, where B1 and B2 are the selected areas in B. Distribution of polar lipids in milk fat globule membrane stained by Rh-DOPE fluorescent dye (red colour in C and D): (C) Raw milk, where C1 and C2 show the heterogeneous distribution of phospholipids. (D) UHT processed milk, where D1 and D2 are two typical forms of membrane enveloping the fat droplets.

Polar lipids, the important structural constituent of MFGM, were investigated by fluorescent Rh-DOPE and were found to be mainly distributed at the periphery of fat droplets in raw and UHT milk. In raw milk, a homogeneous distribution of fluorescent Rh-DOPE, shown as an intact red circle in two dimensions, was found in MFGM surrounding most of the globules (Figure 10C). In the case of fat globules (Figure 10C2), the fluorescent dye was not fully dispersed into the red ring of the MFGM, with some black areas when viewed from the equatorial plane of the globules. When viewed from the superior or interior section of the globules, the fluorescent Rh-DOPE showed a heterogeneous distribution in MFGM with the presence of some orbicular non-fluorescent domains (Figure 10C1). For processed UHT milk, some fat globules were represented as integral red rings labelled by Rh-DOPE, which indicates that the newly formed globules might be fully covered by the original membranes (Figure 10D1). In some cases, fat globules were stained by a discrete red fluorescence dispersed at their surface, meaning they were partly covered by the thinner MFGM from the raw milk (see example in Figure 10D2).

Regarding lipid analysis, the fat content decreased significantly, from 4.82% in raw milk to 1.59% in processed UHT milk, after fat standardisation (Table 4). A significant decrease in TAG proportion, accompanied by increases in the relative percentages of PL and FFA, was observed in processed UHT milk compared with raw milk. Some FA, *i.e.* medium-chain FA (C12:0, C14:0), trans-C18:1 and some PUFA (C18:2(n-6), C18:3(n-3), CLA c9t11), showed increases in relative proportions with decreases in the percentages of C16:1 c9 and C18:1 c9.

The CLSM analysis revealed characteristics of size distribution and native shape of fat globules in raw milk that are in agreement with previous findings (Lopez, Madec, & Jimenez-Flores, 2010; Lopez *et al.*, 2011). In processed UHT milk, the majority of droplets were uniform in size and homogeneously distributed, with diameter ranging from 0.2 μm to 1 μm. A minority of coalesced droplets had diameter >1 μm, mainly attributable to the process of homogenisation, which can break down the large globules in raw milk into smaller globules (Lopez, 2005). The two and three-dimensional diagrams proposed by Lopez *et al*. (2010) show the distribution of polar lipids in the outer bilayers of MFGM. Rh-DOPE fluorescent dye can integrate into the bilayer with the domains rich mostly in glycerophospholipids (phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS)), excluding the rigid sphingolipid-rich and cholesterol compounds in the liquid-ordered phase (circular black areas in Figure 10C1). Due to the processes of homogenisation and heat treatment, the intact tri-layer structure of MFGM might be disrupted or split to cover the considerably increased interfacial surface

areas caused by increased numbers of globules in UHT milk. The membrane materials from raw milk do not have the capacity for full coverage of the new-formed globules, and thus some denuded fat can be created. It is prone to forming a new surface layer by absorption of proteins and coalescence, which is likely to compress the layer and increase its thickness (Walstra, Jenness, & Jennes, 1984; Dalgleish, Spagnuolo, & Douglas Goff, 2004)

Table 4. *Least square means of lipid classes and fatty acid composition in processed UHT milk compared with raw milk (Tukey test for group comparisons, P≤0.05)*

	Raw milk	UHT milk
Fat content (%)	4.82±0.19	1.59±0.16
Lipid class (%)		
Phospholipids	3.25	3.89
Free fatty acids	3.13	3.56
Triacylglycerol	79.8	78.4
FA (%)		
C12:0	2.75	2.90
C14:0	12.2	13.3
C16:1 c9	1.79	1.76
C18:1 t11	1.65	1.72
C18:1 c9	23.8	23.6
C18:2(n-6)	1.66	1.80
C18:3(n-3)	0.48	0.52
CLA c9t11	0.48	0.51

Standard error of the mean (S.E.M) for n = 36 measurements.

The changes in fat globule size and membrane structures resulted in corresponding changes in lipid composition. The processes of fat standardisation and homogenisation might explain the decrease in TAG proportion and increase in PL proportion. Larger fat globules containing higher amounts of TAG are prone to creaming faster than smaller globules, resulting in this being the primary constituent removed during the process. On the other hand, some processes, *e.g*. centrifugation, churning and homogenisation, disrupt the membrane construction, which could result in the loss of PL loosely bound on the surface of MFGM in the cream phase and increase the proportion of PL in the milk serum. This explanation is proposed by Contarini & Povolo (2013). Furthermore, lipolytic activities enhanced by physical damage to MFGM and some thermo-resistant bacteria could accelerate the lipolysis of TAG and some PL to produce more FFA in UHT milk (Deeth, 2006). A similar pattern of increases in C12:0, C14:0, C18:1 t11, C18:2(n-6), C18:3(n-3), CLA c9t11 and a

decrease in C18:1 c9 was observed by Lopez *et al*. (2011), who found these variations in FA in smaller fat globules, but not larger globules. The increases in the proportions of SFA (C12:0, C14:0) and some UFA (C18:2(n-6), C18:3(n-3), CLA c9t11) might be attributable to the increase in FFA in UHT milk, which originated from the lipolysis of TAG and some PL during processing. Low-temperature (50 °C and 65 °C) preheating of fat and aerobic conditions might lead to an increase in trans-isomer C18:1, as relatively low-temperature heating cannot eliminate dissolved oxygen sufficiently and inactive some microorganisms or enzymes, which could lead to lipid oxidation and isomerisation in UHT milk (Herzallah, Humeid, & Al-Ismail, 2005).

4.2.2 UHT processing in pilot plant (Paper III)

In Paper I, the lipid distribution was significantly altered in the start (raw milk) and end products (commercial UHT milk). In order to identify how the UHT processing steps affected the components of milk lipids, four test milk samples were collected from key points during UHT milk production in a pilot plant (Paper III).

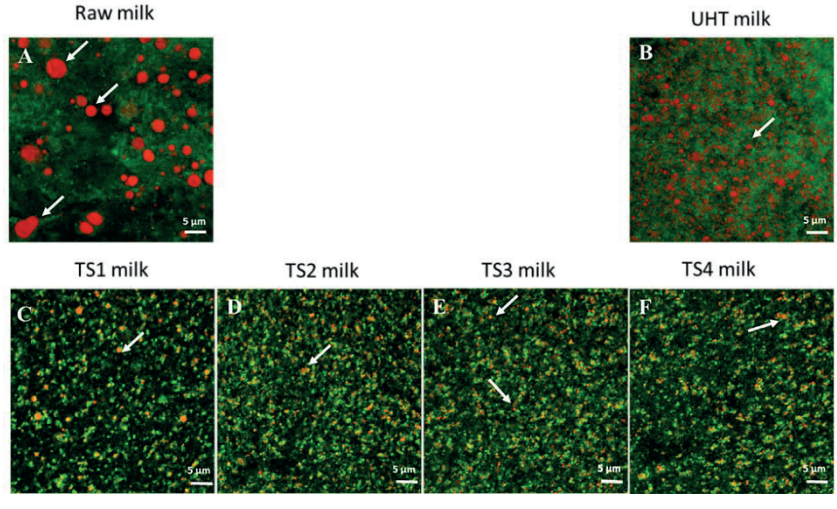

Figure 11. Confocal laser scanning microscopy (CLSM) images showing microstructural organisation of fat globules stained by Nile Red and protein network by Fast Green in (A) raw milk and (B) corresponding commercial UHT milk from Norrmejerier dairy; and in four test milk samples from Tetra Pak (Lund): (C) pasteurised homogenised whole milk from Skånemejerier, TS1; (D) second-stage homogenised milk, TS2; (E) stabilised milk, TS3; and (F) final UHT milk, TS4.

The microstructural organisation of fat globules and the protein network was examined in commercial products and in test samples from pilot plant using

microscopy techniques (CLSM and TEM). Native fat globules in unprocessed raw milk were evident as spherical shapes with a wide size variation (Figure 11A). In the four test samples, fat globule size was smaller in milk samples TS2 (Figure 11D), TS3 (Figure 11E) and TS4 (Figure 11F) than in the TS1 sample (Figure 11C) at the same magnification, while there was no obvious difference in size between the TS2, TS3 and TS4 samples. This indicates that the second processing stage, homogenisation, which affected the TS2 samples compared with TS1 samples, could significantly decrease the diameter of fat globules, whereas heat treatment possibly had little impact on globule size. Comparing milk samples from the pilot plant and commercial milk revealed significant decreases in the size of fat globules in TS1 and TS4 samples, compared with the raw and corresponding UHT milk, respectively. These differences can mainly be attributed to use of higher pressure in second-stage homogenisation in the pilot plant than in commercial production.

Interactions between MFGM and protein were revealed by TEM (Figure 12). The core of neutral lipids (TAG) in fat globules was completely covered with well-defined MFGM (Figure 12A). Some wrinkles on the surface of MFGM in raw milk might be attributable to the milking process or vortexing in TEM sample preparation (Sharma, Oey, & Everett, 2015). Free dispersion of proteins was evident around fat globules and a few were attached to the native MFGM. The combined processing steps of pasteurisation and homogenisation applied in TS1 resulted in more protein adhering to the newly-formed surface of fat globules and it was prone to form long protruding strands connected to fat particles (Figure 12B). After second-stage homogenisation, two main populations of fat globules were observed in TS2 samples: 1) Some large fat globules were partly covered with some original MFGM, which led to more sites being exposed to adherence of proteins. Compared with TS1, the protein seemed larger and denser. 2) Some smaller fat globules were inclined to aggregation and shared protein with adjacent particles (Figure 12C). During the process of stabilisation, fat globules showed no significant changes: some larger fat globules still remained part of MFGM materials on the surface, with a cover of a thicker layer of proteins. In addition, there were some aggregated small fat globules, together with protein made up of lipoproteins (Figure 12D). Although some fat globules became free fats with non-globular shapes, MFGM materials still covered most of the globules. However, when the milk underwent UHT treatment, the fat globules became more irregular, with an opened-up structure in the core and release of MFGM fragments from the surface. The fat globules were completely entrapped by a protein network through filamentous assemblages (Figure 12E). Comparing the two final UHT products, the fat globules made in the commercial production process were better able to maintain

integrity of globular shape and MFGM materials, with partial absorption of proteins to the surface (Figure 12F).

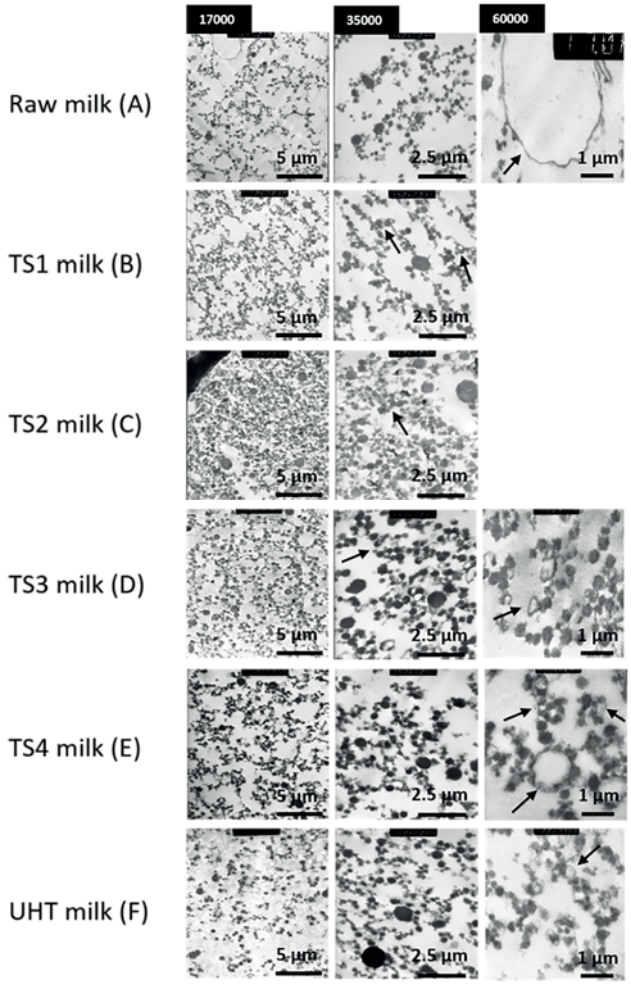

Figure 12. Transmission electron microscopy micrographs (TEM) showing interactions between milk fat globule membrane (MFGM) and proteins in (A) raw milk and (B) corresponding commercial UHT milk from Norrmejerier dairy; and in four test milk samples from Tetra Pak (Lund): (C) pasteurised homogenised whole milk from Skånemejerier, TS1; (D) second-stage homogenised milk, TS2; (E) stabilised milk, TS3; and (F) final UHT milk, TS4.

A similar pattern of wide variation in fat globule size and heterogeneous distribution of polar lipids on intact MFGM in raw milk was observed by Lopez (2011), who found heterogeneous size of naturally dispersed milk fat globules (diameter range 0.1-20 µm, mean 3-5 µm) and suggested a tri-layer structure of

lateral organisation of polar lipids on MFGM. The reduction in fat globule size in TS1 and TS2 samples was mainly due to the combination of pasteurisation and second-stage homogenisation. Although heat treatment alone may have no impact on fat globule size, when it applied before homogenisation it can increase breakdown of large particles into smaller particles by homogenisation through intense mechanical forces, thereby significantly increasing the total surface area (Michalski & Januel, 2006; Liang, Qi, Wang, Jin, & Mcclements, 2017). In addition, heat treatment may cause the thicker deposit of protein on the newly-formed MFGM and the long filamentous assemblages associated with these (Bermúdez-Aguirre, Mawson, & Barbosa-Cánovas, 2008; Jukkola & Rojas, 2017). In particular, the more expanded structure of fat globules and the release of MFGM fragments in the final UHT milk from the pilot plant (TS4) were possibly due to the UHT treatment. According to García-Risco *et al*. (2002), heat treatment can alter MFGM structure, change FA composition and increase the incorporation of hair-like structure proteins with fat globules. Moreover, the cooling process can cause loss of MFGM materials, as suggested by Sharma & Dalgleish (1994). The differences in microstructure organisation of fat globules in UHT products from the two different production lines indicate that the processing regime applied in different dairy industries have clear impacts on physical characteristics of fat in final UHT milks and also affect its lipid distribution.

The total fatty acid (TFA) composition and FA distribution in PL, TAG and FFA were determined to assess the impacts of processing on lipid characteristics during UHT milk production. The amount of TFA, PL and TAG decreased significantly, while the FFA content increased, on comparing the start product of pasteurised milk (TS1) with the final UHT milk (TS4).

Table 5. *Fatty acid (FA) distribution of phospholipids (µg FA/mL milk) in pasteurised, homogenised, stabilised, and final UHT milk samples taken during pilot plant processing*

Fatty acids:	Treatment in series of UHT processing			
	Pasteurised milk	Homogenised milk	Stabilised milk	UHT milk
C14:0	12.2±0.03^{a}	10.6±0.13^{b}	9.57±0.22^{c}	8.92±0.23^{d}
C16:0	44.0±0.14^{a}	41.5±0.19^{b}	40.9±0.28^{c}	36.9±0.11^{d}
C18:0	18.3±0.34^{a}	17.1±0.32^{b}	16.2±0.25^{c}	15.7±0.28^{c}
C18:1 c9	34.7±0.24^{a}	32.1±0.36^{b}	32.0±0.34^{b}	29.6±0.14^{c}
C18:2(n-6)	5.58±0.13^{a}	5.12±0.04^{b}	4.98±0.05^{bc}	4.84±0.02^{c}
Total FA	114.9±0.82^{a}	106.4±0.27^{b}	103.7±0.19^{c}	95.9±0.74^{d}

$^{a–d}$Means within rows with different letters are significantly different ($P<0.05$).

As for FA in PL (Table 5), the amounts of all five FA (C14:0, C16:0, C18:0, C18:1 c9 and C18:2(n-6)) showed similar patterns of significant decreases after pre-heating and homogenisation. A difference then arose, in that the SFA (C14:0, C16:0, C18:0) content decreased continually during stabilisation and UHT treatment, while the UFA (C18:1 c9 and C18:2(n-6)) content was only affected by the UHT treatment, and not by the stabilisation step (Table 5).

Table 6. *Fatty acid distribution of triacylglycerol (µg FA/mL milk) in pasteurised, homogenised, stabilised, and final UHT milk samples taken during pilot plant processing*

Fatty acids:	Treatment in series of UHT processing			
	Pasteurised milk	Homogenised	Stabilised	UHT milk
C8:0	25.7±0.35^{a}	13.9±0.31^{b}	13.2±0.40^{b}	13.1±0.39^{b}
C10:0	228±0.59^{a}	188±0.64^{b}	144±0.52^{c}	141±1.32^{d}
C12:0	304±0.82^{a}	265±4.03^{b}	255±1.63^{c}	252±2.16^{c}
C14:0	1022±5.34^{a}	939±5.48^{b}	928±1.61^{c}	822±2.59^{d}
C14:1 c9	83.3±0.27^{a}	82.2±0.39^{b}	81.7±0.37^{b}	81.7±0.43^{b}
C15:0	102±0.18^{a}	100.0±0.67^{b}	99.5±0.49^{b}	99.3±1.05^{b}
C15:1 c10	18.6±0.37^{a}	18.5±0.36^{a}	18.1±0.39^{a}	18.0±0.42^{a}
C16:0	3157±3.26^{a}	2922±2.05^{b}	2872±3.78^{c}	2575±5.30^{d}
C16:1 c9	179±0.62^{a}	161±1.92^{b}	156±1.78^{c}	155±0.78^{c}
C17:0	44.1±0.35^{a}	40.2±0.45^{b}	39.7±1.28^{b}	34.2±1.10^{c}
C17:1 c9	3.87±0.15^{a}	3.66±0.08^{ab}	3.55±0.13^{bc}	3.34±0.06^{c}
C18:0	1068±9.16^{a}	1004±4.08^{b}	996±1.56^{b}	905±1.76^{c}
C18:1 t11	175±1.45^{a}	163.7±1.42^{b}	159.0±1.39^{c}	152.9±1.84^{d}
C18:1 c9	2195±13.8^{a}	2104±8.97^{b}	2065±10.6^{c}	1860±3.66^{d}
C18:2(n-6)	195±1.61^{a}	182±0.82^{b}	180±5.27^{b}	159±3.31^{c}
C18:3(n-3)	50.3±0.76^{a}	50.2±1.01^{a}	47.8±0.67^{b}	42.5±0.41^{c}
C20:0	15.4±0.48^{a}	15.1±0.95^{a}	14.0±0.60^{ab}	12.9±0.49^{b}
CLA c9t11	50.8±0.46^{a}	48.1±0.55^{b}	47.2±0.47^{b}	42.8±0.42^{c}
CLA t10c12	13.4±0.34^{a}	12.4±0.29^{ab}	12.2±0.59^{ab}	11.1±1.06^{b}
C20:4(n-6)	8.80±0.27^{a}	8.61±0.37^{ab}	8.15±0.10^{b}	8.13±0.06^{b}
C22:5(n-3)	5.65±0.05^{a}	5.29±0.12^{b}	5.28±0.13^{b}	4.99±0.12c
∑SFA	5971±10.9^{a}	5493±8.87^{b}	5367±0.93^{c}	4859±3.93^{d}
∑MUFA	2663±14.4^{a}	2540±8.57^{b}	2490±9.42^{c}	2279±3.90^{d}
∑PUFA	336±1.85^{a}	318±0.35^{b}	311±4.98^{b}	279±4.56^{c}
∑CLA	64.3±0.64^{a}	60.4±0.47^{b}	59.4±0.55^{b}	53.9±0.66^{c}
Total FA	8970±11.8^{a}	8351±17.6^{b}	8169±14.7^{c}	7417±4.14^{d}

$^{a–d}$Means within rows with different letters are significantly different ($P<0.05$).

In TAG (Table 6), the amount of SFA and MUFA decreased significantly. In particular, the dominant FA C14:0, C16:0, C18:0 in SFA and C18:1 c9, C18:1 t11 in MUFA were affected by each key step during production. Most PUFA (C18:2(n-6), CLA c9t11, C22:5(n-3)) followed similar patterns, with a decrease in FA amount brought about by all processes except stabilisation. A minor difference was found in C18:3(n-3), the amount of which was not significantly affected by the process of homogenisation.

The processing steps resulted in the loss of TFA in the final UHT milk. The significant decrease in PL and TAG and increase in FFA mainly contributed to lipid lipolysis, induced by lipases hydrolysing PL and TAG to form free fats (Pereda *et al*., 2008). It is obvious that the second-stage homogenisation significantly altered the FA distribution in PL. This indicates that homogenisation may disrupt the bilayer structure of polar lipids, leading to loss of MFGM components in processed UHT milk (Morin, Jiménez-Flores, & Pouliot, 2007). This result was supported by the TEM micrographs. The FA profile in MFGM may be sensitive to heat, with the PL component migrating from MFGM to the serum upon heating (Singh, 2006). The findings in this thesis show that SFA in PL seems more likely to be affected by high temperature than UFA in PL. It is well-known that polar lipids of PC and SM contain higher proportions of SFA, which are mainly located in the outer layer of MFGM, while PE, PC and PI contain higher proportions of UFA, which are mainly distributed in the inner layer of the membrane (Michalski, 2009). It is uncertain that the location of FA in polar lipids of MFGM led to the difference in FA release, but this aspect is worthy of further examination. The decrease in FA in TAG indicates that damage to the core of fat globules triggered by homogenisation could lead to the loss of TAG components. In addition, the marked decreases in the amounts of C14:0, C16:0, C18:0, C18:1 c9 and C18:2(n-6) in milk during production possibly contributed, for two reasons: (1) The three available positions in TAG backbones favour esterification of these five FA, which leads to them occurring in higher proportions in TAG (Jensen, 2010; Tzompa-Sosa, van Aken, van Hooijdonk, & van Valenberg, 2014); (2) a combination of homogenisation and thermal treatment (especially UHT treatment) can lead to breakdown of much smaller fat globules than homogenisation alone (García-Risco *et al*., 2002), greatly increasing the total surface area and providing more binding sites for related lipases hydrolysing FA from TAG. Interestingly, the changes in percentage sum of SFA, MUFA, PUFA and CLA from start milk to final UHT milks produced by two different productions lines followed different patterns. These differences in FA indicate that not only the microstructural organisation of fat, but also FA distribution, tends to be affected by the processing regime applied in different industrial processes.

4.3 Storage time and temperature effects on milk lipids

Storage time and temperature significantly affected lipid quality, and thereby lipid classes, FA composition and oxidation in UHT milk (Table 7). There were significant decreases in the proportions of PL (-0.73% to -1.64%) and TAG (-1.07% to -3.05), accompanied by significant increases in FFA (1.33% to 2.70%) in UHT milk stored for longer than one year. Increases in ∑SFA and decreases in ∑MUFA, ∑PUFA, ∑CLA and UI proportions in PL and TAG with increasing storage duration were detected, which is in agreement with the trends observed in total FA in UHT milk. Similar patterns of significant decreases in PL, TAG, ∑MUFA, ∑PUFA, ∑CLA and UI in PL and TAG, and increases in FFA, were found at the higher storage temperatures tested (20 °C, 30 °C and 37 °C).

Table 7. *Least square means of differences in lipid profile, including lipid class and fatty acid composition, in UHT milk stored for four different periods at four different temperatures, as analysed by GLM mode*

	Storage time (month)				Storage temperature (°C)			
	3	6	9	12	4	20	30	37
Lipid class:								
TAG	-1.07^{a}	-1.32^{b}	-2.13^{c}	-3.05^{c}	-1.30^{a}	-1.72^{b}	-2.23^{c}	-2.23^{c}
PL	-0.73^{a}	-1.16^{b}	-1.33^{c}	-1.64^{d}	-1.01^{a}	-1.20^{b}	-1.23^{b}	-1.42^{c}
FFA	1.33^{a}	1.82^{b}	1.79^{b}	2.70^{c}	1.45^{a}	1.75^{b}	2.04^{c}	2.40^{d}
Fatty acids:								
TAG:								
∑SFA	0.56^{a}	1.22^{b}	1.92^{c}	3.11^{d}	0.88^{a}	1.58^{b}	2.12^{c}	2.22^{c}
∑MUFA	-0.30^{a}	-0.79^{b}	-1.37^{c}	-2.45^{d}	-0.69^{a}	-1.17^{b}	-1.50^{bc}	-1.59^{c}
∑PUFA	-0.27^{a}	-0.43^{b}	-0.55^{c}	-0.67^{d}	-0.19^{a}	-0.47^{b}	-0.62^{c}	-0.63^{c}
∑CLA	-0.06^{a}	-0.09^{b}	-0.10^{b}	-0.11^{c}	-0.04^{a}	-0.09^{b}	-0.11^{c}	-0.11^{c}
UI	-0.92^{a}	-1.78^{b}	-2.64^{c}	-4.01^{d}	-1.15^{a}	-2.21^{b}	-2.95^{c}	-3.06^{c}
PL:								
∑SFA	1.43^{a}	3.26^{b}	6.06^{c}	7.92^{d}	2.48^{a}	4.44^{b}	5.65^{c}	6.10^{d}
∑MUFA	-1.07^{a}	-2.53^{b}	-4.95^{c}	-6.40^{d}	-2.00^{a}	-3.56^{b}	-4.49^{c}	-4.90^{d}
∑PUFA	-0.36^{a}	-0.73^{b}	-1.11^{c}	-1.52^{d}	-0.48^{a}	-0.87^{b}	-1.16^{c}	-1.20^{d}
UI	-1.79^{a}	-3.99^{b}	-7.17^{c}	-9.43^{d}	-2.96^{a}	-5.31^{b}	-6.81^{c}	-7.31^{d}

All data transformed and shown as the difference between fresh sample and treatment. $^{a–d}$Means within rows with different letters are significantly different ($P<0.05$). Standard error of the mean (S.E.M) for n = 144 measurements. For abbreviations, see Table 3.

The significant increase in the percentage of FFA in UHT milk was mainly attributable to lipid hydrolysis of TAG and PL, probably catalysed by some endogenous lipases, phospholipases and some bacteria lipases during storage (Toušová *et al*., 2013). Time-related changes were observed. After the homogenisation and UHT steps, the physical structure of MFGM was disrupted and damaged, which could result in greater absorption of protein on the surface and fat coalescence, maintaining the stability of fat globules. With increased storage duration, proteins are prone to sedimentation (Malmgren *et al*., 2017), which means that fewer proteins would be available for fat stabilisation, thereby leading to a greater increase in lipase-accessible free fat. On the other hand, some thermo-resistant bacteria might survive and continuously hydrolyse more TAG and PL during storage. Storage temperature also influences the rate of lipolysis. The higher storage temperatures tested in this thesis (*i.e*. 20 °C, 30 °C and 37 °C) might be associated with microorganism activities and accelerate the degree of lipid lipolysis (Deeth, 2006).

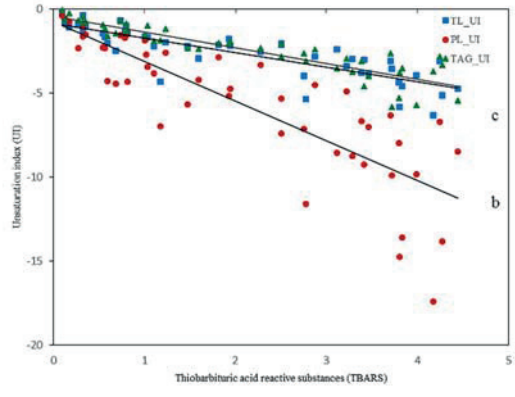

Figure 13. Linear relationship and Spearman correlation coefficient between thiobarbituric acid reactive substances (TBARS) and unsaturation index (UI) in (a) total lipids (TL), (b) phospholipids (PL) and (c) triacylglycerol (TAG). Each data point represents mean value of observations (N=3).
Equation of TL: y=-0.8686x-0.847, R2=0.6961.
Equation of PL: y=-2.3608x-0.7736, R2=0.6968.
Equation of TAG: y=-0.936x-0.429, R2=0.8059.
**Indicates significant correlation at P<0.01

The significant decreases in ∑MUFA, ∑PUFA and UI indicate that a greater degree of lipid oxidation occurred when UHT milk was stored for longer periods and at higher temperatures. In general, the rate of lipid oxidation is affected by several factors (Shahidi & Zhong, 2010): (1) Fatty acid profile: PUFA, with the

highest degree of unsaturation, were the most susceptible to oxidation, which shows their compromised oxidative stability. (2) The location of PUFA in the sn-2 position in the backbone of TAG can prevent oxidation. (3) Different lipid classes show variation in oxidative stability, FFA>PL>TAG. (4) Some antioxidants, for example, fat-soluble vitamin E, have enhanced antioxidant efficiency in the presence of phospholipids as a synergist. (5) Environmental factors, *i.e*. high temperature, light, oxygen and metals, to which lipid is exposed during processing and storage.

In this thesis, the oxidative status of UHT milk during storage was characterised by higher concentrations of TBARS and changes in UI. During the first three months of storage, the concentration of TBARS increased slowly (Paper II), because initiation of lipid oxidation requires formation of free radicals as the initiators or catalysts to remove the hydrogen atom in the lipid molecule (Barden & Decker, 2013). With increased storage duration, the concentration of TBARS significantly increased. Lipid oxidation is a self-propagating and self-accelerating process, as peroxyl radicals drive this rapid reaction by attacking new lipid molecules. The concentration of TBARS in this stage might be influenced by *e.g*. the concentration of tocopherol (VE) in stored UHT milk, decomposition of primary products and accumulation of secondary products (Citta *et al*., 2017). As expected, the concentration of TBARS was strongly negatively correlated with UI in PL and TAG, and PL was oxidised faster than TAG (Figure 13). The higher oxidative reactivity of PL is mainly attributable to: (1) Higher proportion of PUFA in PL composition compared with TAG to retain the fluidity of MFGM; (2) the distribution of PL at the surface of MFGM facilitating the propagation of oxidation; and (3) nearness to catalytic locations of oxidative enzymes (Shahidi & Zhong, 2010). In addition, the higher storage temperature could accelerate the rate of oxidation, with greater decreases in the amount of PUFA. These results in this thesis are in agreement with previous findings of higher amounts of lipid oxidative products in milk products stored at higher temperatures (Romeu-Nadal, Chávez-Servín, Castellote, Rivero, & López-Sabater, 2007; Ranalli, Andrés, & Califano, 2017; Mahmoodani, Perera, Abernethy, Fedrizzi, & Chen, 2018).

5 Main findings and conclusions

This thesis evaluated seasonal variations in lipid composition in raw milk and in fresh and stored UHT milk and assessed the impacts of storage duration and temperature on lipid class and FA composition in UHT milk during storage, using HPTLC and GC-FID analysis. Microstructural changes in fat globules and lipid profiles in UHT milk from a commercial plant and a pilot plant were also examined.

The main findings and conclusions of the thesis are:

- Seasonal variations in lipid profiles in raw milk persist in corresponding processed UHT milk, with higher proportions of PL, FFA and UFA and lower proportions of TAG and SFA in summer milk (June and July) than in milk from other seasons.

- The commercial UHT production process influences the microstructure of fat globules, resulting in smaller diameter and thinner globule membrane. It thereby changes the distribution of main constituents in globules and membranes, with decreases in the proportion of TAG and increases in PL and some UFAs in final UHT milk compared with raw milk.

- UHT milk produced in summer contains higher levels of UFA and is more prone to oxidation during storage than milk from other months. This suggests that the influence of milking month on lipid properties should be considered when assessing the shelf-life of UHT milk.

- Separation of the fat phase increases in stored UHT milk and shows greater oxidation and lipolysis with increasing storage duration up to 12 months. This novel information on storage time effects on lipid properties can help the dairy industry maximise the initial oxidative lag phase, lowering the rate of milk spoilage and prolonging the shelf-life.

- Higher storage temperature (20 °C, 30 °C, 37 °C) for UHT milk results in a higher degree of fat aggregation, lipid oxidation products and lipolysis from PL and TAG compared with refrigerated storage (4°C).

- Second-stage homogenisation greatly reduces fat globule size and disrupts MFGM structure. Combined with prior pasteurisation, homogenisation can cause casein micelles to form chains and adhere to the new surfaces in processed UHT milk.

- UHT treatment may aggravate the damage to MFGM structure and open up the core of fat globules, resulting in changes in FA distribution in PL, TAG and FFA caused by lipolysis.

- This is the first body of work to investigate the microstructural organisation of fat globules and lipid distribution in different lipid classes in processed UHT milk collected from a pilot production line.

- Comparisons of fat microstructure and changes in lipid composition from start milk to end products from different industrial processes suggest that processing differences have impacts on the characteristics of final UHT milk. These affect the stability of fat in commercial UHT milk and should be considered by dairy industries seeking to improve their products.

6 Future Perspectives

This thesis examined the influence of seasonal variations originating from raw milk, UHT processing, and storage conditions (time and temperature) on lipid profiles in UHT milk. Based on the findings, the following research areas are of interest to provide comprehensive insights into the mechanisms determining UHT milk stability during milk production and storage.

- Merging data from this project for integrated analysis of lipid, other milk composition, bacteria amount and proteolytic activities, to re-evaluate whether the seasonal variation in raw milk can be observed in corresponding UHT milk.

- It is worth conducting a thorough comparison of upstream and downstream homogenisation effects on milk fat properties, *i.e*. fat globule size, membrane composition, lipolysis and oxidation during UHT production and storage.

- Heat treatment can accelerate the binding of proteins to MFGM. Different heating systems (direct and indirect) and induced interactions between proteins and MFGM should be further investigated.

- In UHT milk, proteins were attached to the MFGM, maintaining the stability of fat globules. Combined analysis of lipid lipolysis and protein enzymatic hydrolysis should be conducted to evaluate the destabilisation of UHT milk, as protease destabilises protein and may have indirect effects on fat creaming and hydrolysis during storage.

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Popular science summary

Bovine milk has a long tradition as a source of nutrients in the human diet. Fresh milk is preferred because of its natural fresh taste and nutritional value. However, fresh milk is also a good medium for bacterial or microbial growth and this can produce toxic or undesired compounds which have detrimental effects on human health. Heat treatment was a key development in the history of the dairy industry, as relatively high temperature can kill harmful microorganisms and greatly prolong the shelf-life of milk.

Ultra-high temperature (UHT) milk is a product categorised by the type of heat treatment. Raw milk is subjected to ultra-high temperature heating at 135-140 °C for a few seconds to inactive enzymes and detrimental microbial activities, aiming to achieve long shelf-life of the product during ambient distribution and storage conditions. This thesis explored the relations between raw milk quality, UHT processing and storage conditions, and the effects on the shelf-life of UHT milk.

In one study, pooled raw milk (80 farms) was sampled for six months (March, May, June, July, September and November) and corresponding UHT milk was produced. Analysis revealed that seasonal variations in the lipid composition of the raw milk persisted in the corresponding UHT milk, with *e.g.* higher proportions of phospholipids and unsaturated fatty acids in summer milk.

In another study, fresh UHT milk produced in May, July and November was stored at the dark at a controlled temperature of 4 °C, 20 °C, 30 °C or 37 °C for 3, 6, 9 or 12 months. Fresh raw milk from July was found to contain higher proportions of phospholipids and unsaturated fatty acids, which resulted in greater decreases in these compounds during storage, through lipid lipolysis and oxidation, than in milk from other months. The findings indicated that seasonal variations in lipids persisted at the farm, during UHT processing and throughout storage. Significant fat aggregation was observed, together with a higher degree of lipolysis and oxidation, when UHT milk was stored for up to one year (particularly after 6 months). Refrigerated storage (4 °C) lowered the rate of

creaming, hydrolysis of phospholipids and triacylglycerols and oxidative reactions compared with high-temperature storage (20 °C, 30 °C and 37 °C).

Comparison of raw and UHT milks revealed smaller size of fat globules and thinner membrane in the final UHT product, together with some changes in lipid composition. In order to identify the influence of specific steps in UHT processing on milk lipids, milk samples were collected from four key steps (pasteurisation, homogenisation, stabilisation, final UHT) in a pilot-scale processing system. It was found that homogenisation greatly decreased the size of fat globules and disrupted globule membrane structure in processed UHT milk from the pilot plant. A combination of pasteurisation followed by homogenisation increased the amount of protein attaching to new-formed fat globule membranes and accelerated chain formation by freely dispersed protein. Subsequent UHT treatment possibly aggravated the damage to globule membranes, which can lead to increased hydrolysis of phospholipids and triacylglycerols.

This is the first set of studies to investigate the influence of key processing steps on microstructural organisation of fat globules and fatty acid distribution in different lipid classes of phospholipids, triacylglycerols and free fatty acids in UHT milk collected during the production process. Comparison of fat microstructure and lipid composition in UHT milk products from two different production lines suggested that the characteristics of lipids are affected by different processes applied in dairy industries, the effects of which should be considered.

This thesis provides evidence that seasonal variations in lipid profiles observed in raw milk can persist during UHT processing and throughout the shelf-life of stored UHT products. Changes in microstructural organisation of fat globules and lipid distribution are caused by key processing steps and storage conditions. These results could provide a better understanding of lipid characteristics for farmers and dairy industries seeking to improve UHT milk processing and prolong product shelf-life.

Populärvetenskaplig sammanfattning

Komjölk är av tradition en viktig källa till näringsämnen för människan, särskilt i Norden är mjölkkonsumtion populär. Färsk mjölk är omtyckt för sin naturliga goda smak och bra näringsvärde. Emellertid är färsk mjölk också ett bra medium för tillväxt av mikroorganismer som kan leda till både oönskad smak och också bilda ämnen med skadlig effekt på människors hälsa. Följaktligen är värmebehandling en milstolpe i utvecklingen av mejeriindustrin, eftersom hög temperatur kan döda de skadliga mikroorganismerna för att göra mjölken drickbar.

Ultrahög temperaturbehandlad (UHT) mjölk är en mjölkprodukt som har genomgått en särskild värmebehandling. UHT-mjölk genomgår en extremt hög temperatur av 135-140 ° C under några sekunder för att inaktivera enzymer och mikrobiella aktiviteter, med målet att uppnå en lång hållbarhet hos produkten under distribution och lagring vid rumstemperatur. I denna avhandling undersökte vi hur mjölkkvaliteten, UHT-processen och lagringsförhållandena påverkar den slutliga UHT- produkten, särskilt fettfraktionen.

I första arbetet, papper I, undersöktes den färska mjölk-råvara i samlingsprov (80 gårdar) från sex olika månader (mars, maj, juni, juli, september och november) och dess motsvarande UHT-mjölk. Vi fann att liknande säsongsvariation i lipidsammansättning från mjölk-råvaran kvarstår i motsvarande UHT-mjölk, och med högre andel membranfett, fosfolipider, PL, och omättade fettsyror, UFA, i sommarmjölk. Dessutom lagrades UHT-mjölk producerad i maj, juli och november i mörka kontrollerade temperaturrum på 4° C, 20° C, 30° C och 37° C under 3, 6, 9 och 12 månader (papper II). UHT-mjölk (juli) innehöll högre andel PL och UFA i utgångsproven, vilket resulterade i större minskningar av PL och UFA orsakade av lipidlipolys och oxidation jämfört med mjölk från andra månader under lagring. Dessa resultat indikerade att säsongsvariationen av lipider kunde urskiljas på gårdsnivå, under UHT-

bearbetning och under hela lagringstiden. Dessutom observerades en markant fettaggregering tillsammans med högre grad av lipolys och oxidation när UHT-mjölk lagrades under längre tid (särskilt efter 6 månader) upp till ett år. Lagring vid kylförvaring (4° C) kan sänka graden av gräddsättning, hydrolys av PL och lagringsfettet triacylglycerol, TAG, och oxidativa reaktioner jämfört med högtemperaturlagring (20° C, 30° C och 37° C) i viss utsträckning.

I jämförelse med icke UHT-processad mjölk (papper I) hittade vi mindre storlek på fettdropparna och tunnare membran i den slutliga UHT-produkten, tillsammans med några förändringar i lipidkompositioner. För att identifiera påverkan av specifikt steg i UHT-processen på mjölklipider (papper III) samlades fyra mjölkprover från nyckelstegen i pilotskaleanläggning (pastöriserad mjölk, homogeniserad mjölk, stabiliserad mjölk och slutlig UHT-produkt). Det observerades att homogenisering i stor utsträckning minskade storleken på fettdropparna och störde membran-strukturen i processad UHT-mjölk från pilotanläggningen. Kombinationen av pastörisering före homogenisering kan medföra att kaseinmiceller fäster mer vid de nybildade membranen och påskyndar kedjebildning av fritt dispergerade kaseinmiceller. Dessutom kan UHT-behandling efteråt förvärra skadorna på membranen, vilket kan leda till ökad hydrolys av PL och TAG. Så vitt vi vet är det första gången någon undersöker påverkan av viktiga bearbetningssteg på den mikrostrukturella organisation av fettdroppar och fettsyreförändringar i olika lipidklasser av PL, TAG och FFA vid processning av mjölk som samlats in i olika ställen av UHT-produktionslinjen.

Denna studie gav bevis för att säsongsvariationer i lipidprofiler som härrör från den färska mjölk-råvaran kan kvarstå i motsvarande processad UHT-mjölk och under hela lagringen av UHT-produkterna. Dessutom studerades förändringarna i fettdropparnas mikrostruktur och lipidfördelning, orsakad av viktiga bearbetningssteg och lagringsförhållanden. Dessa resultat kan ge bättre förståelse för fettets betydelse för mjölkens kvalitet i processer till mejeriindustrier och mjölkproducenter som kan ge instrument att förbättra kvaliteten och därmed förlänga hållbarheten.

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